Differential effects of progestogens on HIV-1 replication and host gene expression in primary PBMCs and cervical tissue explants

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Declaration

I, **Roslyn Michelle Ray**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorise the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature:…… Date:……..

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Abstract

The synthetic progestogens, medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN), are widely used in developing countries as injectable contraceptives, where disease burden is high. Levonorgestrel (LNG) is a common progestogen used in oral contraceptives and intrauterine devices. Some studies suggest that MPA, unlike NET, increases HIV-1 acquisition in women, while few studies have reported on the effects of LNG on HIV-1 acquisition. Whether MPA, NET and LNG differentially affect HIV-1 infection and the expression of key genes relevant to HIV-1 acquisition via differential molecular mechanisms is key to understanding choice of progestogen contraceptive in young women at high risk for HIV-1 acquisition.

The central hypothesis of this study is that the differential effects on host gene expression and HIV-1 replication by the different progestogens is due to their differential selectivity to towards different steroid receptors, in particular the glucocorticoid receptor (GR). In order to investigate this hypothesis, the regulation of selected genes was investigated in cervical tissue explants from premenopausal, HIV-1 negative, and contraception negative women and peripheral blood mononuclear cells (PBMCs) from women, by real time quantitative PCR, western blotting and Luminex assays, in response to physiologically relevant doses of progestogens. Infection assays were performed in the absence and presence of HIV-1 using HIV-$1_{BAL\text{-}RENILLA}$ or $HIV_{pNL4.3}$ infectious molecular clones (IMCs). The GR antagonist RU486 or GR siRNA knockdown was used to determine the role of the GR in modulating ligand-specific effects.

PBMCs and primary cervical explants were chosen as useful models to assess the direct effects of these progestogens in both the systemic and in the local mucosal immune environments. In PBMCs, MPA like dexamethasone (DEX, a GR specific agonist), showed anti-inflammatory effects, decreasing pro-inflammatory interleukin (IL) 6, IL8 and regulated upon activation, normal T cell expressed and secreted (RANTES) levels and increasing anti-inflammatory glucocorticoid interacting leucine zipper (GILZ) gene expression levels, while NET, progesterone (P4) and LNG did not, after 48 hours. In primary ectocervical tissue explants, DEX, cortisol, MPA and P4 significantly repressed IL6 while only DEX, cortisol and MPA significantly repressed IL8 and increased GILZ gene expression levels after 48 hours. Steroid receptor expression was characterised in both PBMCs and ectocervical explants. GR was the only detectable steroid receptor protein expressed in PBMCs, while ectocervical explants expressed all the steroid receptors. The progesterone and estrogen receptor levels were higher in ectocervical explants from donors that were in the follicular phase compared to ectocervical explants from donors in the luteal phase of the menstrual cycle. In PBMCs, results suggested that differential gene expression by MPA versus NET and P4 is mediated via the GR after 48 hours. Furthermore, it was observed that MPA and DEX, unlike NET, LNG and P4 increases HIV-1 replication in viable PBMCs, in the majority of donors. The increase in HIV-1 replication in the MPA treated PBMC samples correlated significantly with an increased in IL6 mRNA levels.

Interestingly, IL6 mRNA expression was significantly elevated by MPA in 7 out of 10 of the donor samples, in the absence and presence of HIV-$1_{BaL\text{-}Renilla}$ after 9 days, in PBMCs. IL6, IL8 and RANTES repression by DEX and MPA after 48 hours was lost after 3 – 7 days of hormonal incubation, while GILZ mRNA upregulation was maintained in PBMCs. Although the mechanism whereby MPA- and DEX- dependent repression of IL6, IL8 and RANTES mRNA expression in PBMCs is lost is yet to be determined, the results suggest that time of exposure to MPA could determine whether anti- or pro-inflammatory effects occur.

In primary cervical explants, MPA, but not NET increased $HIV_{pNL4.3}$ replication in 5 out of 8 donor samples, and increased HIV-$1_{BaL\text{-}Renilla}$ replication in 3 out 6 donor explant samples. The data suggest that MPA exhibits differential and direct effects on HIV-1 replication in primary cervical explants, with the majority of donor samples showing an increase in HIV-1 replication with MPA, but not NET, similar *in vivo* effects reported in some observational studies.

The results suggest that MPA, unlike NET and LNG, is likely to exert significant effects on host immunomodulatory gene expression and HIV-1 replication, at peak serum concentrations in contraceptive users, directly on cells and tissue in both the systemic and local mucosal environments. Collectively, the data suggest that NET, unlike MPA, would be a safer choice of injectable progestin contraceptive in young women in high risk areas for HIV-1 infection. Additionally, this study suggests that LNG has a lower risk profile in comparison to MPA, but similar to NET. The molecular basis for this choice most likely involves differential effects of MPA as compared to NET and P4, on transcription of immunomodulatory genes, due to their differential actions via the ubiquitous GR.

List of Abbreviations

ANOVA	analysis of variance
AP-1	activator protein 1
AR	androgen receptor
ATCC	American type culture collection
basic FGF	basic fibroblast growth factor
BCG	Bacillus Calmette–Guérin
BD-2	beta-defensin 2
bp	base pair
BV	bacterial vaginosis
CCL	chemokine (C-C motif) ligand
CCR5	C-C chemokine receptor type 5
cDNA	complementary DNA
CHX	cycloheximide
CI	confidence interval
CMV	cytomegalovirus
COC	combined oral contraceptive
CORT	cortisol
CROI	Conference on Retroviruses and Opportunistic Infections
CTRL	control
CVL	cervico-vaginal lavage
CXCL	chemokine (C-X-C motif) ligand
CXCR4	C-X-C chemokine receptor type 4
DC	dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DEPC	diethylpyrocarbonate
DEX	dexamethasone
DMEM	dulbecco’s modified eagles medium
DMPA	depo-medroxyprogesterone acetate
DNA	deoxyribonucleic acid
E2	estradiol
EC	epithelial cell
ECL	enhanced chemiluminescence
EC50	effective concentration required for 50% of maximal response

EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERK	extracellular-signal-regulated kinases
EtOH	ethanol
EtBr	ethidium bromide
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FGT	female genital tract
FSH	follicle stimulating hormone
GAG	group-specific antigen protein (HIV-1 viral protein)
GAPDH	glyceraldehyde phosphate dehydrogenase
GC	glucocorticoid
G-CSF	granulocyte-colony stimulating factor
GILZ	glucocorticoid induced leucine zipper
GM-CSF	granulocyte macrophage - colony stimulating factor
GR	glucocorticoid receptor
GRIP-1	glucocorticoid receptor interacting protein 1
GRE	glucocorticoid response element
HC	hormonal contraception
HCl	hydrochloric acid
HIV-1	human immunodeficiency virus subtype 1
HIVR4P	HIV research for prevention conference
HNP	human neutrophil peptide
hrs	hours
HR	hazard ratio
HSV	herpes simplex virus
IκBα	Inhibitor kappa B alpha
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IP-10	interferon gamma-induced protein 10
IUD	intrauterine device
JNK	c-Jun N-terminal kinase
K_D	equilibrium dissociation constant
K_i	dissociation constant

kDa	kiloDalton
LC	Langerhan cell
LBD	ligand-binding domain
LL-37	human cathelicidin
LH	luteinising hormone
LNG	levonorgestrel
Luc	luciferase
LPS	lipopolysaccharide
M	Molar
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MDMs	monocyte derived macrophages
MIP	macrophage inflammatory protein
MOPS	4-morpholine-propanesulfonic acid
MPA	medroxyprogesterone acetate
mRNA	messenger RNA
MR	mineralocorticoid receptor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NCoR	nuclear receptor co-repressor 1
NET	norethisterone
NET-A	norethisterone acetate
NET-EN	norethisterone enanthate
NF-AT	nuclear factor of activated T-cell
OC	oral contraceptive
P4	progesterone
p24	HIV-1 viral core protein
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pDCs	plasmacytoid dendritic cells
PDGF-BB	platelet-derived growth factor type BB
poly I:C	polyinosinic:polycytidylic acid
PR	progesterone receptor
PrEP	Pre-exposure prophylaxis
RT-qPCR	reverse transcription quantitative PCR
RANTES	regulated upon activation, normal T cell expressed and secreted

RBA	relative binding affinity
RCT	randomised controlled trial
Rel.	relative
RLU	relative light units
RPMI	Roswell Park Memorial Institute
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SHIV	simian-human immunodeficiency virus
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
SLPI	secretory leukocyte proteinase inhibitor
SMRT	silencing mediator for retinoid or thyroid-hormone receptor
SR	steroid receptor
SRC-1	steroid receptor co-activator 1
SRE	steroid response element
STAT5	signal transducer and activator of transcription 5
STI	sexually transmitted infection
TBS	TRIS-buffered saline
TBST	TRIS-buffered saline-tween
T h	T helper
TF	transcription factor
TNF-α	tumour necrosis factor-α
T reg	T regulatory
WHO	world health organisation
w/v	weight per unit volume
v/v	volume per unit volume
VEGF	vascular endothelial growth factor
VMMCs	vaginal mucosal mononuclear cells
VOICE	Vaginal and Oral Interventions to Control the Epidemic
VpR	HIV-1 viral accessory protein R
VZV	varicella zoster virus

Thesis Outline

The thesis is comprised of six chapters, of which two are results chapters, as well as five appendices.

Chapter 1: Progestogens, Immunity and HIV-1 infection: The role of progestin contraceptives and endogenous P4 on immunomodulatory gene regulation and HIV-1 acquisition. This chapter is a literature review and gives a brief overview on the different types progestogens used in contraception and their differential mechanisms of actions through different steroid receptors, particularly the glucocorticoid receptor (GR). This review focuses on three progestogens, medroxyprogesterone acetate (MPA), norethisterone acetate (NET-A) and levonorgestrel (LNG). Furthermore the review focuses on the current knowledge of the different progestins and their effects on disease susceptibility, particularly, HIV-1, and immune gene function in both the systemic and local genital mucosa immune environments. This chapter also outlines the research aims and hypotheses of this current study.

Chapter 2: Methods and Materials. This chapter provides detailed technical methodology used to obtain the results in the research chapters.

Chapter 3: MPA, unlike NET, P4 and LNG, regulates immunomodulatory gene expression via the GR and increases HIV-1 replication in the majority of primary human PBMCS. This chapter focuses on the differential regulation of select immunomodulatory genes by the different progestogens (MPA, P4, NET and LNG) and a glucocorticoid (DEX) over time in PBMCs. This chapter also investigates the role of the GR in mediating some of these responses. Furthermore, this chapter investigates the direct effects of these progestogens on HIV-1 replication in PBMCs.

Chapter 4: The effects of progestogens on immunomodulatory gene expression and HIV-1 replication *ex vivo* in human cervical explants. This chapter focuses on the effects of different progestogens (MPA, P4, NET and LNG) and glucocorticoids (DEX and CORT) on immune gene expression in cervical explants that are models for the local mucosa. This chapter also investigates the role of the menstrual cycle on regulating the steroid receptors as well the effects of progestogens on SR mRNA expression. Furthermore this chapter investigates the direct effects of the progestins MPA and NET on HIV-1 replication in tissue explants.

Chapter 5: Conclusions and Future Perspectives. This chapter synthesizes the results from chapters 3 and 4 and draws conclusions in the context of the field. Suggestions into future research on these topics are also discussed.

Chapter 6: Reference list. This contains all the references used throughout this thesis in alphabetical order.

Appendices:

Appendix A: Supplementary Data. This appendix consists of relevant supplementary data used to support the findings in chapters 3 and 4**.**

Appendix B: Repeated Measure two way ANOVA analysis for ectocervical explant HIV-1 infection time course analysis. This appendix contains the statistical output used to analyse the effects of HIV-1 replication over time in chapter 4.

Appendix C: Donor Information. This appendix provides a table with detailed information on the donors used in chapter 4, including hormone levels, age, HSV-1, HSV-2, HIV-1 and HPV status. Additionally the table provided the information on what experiments in chapter 4 were performed on each donor's tissue.

Appendix D: Buffers and Solutions. This appendix provides details for common solutions used throughout the study and mentioned in chapter 2.

Appendix E: Optimisation results used to determine the methodology employed in this study. This appendix provides information on the steps used to determine the optimal conditions used in this study in both chapters 3 and 4.

Appendix F: Research articles. This appendix contains PDF versions of the research articles co-authored by the candidate and listed below.

The following are peer reviewed publications arising from this thesis and consist of both reviews and original research articles:

1. Govender, Y., Avenant, C., Verhoog, N.J.D., **Ray, R.M**., Grantham, N.J., Africander, D.J. & Hapgood, J.P. 2014. The Injectable-Only Contraceptive Medroxyprogesterone acetate, unlike norethisterone acetate and progesterone, regulates inflammatory genes in endocervical cells via the glucocorticoid receptor. *PloS one* 9(5): e96497.

The present author contributed to this study in experimental design and manuscript preparation.

2. Hapgood, J.P., Africander, D.J., Louw, **Ray, R.M.** & Rohwer, J.M. 2013. Potency of progestogens used in hormonal therapy: Toward understanding differential actions. *Journal of Steroid Biochemistry and Molecular Biology*, 142: 39–47.

The present author was involved in preparing the bio-character section of this review, as well as preparing the reference list.

3. Hapgood, J.P., **Ray, R.M**., Govender, Y., Avenant, C. & Tomasicchio, M. 2014. Differential glucocorticoid receptor-mediated effects on immunomodulatory gene expression by progestin contraceptives: Implications for HIV-1 pathogenesis. *American Journal of Reproductive Immunology*, 71(6): 505–512.

This paper is partly a review paper with some limited data. The present author contributed to this paper by writing the rough draft of the results section of the manuscript, helping in the preparation and editing of the manuscript as well as performing all the experiments for the data shown in this publication, all of which are in this thesis.

4. Tomasicchio, M., Avenant, C., Toit, Du, A., **Ray, R.M**. & Hapgood, J.P. 2013. The progestin-only contraceptive medroxyprogesterone acetate, but not norethisterone acetate, enhances HIV-1 Vpr-mediated apoptosis in human CD4+ T cells through the glucocorticoid receptor. *PloS one*, 8(5): e62895.

The present author contributed to this paper by helping in the preparation and editing of the manuscript as well as designing all the steroid receptor primers in this study (also in this thesis) and performing select experiments (5% of the paper).

Chapter 1

Progestogens, Immunity and HIV-1 infection: The role of progestin contraceptives and endogenous P4 on immunomodulatory gene regulation and HIV-1 acquisition

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1. Introduction

Heterosexual transmission of HIV-1 accounts for the majority of infections in women (Padian et al., 1991; Padian et al., 1997). In Sub-Saharan Africa, 59% of all those infected are women (World Health Organisation, 2011). While vaginal intercourse has a low rate of transmission, it contributes to more new cases of HIV than any other type of transmission (Hladik & Hope, 2009). Currently, there is an on-going and contentious debate whether hormonal contraceptives may increase HIV-1 acquisition in women. Synthetic progestins, such as medroxyprogesterone acetate (MPA) or Depo-Provera and norethisterone enanthate (NET-EN) or Nuristerate, are used worldwide as injectable contraceptives. These contraceptives, along with other contraceptive methods provide important health benefits and empowerment to women globally (World Health Organisation, 2014b). In sub-Saharan Africa, 11 million women use injectable contraceptives (United Nations, Department of Economic and Social Affairs, Population Division, 2011), particularly in areas with high HIV-1 prevalence (Morrison et al., 2012). Thus identifying whether injectable or other hormonal contraception methods increases the risk of HIV-1 acquisition in women is imperative and of great public health importance.

1.1. Progesterone and Progestin Contraceptives

The menstrual cycle is under control of a gonadotropin releasing hormone (GnRH) pulsatile signal from the hypothalamus, as part of the hypothalamic-pituitary-gonadal axis (HPG) (Wira et al., 2015). Gonadotropin releasing hormone cells of the pituitary, secrete luteinising hormone (LH) and follicle-stimulating hormone (FSH) (Chabbert-Buffet & Bouchard, 2002). The menstrual cycle consists of several different phases; the follicular, the ovulatory and the luteal phase. The follicular phase refers to the phase of growth of a single follicle, governed by FSH stimulation, with increasing amounts of estrogen (E2) being secreted (Wira et al., 2015). During this phase, the follicle reorganises to become the corpus luteum, and the epithelial cells proliferate in the endometrium, under the control of E2 (Wira et al., 2010). Ovulation is triggered by an LH surge, which occurs mid-cycle. After ovulation, the corpus luteum secretes E2 and progesterone (P4) under the control of LH (Wira et al., 2015), signalling the second phase of the menstrual cycle, known as the luteal phase. During this phase, epithelial cells of the endometrium differentiate under the control of P4 into secretory cells, also known as the secretory phase (Sitruk-Ware et al., 2013). In the absence of pregnancy, the corpus luteum regresses and E2 and P4 secretion decreases. The endometrium sheds leading to menstruation, and FSH levels increase, initiating a new cycle (Chabbert-Buffet & Bouchard, 2002).

Progesterone (P4) is the natural progestin in humans, and is integral for female reproductive function. The concentration of P4 will either lead to inhibition of or activation of ovulation (Stanczyk, 2002). A decrease in the concentration of progesterone leads to menstruation and endometrial repair, while increased levels lead to the inhibition of ovulation during pregnancy (Critchley et al., 2003). It is this quality that makes P4 an

ideal contraceptive candidate. However due to progesterone's short half-life; synthetic progestins were designed to provide both the inhibitory effect of progesterone and maintaining a long half-life *in vivo* (Hapgood et al., 2004; Africander et al., 2011). As such, progestins or progestogens (when including both synthetic and the natural hormone P4) are defined as compounds that have pro-gestational activity (Stanczyk et al., 2013). That is, progestogens are able to maintain pregnancy and transform the endometrium to a secretory phase to support gestation (Stanczyk et al., 2013). Progestogens also prevent the over-proliferation of the endometrial tissue and are used to counteract the proliferative effects of estrogen (Stanczyk et al., 2013; Akison & Robker, 2012). However the extent to which the progestin may exert this effect is based on its anti-estrogenic properties, as well as the dose and duration of the treatment (Sitruk-Ware, 2006b).

Over the years, many progestins have been designed. Of interest to this study, are the synthetic progestins used most often as injectable contraceptives, medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN) and the progestin levonorgestrel (LNG) used in combined oral contraceptives or in intrauterine devices (Sitruk-Ware, 2006a). Their structures are depicted in Figure 1.1.

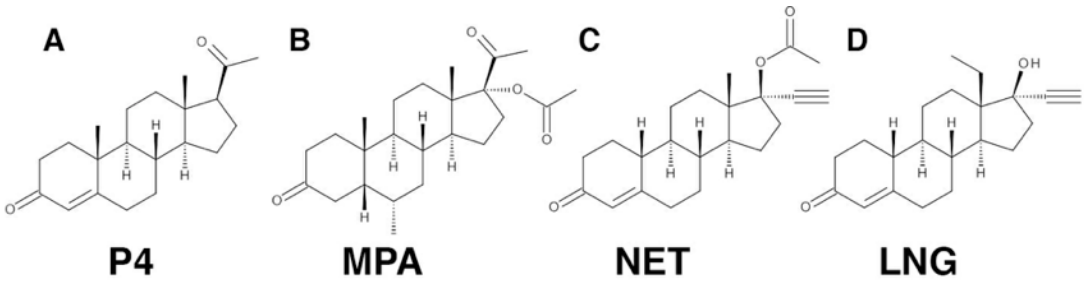


Figure 1.1: Structures of the progestogens. (A) The natural endogenous hormone, progesterone (P4), (B) first generation, progesterone derivative, medroxyprogesterone acetate (MPA), (C) first generation testosterone derivative, norethisterone acetate (NET-A) and (D) second generation testosterone derivative, levonorgestrel (LNG) (Stanczyk et al., 2013).

MPA, administered for contraception as Depo-MPA (DMPA) or Depo-Provera, is a 150 mg three-monthly intramuscular injection that is used by millions of women worldwide (Drey & Darney, 2002), particularly in Sub-Saharan Africa with high HIV-1 prevalence (Morrison et al., 2012). MPA is a first generation progestin, that is one of the first synthetic progestins synthesised, and is a progesterone derivative belonging to the pregnane group of progestins (Stanczyk, 2003). The benefits of MPA as a contraceptive include its high efficacy and safety (Drey & Darney, 2002).

Norethisterone enanthate (NET-EN) is a 200 mg two-monthly injectable that is used less than MPA, although its usage is high in some regions of South Africa (Smit & Beksinska, 2013). NET-A is a testosterone derivative, belonging to the estrane group of progestins (Stanczyk, 2002). However being a derivative of testosterone, it has the ability to exert some androgenic effects via the androgen receptor (AR) (Sitruk-Ware & Nath, 2013). However, these effects are reduced due to the chemical modifications

performed to make NET-A (Stanczyk et al., 2013). Interestingly, NET-A has been shown to convert to ethinyl estradiol (EE) and can exert some estrogenic effects. However this is concentration dependent, as the conversion rate is low (Sitruk-Ware & Nath, 2013). This is the only known progestogen that exhibits any estrogenic effects (Africander, Verhoog, et al., 2011; Stanczyk et al., 2013).

Levonorgestrel (LNG) is a second generation testosterone-derived progestin (Stanczyk, 2003) belonging to the estrane group having weak androgenic activity. LNG is commonly administered as an oral contraceptive with the combination of EE (Sitruk-Ware & Nath, 2011). It is also used as a progestin only contraceptive in intrauterine devices, like the Mirena® (Drey & Darney, 2002) or in implants like Norplant® or Jadelle® (Sitruk-Ware & Nath, 2011). It is a derivative of NET-A with the replacement of the methyl group at carbon 14 with an ethyl group (Stanczyk et al., 2013). Due to the local delivery nature of the implants and intrauterine devices as well as the daily use oral contraceptives, the concentration of LNG is much lower than that administered for the injectable contraceptives (Sitruk-Ware & Nath, 2011). Oral LNG is administered at 20 - 50 µg and the intrauterine device is delivered at 20 µg dose per day (Kuhl, 2011). In addition, LNG has recently been used in combination microbicide-contraception trials as part of developing new disease prevention strategies for women delivered in a Carraguard® vaginal gel (Sitruk-Ware et al., 2007).

While these contraceptives have been designed to act as the natural ligand P4 exerting strong progestational effects via the progesterone receptor (PR), they have different off-target effects than the natural progestin, P4, based on their modifications (see Figure 1.1) (Stanczyk, 2003; Hapgood et al., 2004; Stanczyk et al., 2013). The progesterone receptor belongs to the nuclear receptor super-family which includes the androgen receptor (AR), the mineralocorticoid receptor (MR), the estrogen receptor (ER) and the glucocorticoid receptor (Africander, Verhoog, et al., 2011; Sitruk-Ware, 2006b). There is a high conservation amongst this superfamily of structure and function, acting as ligand-activated transcription factors (Africander et al., 2011; Stanczyk et al., 2013), which mediate a variety of important physiological functions including, stress, reproduction and metabolism (Africander et al., 2011; Hapgood et al., 2004). As such, it is unsurprising that progestins may exhibit differential activity from the natural ligand P4 due to their interaction and binding with other steroid receptors (Africander et al., 2011; Stanczyk et al., 2013; Sitruk-Ware, 2004). Indeed, it is well established that these different synthetic progestins exhibit different biological effects, in particular their extents of glucocorticoid, androgenic, and mineralocorticoid activities, as seen in Table 1.1 (Hapgood et al., 2004; Africander et al., 2011; Africander et al., 2013; Africander et al., 2014; Stanczyk et al., 2013). Consequently, many of the reported side effects with the synthetic progestins are most likely related to their androgenic or glucocorticoid properties (Sitruk-Ware, 2008; Sitruk-Ware et al., 2004; Hapgood et al., 2004; Sitruk-Ware & Nath, 2013; Sitruk-Ware, 2006b; Erkkola & Landgren, 2005; Kuhl & Stevenson, 2006; Toh et al., 2012). The androgenic qualities of LNG exhibited at high concentrations, has been associated with acne, while bloating, weight gain (reviewed in Sitruk-Ware, 2006b; Hapgood et al., 2004) and loss of bone density (Ishida & Heersche, 2002) have been proposed to

be due to the glucocorticoid properties of MPA. As seen in Table 1.1, preclinical studies and binding studies show that MPA, NET and LNG all exhibit some binding to the androgen receptor, with P4 exhibiting weak anti-androgenic activity (Stanczyk, 2003) and all exhibiting some androgenic activity (Sasagawa et al., 2008; Africander, et al., 2011). MPA and NET exhibit very little anti-mineralocorticoid activity at contraceptive doses (Sasagawa et al., 2008; Africander et al., 2013), unlike some reports for LNG (Africander et al., 2011; Africander et al., 2013). However, MPA binds to the GR with an affinity (Kd of 4 nM – 11 nM) similar to that of the endogenous glucocorticoid cortisol, and acts as a full to partial agonist for the GR (Bamberger et al., 1999; Zhang et al., 2000; Thomas et al., 2006; Koubovec et al., 2004; Ronacher et al., 2009; Africander et al., 2011), whereas P4, NET and LNG bind to the GR with about a 50 - 100 fold lower affinity and are very weak partial GR agonists with much lower potency and efficacy (Africander et al., 2011; Koubovec et al., 2004; Koubovec et al., 2005; Ronacher et al., 2009; Govender et al., 2014). Recent studies by Africander et al. (2014) suggest that MPA and NET have moderate to high binding affinity towards the AR (using competitive binding assays in COS-1 cells) to a similar effect as the natural ligand, 5α-dihydrotestosterone, as well as exerting differential transcriptional effects via the AR using androgen receptor response element promoter-reporter assays (Africander et al., 2014). Similarly, the same lab reported that NET-A and MPA had relatively low affinity towards the MR, binding with approximately 100 - fold lower affinity than endogenous P4 in competitive binding assays (Africander et al., 2013). As discussed by Stanzcyk et al. (2013) and Hapgood et al. (2013), the binding affinities of progestogens towards the different steroid receptors may not correlate directly with their bioactivity as determined by bioassays or pre-clinical models (such as ovulation inhibition assays in rabbits for progestenic activity and weight gain in the prostate of male rats for androgenic activity), which are prone to inherent biological variability and methodological limitations (Stanczyk et al., 2013; Hapgood et al., 2013). These differences may be due to the presence of other steroid receptors present in the bioassays, unlike the relative bindings assays that are performed in steroid receptor negative cell lines (reviewed in Hapgood et al., 2013). Additionally, ligands that bind with a strong affinity to a steroid receptor, may have weak to no effects on downstream transcriptional effects, such as RU486, a GR/PR steroid receptor antagonist (Hapgood et al., 2013; Ronacher et al., 2009; Africander et al., 2014; Africander et al., 2013).

Some studies have reported the differential biological effects of synthetic progestins using various animal models to study androgenic, glucocorticoid, mineralocorticoid, and progestenic activity (Winneker et al., 2003; Kumar et al., 2000; reviewed in Sitruk-Ware & Nath, 2011; Stanczyk et al., 2013; Africander et al., 2011; Hapgood et al., 2013). For example, MPA has been shown to have strong progestogenic biological activity, weak androgenic activity, full to partial agonist activity via the GR and weak to no MR agonist activity in various animal models as reviewed in Table 1.1. NET, like MPA has been shown to have strong progestogenic agonist activity, with no anti- mineralocorticoid activity reported (Winneker et al., 2003). However, unlike MPA, NET has no glucocorticoid activity, but has partial androgenic effects via the AR. Similarly, LNG, like NET, has strong progestogenic activity, partial androgenic activity, no glucocorticoid

activity and weak anti-mineralocorticoid activity (Kumar et al., 2000; Stanczyk et al., 2013; Africander et al., 2011; Hapgood et al., 2013).

Table 1.1: Biological activity of progestogens in preclinical models and relative binding assays.

Progestogen	Progestogenic	PR RBA (%)	Androgenic	Anti-androgenic	AR RBA (%)	Anti-mineralocorticoid	MR RBA (%)	Glucocorticoid	GR RBA (%)
P4	+	50[a] 100[a]	±	±	03[a] 1[b] 80[b]	+	100[a] 100[a] 9[b]	±	10[a] 11[a] 2[b]
MPA	++	115[a] 298[a]	±	-	5[a] 36[a] 2[b] 150[b]	-	160[a] 3.1[a] 0.08[b]	+	29[a] 58[a] 39[b]
NET	++	75[a] 134[a]	+	-	15[a] 55[a] 134[b]	-	0[a] 2.7[a] 0.07[b]	-	0[a] 1.4[a]
LNG	++	150[a] 320[a]	+	-	45[a] 58[a]	±	75[a] 17[a]	-	1[a] 7.5[a]

Table adapted from a progestin review by Africander et al. (2011) and Stanczyk et al. (2013). ++ Strongly effective, + effective, - no effect, ± Literature not consistent. Data for these effects were obtained from preclinical studies. "For progestational effects, endometrial transformation tests using the McPhail scale, maintenance of pregnancy, inhibition of ovulation and or the assessment of gestagenic activity in female rabbits or rats was performed. Androgenic activity was measured by an increase in the weight of the ventral prostate or levator castrated male rats, anti-androgenic activity was assessed by treating castrated male rats and measuring feminisation-inducing activity thereafter. Glucocorticoid responses were assessed for production of glycogen and tyrosine transaminase production in rats. Anti-mineralocorticoid activity was measured by feeding rats a low sodium diet and sodium and potassium excretion measured (Africander et al., 2011)." Relative binding assays (RBA) were determined by competitive binding assays, and complied by Stanczyk et al. (2013), with additional binding information included for the AR (Africander et al., 2014) and MR binding (Africander et al., 2013). "(a) Were based on competitive binding assays using a radiolabelled reference ligand and increasing concentrations of unlabelled competitor ligand calculating IC_{50} values, while K_i (equilibrium dissociation constant for an unlabelled competitor or inhibitor ligand competing for the radiolabelled reference ligand to the receptor) values were determined by homologous and heterologous displacement using the Cheng-Prusoff equation" (b) (Stanczyk et al., 2013).

The nature and extent to which these steroid hormones exert their biological functions is not only dependent on the concentration of the ligand, but the expression of the steroid receptors within the cells (Hapgood et al., 2013). Hapgood et al. (2013) addressed the effects of changes in steroid receptor and ligand concentrations by modelling the changes in the form of competition binding curves. The authors observed in the models, that increasing the concentration of the receptor significantly changed the apparent affinity (IC_{50}) of the ligand for the receptor (Hapgood et al., 2013). It has also been observed in several studies that some of the steroid receptors exhibit tissue specific regulation. Moreover, it has been observed that within each cell type, different ratios of the steroid receptors and the ratios of their respective isoforms play an integral role in the site specific gene regulation observed (Lu & Cidlowski, 2006; Beato & Klug, 2000; Mahita & Cidlowski, 2013; Gruver-Yates & Cidlowski, 2013). For example, it has been established that the PR is predominantly expressed in the female reproductive tract, mammary gland, brain and pituitary gland (Lu et al., 2006). The PR has two isoforms, the dominant negative PRA and the active form PRB (Africander et al., 2011; Akahira et al., 2002; Richer et al., 2002; Conneely et al., 2002; Conneely et

al., 2001). These isoforms have been shown to be differentially expressed in different tissue types, (Akahira et al., 2002; Tan et al., 2012, Africander 2011, Stanczyk 2013), have differential sub-cellular localisation (Lim et al., 1999) and that the isoforms display gene specific regulation (Beato & Klug, 2000). Similarly, the ER, AR and MR have been found to be expressed in select tissues (Reviewed in Africander et al., 2011; Stanczyk et al., 2013). Like the PR, the ER has two predominant isoforms, ERα and ERβ (Stanczyk et al., 2013). These isoforms also have tissue specific regulation with the ERα predominantly expressed in reproductive tissues while ER β is predominantly expressed in the ovary (reviewed in Faulds et al., 2012). The GR has been found to be expressed in all tissue types (Lu et al., 2006; Mahita & Cidlowski, 2013), and expresses several isoforms (Lu & Cidlowski, 2006; Gruver-Yates et al., 2014), each of which have shown distinct transcriptional expression patterns in *in vitro* models (Lu & Cidlowski, 2006; Oakley & Cidlowski, 2011; Gruver-Yates & Cidlowski, 2013). Thus the context of the cellular environment plays an important role in determining the physiological effects exerted by the progestogens, and could be particularly important when assessing the effects of the progestogens on gene transcriptional effects.

Taken together, the nature and extent to which synthetic progestins affect biological functions, other than their intended reproductive effects, needs to be carefully considered when assessing the choice of contraceptive for women. Moreover, a greater understanding in their underlying mechanism of action needs to be investigated if more informed choices are to be made.

1.1.1. Modes of Action of Steroid Receptors

At the cellular level, progestogens and steroid hormones mediate their effects via alterations in transcription of specific genes via binding to and regulating the activity of steroid receptors (Africander et al., 2011). As seen in Figure 1.2, ligands such as the progestogens are able to diffuse though the plasma membrane from the blood and enter the cytoplasm of the cell (Mahita & Cidlowski, 2013; Moore et al., 2011). Once inside, the ligand is able to bind to its inactive steroid receptor (SR) complexed with chaperones (such as heat shock protein 90) (Mahita & Cidlowski, 2013; Wan et al., 2001; Brinkmann et al., 1999). Ligand-binding to the inactive SR induces conformational changes of the SR and induces the removal of the chaperone complex (Griekspoor et al., 2007). The ligand-bound SR is subsequently hyper-phosphorylated by kinases in the cytoplasm as several sites in the SR. The now active SR is then able to translocate to the nucleus where it can exert genomic effects on target genes. Ligand-bound SRs are able to dimerise and bind directly to specific DNA sequences, known as steroid receptor response elements (Beato & Klug, 2000; Mahita & Cidlowski, 2013; De Bosscher et al., 2010; Moore et al., 2011; Claessens & Tilley, 2014; Williams & Sigler, 1998; Gruver-Yates et al., 2014). DNA binding is mediated by the DNA-binding domain (DBD) of the SR where it recognises specific palindromic sequences associated with the SR (Wan et al., 2001). However, due to the similarity in DBDs of the SRs, it is known that some SREs may be recognised by more than one type of SR (Wan et al., 2001; Griekspoor et al., 2007; Mahita & Cidlowski, 2013; Moore et al., 2011; Africander et al., 2011). Once the ligand-bound SR dimer is bound to its cognate SRE, it is able to recruit several co-factors, known as co-activators to the promoter site, and increase transcription of the

target gene (Moore et al., 2011; Africander et al., 2011; Wu et al., 2005; Brinkmann et al., 1989). This is known as transactivation. Steroid receptors are also able to reduce transcription of target genes (Fuller et al., 2012; De Bosscher et al., 2003; Africander et al., 2011; Moore et al., 2011). The most common method is known as tethering, whereby a ligand-bound SR monomer tethers to other transcription factors, bound to their canonical sites in the promoter of target genes, and inhibits the recruitment of transcriptional machinery to the promoter, thereby reducing gene transcription (De Bosscher et al., 2003). This is known as transrepression. These mechanisms of differential gene transcription by SRs are how these SRs are able to exert their distinct biological effects in the body.

Ligands bound to steroid receptors are also able to mediate rapid differential responses through non-genomic actions (Norman et al., 2004). These responses are independent of nuclear translocation and direct DNA binding of the ligand bound SR to SREs or other transcription factors (Bennett et al., 2010; Mahita & Cidlowski, 2013). These rapid effects occur through the activation of protein-kinase cascades to regulate biological responses (Norman et al., 2004). This includes post-translational modifications, such as phosphorylation, protein-protein interactions with G proteins, second messengers and kinases (reviewed in Banerjee et al., 2014; Bennett et al., 2010; Mahita & Cidlowski, 2013; Mueck et al., 2014; Norman et al., 2004). For example, some studies have found that synthetic progestins bound to membrane-bound PR are able to activate protein-kinase cascades and regulate proliferation in breast cancer cell lines (Ruan et al., 2012; Zhou et al., 2013; reviewed in Mueck et al., 2014). It has also been observed in several studies, that ligand bound GR is able to exert non-genomic actions through the mitogen-activated protein kinase (MAPK) pathway to affect the cardiovascular, immune and neuroendocrine pathways (Widén et al., 2000; Bruna et al., 2003; Solito et al., 2003; reviewed in Mahita & Cidlowski, 2013). Additionally, non-genomic actions via ligand bound ER in vascular endothelial cells has been shown to mediate endothelial cell repair via activation of endothelial NO synthase (eNOS), in the cytoplasm (Cheng et al., 1999; Florian et al., 2004; reviewed in Banerjee et al., 2014).

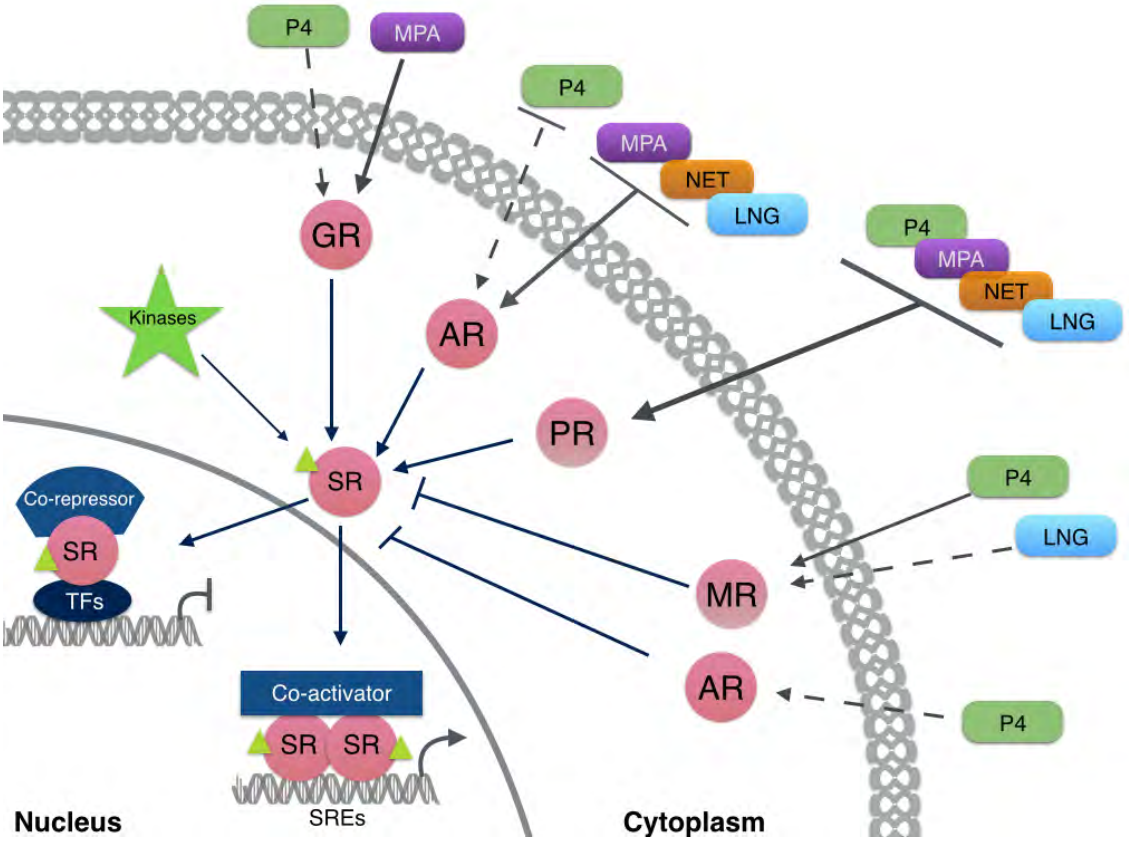


Figure 1.2: Schematic diagram adapted from Stanczyk et al. (2013) illustrating the different genomic actions of progestogens on steroid receptors. As presented in Table 1.1, the different progestogens have different biological activities for the different steroid receptors (SRs). Once the progestogens bind to their target receptors as well as to other SRs with varying binding affinity (see Table 1.1), they exert differential effects on gene transcription. In some cases, the SRs become activated through a series of phosphorylation events performed by various kinases (green stars) in the cytoplasm. Once phosphorylated (denoted by the green triangles), the ligand-bound SR translocates into the nucleus where it can affect changes on gene transcription. Ligand activated SRs can bind as dimers to their cognate steroid receptor response elements (SREs), recruit co-activators and increase transcription (transactivation). Ligand bound SRs can also bind as monomers to other transcription factors, recruit co-repressors, and prevent gene transcription (transrepression). Alternatively, the ligand bound SR may prevent SR activation and subsequently prevent SR-dependent transcriptional effects (e.g. the anti-mineralocorticoid effects that P4 and LNG exert via the MR). P4, unlike the other progestogens, has been found to exhibit both weak androgenic and anti-androgenic effects (Table 1.1). Thick grey arrows denote strong biological activity of the progestogen via the SR, while dashed grey arrows represent weak biological activity of the progestogen via the SR. Blue arrows indicate the order of effects, while blue lines with perpendicular lines at the end represent a blocking effect.

Taken together the data suggests the different progestins may exert differential biological activity through binding to different steroid receptors to different extents, which results in a unique downstream pattern of genomic and non-genomic effects. Of interest to this study is the GR, which discriminates between MPA, NET, LNG and P4 unlike the other SRs.

1.1.1.1 Glucocorticoid Receptor Signalling Pathway

Glucocorticoids regulate numerous physiological functions such as growth, development, stress responses and immune homeostasis (Cruz-Topete & Cidlowski, 2015; Mahita & Cidlowski, 2013), and their expression is under the control of the hypothalamic-pituitary-adrenal (HPA) axis (Zhou & Cidlowski, 2005). Glucocorticoids, like cortisol (CORT) or the synthetic GC dexamethasone (DEX), bind to inactive GR in the cytoplasm. The GR is a modular protein consisting of several domains, the N-terminal transactivation domain, the DNA binding domain and the C-terminal ligand binding domain (Oakley & Cidlowski, 2011). The GR remains inactive in the cytoplasm complexed with several chaperone proteins, including heat shock proteins and several immunophilins (Baschant & Tuckermann, 2010). Upon ligand binding, several conformational changes occur within the GR, resulting in the dissociation of the chaperone complex, and subsequent exposure of the nuclear localisation signal (Oakley & Cidlowski, 2011). While in the cytoplasm, the ligand bound GR is hyper-phosphorylated at several target serine residues (namely, S203, S211 and S226), important in modulating GR activity (Avenant, et al., 2010; Avenant et al., 2010; Beck et al., 2009). The ligand bound GR is subsequently translocated into the nucleus, where ligand bound GR can exert differential effects on gene transcription (Oakley & Cidlowski, 2011). Ligand bound GR can bind as a dimer to target glucocorticoid response elements (GREs) found in the promoter of target genes (Zhou & Cidlowski, 2005). The bound GR dimer recruits several co-activators resulting in positive transcriptional regulation (Mahita & Cidlowski, 2013; Cruz-Topete & Cidlowski, 2015; Newton, 2000). Further, ligand bound GR dimers can bind to negative GREs (nGREs) in promoters of target genes to suppress transcription, however this mechanism is not well defined (Zhou & Cidlowski, 2005). One of the most well studied mechanisms of transrepression is the GR tethering to other transcription factors. Monomeric ligand-bound GR can negatively regulate transcription by interfering either directly or indirectly to other DNA bound transcription factors, like Nuclear Factor κ B (NFκB), signal transduction and activator of transcription (STAT) or activator protein -1 (AP-1) and prevents NFκB, STAT and AP-1 gene dependent transcription (Zhou & Cidlowski, 2005; De Bosscher & Haegeman, 2009; De Bosscher et al., 2008). Further it has been well established that cross talk between the GR and the tumour necrosis factor (TNF) signalling pathway is the major mechanism whereby inflammation is controlled (Van Bogaert et al., 2010; De Bosscher et al., 2003; De Bosscher et al., 2008). It has also been established that there are cell type specific effects of the GR, with glucocorticoids inducing tolergenicity in dendritic cells, and induction of apoptosis in T cells (Baschant & Tuckermann, 2010).

The biochemical evidence and preclinical assays strongly suggest that there are distinct differences in steroid receptor affinity and biological effects for the different progestogens (Hapgood et al., 2004; Africander et al., 2011; Stanczyk et al., 2013). Of importance is the discrimination for MPA by the GR, compared to the other progestogens. This key difference may play a role in the regulation by MPA of immune function associated with the GR. It is likely that MPA could exert much more potent and efficacious effects on gene promoters involved in immune function than P4, NET or LNG via the GR.

1.2. Identifying useful models to study HIV-1 infection

Blish and Baeton (2010) hypothesised that hormonal contraceptives may increase the risk of HIV-1 infection by several different mechanisms. As seen in Figure 1.3, the authors proposed that hormonal contraceptives may change the local mucosal environment, by increasing susceptibility to other STIs or bacterial vaginosis, and/or could change the integrity of the local mucosal epithelial barrier, and/or changing systemic and local immune cells (Blish & Baeten, 2010). Indeed, Mestecky (2007) proposed that HIV-1 infections depend on a number of factors such as transcytosis through the epithelial barrier, and uptake of the virus by target cells, such as CD4+ T cells and dendritic cells (Mestecky, 2007). Furthermore, it has been suggested that HIV-1 is primarily a mucosal disease, as the majority of HIV-1 infections occurs at a mucosal site during vaginal, anal or oral sex encounters (Mestecky, 2007). Upon infection, in the mucosal environment, draining lymph nodes become important sites of viral replication, where CD4+ T cell depletion occurs (Alimonti et al., 2003). As such, if progestogens affect CD4+ and CD8+ T cell distribution and proliferation, they may compromise humoral and cellular responses both in the mucosa and systemically, thereby changing the susceptibility profile to HIV-1 infection (Mestecky, 2007).

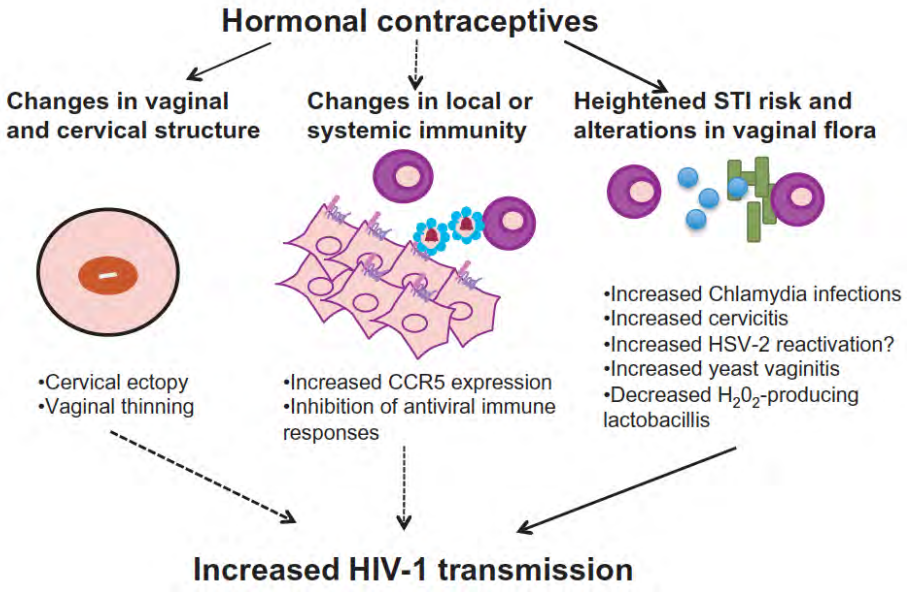


Figure 1.3: Proposed mechanisms whereby hormonal contraceptives may affect HIV-1 transmission. The schematic diagram was taken from a review by Blish and Baeten (2010) (Blish & Baeten, 2010).

HIV-1 entry occurs via binding to CD4+ cells and co-receptor dependent [(C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4)] fusion, predominantly expressed on memory T cells, dendritic cells and macrophages (McDonald et al., 2002; Murphy et al., 2012). The viral envelope protein first binds to the CD4 receptor and undergoes a conformational change to allow it to bind to a second receptor (named the co-receptor) (Cullen, 2001). Predominantly, viral infections occur through binding to the chemokine co-receptor, CCR5, while a small percentage of infections occur via CXCR4 co-receptor binding (Cullen, 2001; Cullen, 2014). The ability of HIV-1 to enter the cell through a specific co-receptor is

known as tropism, and the virus can be defined as being R5-tropic, X4-tropic or dual tropic (Murphy et al., 2012). Co-receptor binding initiates a second conformational change (Cullen, 2001), where HIV-1 fuses into the membrane, where after several events occur. These include virion shedding and reverse transcription of the viral RNA to cDNA, and the insertion of the HIV-1 genome into the genome of the cell (Cullen, 2001; McDonald et al., 2002) through the aid of the viral protein intergrase (Murphy et al., 2012). It appears as though R5-viruses are preferentially transmitted via sexual contact, as the mucosa environment is rich in activated CCR5-expressing immune cells that the virus may infect (Murphy et al., 2012).

The female genital tract (FGT) is a remarkable and intricate environment, consisting of many cell types important in the protection against pathogens. The FGT is sensitive to hormonal fluctuations in the menstrual cycle that change the levels of cytokines, immunoglobulin (IgG) transport, cell type distributions and antigen presentation (reviewed in Wira et al., 2005). The lower female genital tract consists of the ectocervix and the vagina (Miller & Shattock, 2003), while the endocervix is the start of the upper female genital tract (Wira et al., 2014; Wira et al., 2015). Multi-layered squamous epithelial cells cover the ectocervix and the vaginal mucosa and offer mechanical protection against pathogen invasion (Hladik & Hope, 2009). The endocervix is composed of mucus-secreting, simple columnar epithelium covering the lamina propria (Miller & Shattock, 2003). The zone between the ectocervix and the endocervix is a fragile single layered columnar epithelial layer known as the transition or transformation zone, and is thought to be the most susceptible to HIV transmission (Hirbod & Broliden, 2007). The FGT produces a thick hydrophobic glycoprotein mucosa that acts as a physical barrier preventing pathogen entry into cellular targets. Additionally, the FGT produces a plethora of innate antimicrobial peptides and interferons that actively inhibit and prevent pathogen infections (Ferreira et al., 2014). Underlying the epithelial layer is a dense layer of stromal fibroblast cells that provide support and structure. This layer also contains a dynamic distribution of leukocytes (Wira et al., 2015). In order for HIV-1 to establish infection, it must evade and overcome mechanical, chemical and biological barriers present in the FGT (Kaushic et al., 2010). Traversing the epithelial barrier by HIV-1 is in part dependent upon the state of the epithelial barrier (Pope & Haase, 2003), which is subject to weakening due to hormonal changes and ageing or through ulcerations or infections (Iqbal & Kaul, 2007). In addition, it is possible for HIV-1 to traverse intact epithelial layers through mechanisms such as transcytosis (Pope & Haase, 2003; Kaushic, 2011; Ferreira et al., 2014), uptake of HIV-1 by intraepithelial dendritic cells (Margolis & Shattock, 2006) or through the disruption of the tight junctions within the endocervix to allow viral entry (Nazli et al., 2010). Once HIV-1 has passed this barrier, it is able to infect and replicate in CD4+ cells found in the submucosae. While it has been found that HIV-1 does not actively replicate in epithelial cells (Dezzutti & Hladik, 2012; Ferreira et al., 2014), it has been observed in macaques that after initial SIV-1 infection, a small population of resident target cells in the submucosae that have been infected undergo viral expansion, before dissemination into the lymphatic tissues 5 to 6 days post infection (Miller et al., 2005). Hladik et al. (2007) found that the initial resident viral target cells were mainly intraepithelial Langerhan cells (or CD1a+ dendritic cells) and CD4+ T cells in *ex vivo* vaginal organ culture models (Hladik et al., 2007).

Pudney et al. *(*2005) identified cells involved in the cell-mediated immunity in the female genital tract and found distinct populations in each region. The authors found that in the patients observed, most ectocervical tissue samples contained more CD1a+ dendritic cells, CD4+ T cells and CD8+ T cells than the vaginal tissue samples. The authors observed using immunohistochemistry (IHC), that the majority of the lymphocytes observed in the ectocervix and the vagina were located along the stroma-epithelium interface. The transformation zone contained the highest concentration of macrophages, CD4+ and CD8+ T lymphocytes, with CD1a+ dendritic cells located near the ectocervical side of the transformation zone. The authors stated that the ectocervix and the transformation zone could be the most susceptible to HIV-1 infection, due to the abundance of HIV-1 target cells present in these areas. The authors found that the endocervix contained the lowest levels of CD4+ and CD8+ T lymphocytes compared to the vagina, ectocervix and the transformation zone. In addition, the authors found that macrophages but not CD1a+ dendritic cells were observed in the endocervix (Pudney et al., 2005). Interestingly, when inflammation was observed in the patients, the distribution of CD8+ lymphocytes in the vagina was different, with more CD8+ cells observed within the epithelium and an increase in both CD8+ and CD4+ lymphocytes observed in the ectocervix (Pudney et al., 2005).

The data suggest that it is important to use organ culture, or *ex vivo* explants, containing tissue from regions in the lower female genital tract, as it is reflective of the target site of initial infection in heterosexual transmission of HIV-1. *Ex vivo* explant cultures are powerful tools that allow one to answer key questions in HIV-1 infection and potential modulators thereof. Using explants from the ectocervix, endocervix or vagina, researchers have been able to establish HIV-1 distribution within these cell types (Collins et al., 2000; Greenhead et al., 2000). Collins et al. (2000) found that memory T lymphocytes (CD45RO+), dendritic cells and macrophages were present in cervical tissue explants, with memory T cells the most abundant of the cell types assayed. The authors also observed that the cell type distributions did not change over the duration of the time course (6 days) nor did HIV-1 infection alter the cell type distributions. Greenhead et al. (2000) found that that HIV-1 infection (measured by the presence of the viral protein subunit p24) was associated with CD14+CD68+ macrophages and CD3+ lymphocytes located in the sub-epithelial mucosa of the cervical tissue. Interestingly in this study, the authors observed that no p24 positive cells were observed in the epithelium or in epithelial cells (Greenhead et al., 2000). Maher et al. (2005) observed in cervical explants that HIV-1 infected cells were detected 3 to 4 days post infection within the submucosa of the explant. The authors also found that the cervical mucosa contributed to the complexity of infection, with cervical mucous trapping seminal cells (representing cell associated HIV-1) and HIV-1 virions (representing cell free virus). When visualised using confocal microscopy, the authors found that HIV-1 virions were able to penetrate beneath the cervical epithelium and bind to the epithelial surface, despite cervical mucosal trapping of HIV-1 (Maher et al., 2005).

Explants obtained from the lower FGT can be further used to explore the role of select cell types in HIV-1 infection, by digesting the tissue and isolating or purifying different cell types through filtration techniques (Hladik et al., 2007) or by isolating different tissue sections through suction blistering (Hladik et al., 2007). For example, Hladik et al. (2007), processed vaginal explant tissue using suction blistering and isolated the vaginal epithelial sheaths, containing intraepithelial Langerhan and CD4+ T cells. The authors found that exposure to HIV-1 of these vaginal epithelial sheaths, resulted in rapid penetration of HIV-1 into intraepithelial CD4+ T cells and Langerhan target cells, through CD4 and CCR5 receptor mediated binding and endocytosis, respectively (Hladik et al., 2007). Furthermore, utilising purified primary epithelial cells from the endocervix of the FGT, researchers have observed that HIV-1 disrupts the tight junctions formed within the endocervix (Nazli et al., 2010) and utilises transcytosis to cross the epithelial barrier (Ferreira et al., 2014).

Thus, utilising these techniques in primary cervical explants to determine the effects of progestogens on HIV-1 replication in the local genital mucosa will be highly informative and useful. Explant tissue culture is an incredibly useful tool in which to assay the safety and efficacy of microbicides on HIV-1 infection (Fletcher et al., 2006; Fletcher et al., 2008; Fletcher & Shattock, 2008; Dodou et al., 2014; Harman et al., 2012) as well as assessing the effects of entry inhibitors on HIV-1 infection (Hu et al., 2004). However, tissue explants also present challenges when assaying the effects of HIV-1 infection over time. It has been observed that susceptibility to infection may be dependent on the phase of menstrual cycle (Anderson et al., 2010; Carias et al., 2013; Saba et al., 2013). Saba et al., 2013 observed that tissue explants harvested from women in the luteal phase were more permissible to HIV-1 infection compared to tissue harvested from women in the follicular phase (Saba et al., 2013). Thus some tissue explants harvested from donors at different stages of the menstrual cycle, may exhibit different levels of HIV-1 infection levels that may compound the analysis. Furthermore, explant tissue has been shown to be prone to disintegration over time, with the epithelial layer often sloughed within 48 hours of tissue culture (Anderson et al., 2010), that may further exacerbate HIV-1 infection within the culture, compared to samples with an intact epithelium. Additionally, *ex vivo* explants may provide limited information on HIV-1 transmission, due to the difficulties associated with maintaining an intact and polarised epithelium in the explant (Hladik et al., 2007; Anderson et al., 2010; Merbah et al., 2011). Further it is not possible to adequately assess transmission of HIV-1 in the mucosa to target cells using explants, as recruitment of these target cells from surrounding vessels cannot occur in an isolated *ex vivo* tissue explant. As such, most explant studies focus on early HIV-1 infection in this model (Hladik et al., 2007). Lastly, it has been observed that the state of inflammation may play a role in HIV-1 infectivity (Roberts et al., 2012; Masson et al., 2014; Masson et al., 2015). Thus patient information on sexually transmitted infections (STIs) is important in order to make appropriate conclusions about the effect of treatment on HIV-1 infection in *ex vivo* tissue explants.

The role of inflammation is an important determinant of HIV-1 susceptibility (Roberts et al., 2012; Masson et al., 2014; Masson et al., 2015). Explant tissue is host to multiple cell types, as such, a plethora of cytokines,

chemokines and other signalling molecules are expressed (Murphy et al., 2012). Some studies suggest that elevated cytokine levels in the local female genital tract may facilitate viral infection, by recruiting key target cells, like CD4+ T cells for HIV-1 infection, to the local site of infection (Roberts et al., 2010; Masson et al., 2014). Indeed, Greenhead et al. (2000) found that explants treated with PHA to facilitate immune activation enhanced HIV-1 infection (Greenhead et al., 2000), while Pudney et al. (2005) found that more target cells for HIV-1 infection were present in the ectocervix and vaginal biopsy samples from women with inflammation (Pudney et al., 2005). Masson et al. (2014) found that an elevated cytokine profile was associated with women who were chlamydia or gonorrhoea positive. Interestingly, they found that the inflammation was only present in the cervico-vaginal secretions, and did not correlate to the serum cytokine levels in the same women (Masson et al., 2014). This study suggests that there may be compartment specific effects that change the susceptibility profile of women to HIV-1, and that all of these compartments should be assessed in order to elucidate the full effects of progestogens on infection risk in women. Masson et al., (2015) also identified that HIV-1 seroconversion was associated with an increased genital inflammation. The authors proposed that increased levels in macrophage inflammatory factor (MIP)-1α, MIP-1β and interferon-ϒ inducible protein -10 (IP-10) contributed towards this increased association with HIV-1 seroconversion (Masson et al., 2015). Interestingly, this increased inflammatory profile in the young women assayed was not associated with STIs tested (Masson et al., 2015). While these studies suggest that inflammation may be important in increasing HIV-1 infection (Greenhead et al., 2000) or seroconversion (Masson et al., 2014; Masson et al., 2015), the role of inflammation to increase HIV-1 susceptibility has not been fully elucidated. Indeed in a study by Lehman et al. (2014), systemic cytokine levels were not associated with HIV-1 acquisition in women (Lehman et al., 2014).

Upon infection, HIV-1 is disseminated into the lymphatic system through the transport of virus-capturing dendritic cells or infected CD4+ T cells from the mucosa. Once in the lymph nodes, memory CD4+ T cells are predominantly infected and produce the majority of the virus *in vivo* (Pope & Haase, 2003). In addition it has been found that within this population, activation of these cells plays an important role in propagating virus, with activated CD4+ T cells producing 5 fold more virus than resting T cells (Zhang et al., 1999). Interestingly, the authors found that resting T cells may provide the initial target of HIV-1 infection, as well as the initial target cells for latency. After initial infection of resting T cells, activated T cells are recruited through the initiation of immune activation by viral infection, and subsequent viral propagation and dissemination of infection (Zhang et al., 1999). Murooka et al. (2012) found using humanised mice models, that CD4+ T cells that were infected with HIV-1 had reduced motility, formed syncytia and were able to facilitate cell-to-cell virus transfer through the formation of virological synapses. The authors also observed two types of populations of infected CD4+ T cells, those located within the lymph nodes to increase local viral loads, and those that were re-circulated through the tissues promoting efficient systemic viral spread *in vivo* (Murooka et al., 2012). Collectively, results to date suggest that the systemic immune system plays a pivotal role in viral load setup, maintenance of HIV-1 infection and viral dissemination (Pope & Haase, 2003; Haynes & Shattock, 2008). Joag et al. (2014) assessed the effects of a CCR5-tropic HIV-1

pseudovirus on cervical and systemic CD4+ T cells, and found that while viral entry was 3 fold higher in cervical CD4 + T cells compared to systemic CD4+ T cells, viral entry was strongly correlated between the two compartments for each participant (Joag et al., 2014). The authors suggest that this may indicate that there are shared host determinants of viral entry (Joag et al., 2014), which supports the relevance of using systemic PBMCs to study HIV-1 infection.

While CD4+ T cells are important cellular reservoirs for HIV-1, many other cell types contribute to the distribution of HIV-1 to other target sites. HIV-1 has been shown to bind to the surface of dendritic cells to a C-type lectin known as DC-SIGN, or be internalised by endosomes (Murphy et al., 2012). HIV-1 attached dendritic cells are able to move from the submucosa and enter the draining lymph nodes, where they can transfer HIV-1 to CD4+ T cells via an immunological synapse (Murphy et al., 2012). Macrophages have also been shown to be important viral reservoirs in early macrophage-tropic HIV-1 infection (Shen et al., 2009) as well as being important in viral dissemination (reviewed in Fantuzzi et al., 2003). Macrophages and monocytes are important mediators of cellular immunity and produce large amounts of key cytokines such as interleukin (IL) 6, IL8 and tumour necrosis factor (TNF)-α (Fantuzzi et al., 2003), while an increase in IL6 and TNF-α expression has been associated with an increase in HIV-1 viral load (Masson et al., 2014; Roberts et al., 2012; Birx et al., 1990). Some studies utilise the expression of select signalling molecules as markers for cellular activation and function (Huijbregts et al., 2013; Huijbregts et al., 2014; Michel et al., 2015), as such signalling molecules produced by key immune cells could provide insight into cellular activation and function. Roberts et al. (2012) found that a higher viral set point (or viral load) and a decrease in CD4+ lymphocytes, was associated with higher expression levels of several pro-inflammatory cytokines in women, suggesting that changes in the inflammatory profile could alter the susceptibility to infection and disease progression (Roberts et al., 2012). Additionally McKinnon et al. (2015) found that early HIV infection was associated with an increase in expression of the chemokines, regulated upon activation, normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein (MIP)-3α (McKinnon et al., 2015).

Thus, when investigating the effects of progestogens on HIV-1 infection it may be imperative to assay the multicellular effects in *ex vivo* systems from the ectocervix and endocervix as well as in the systemic system using peripheral blood mononuclear cells (PBMCs), as both compartments are important in the viral infection cycle and the progestogens may have distinct effects on gene expression, cell type regulation and HIV-1 replication within these systems.

1.3. Contraception and HIV-1

1.3.1. Observational studies

Contraception is a valuable resource in family planning. Contraception use significantly decreases the mother and infant morbidity and mortality rate, as well as empowering women in choice of family planning

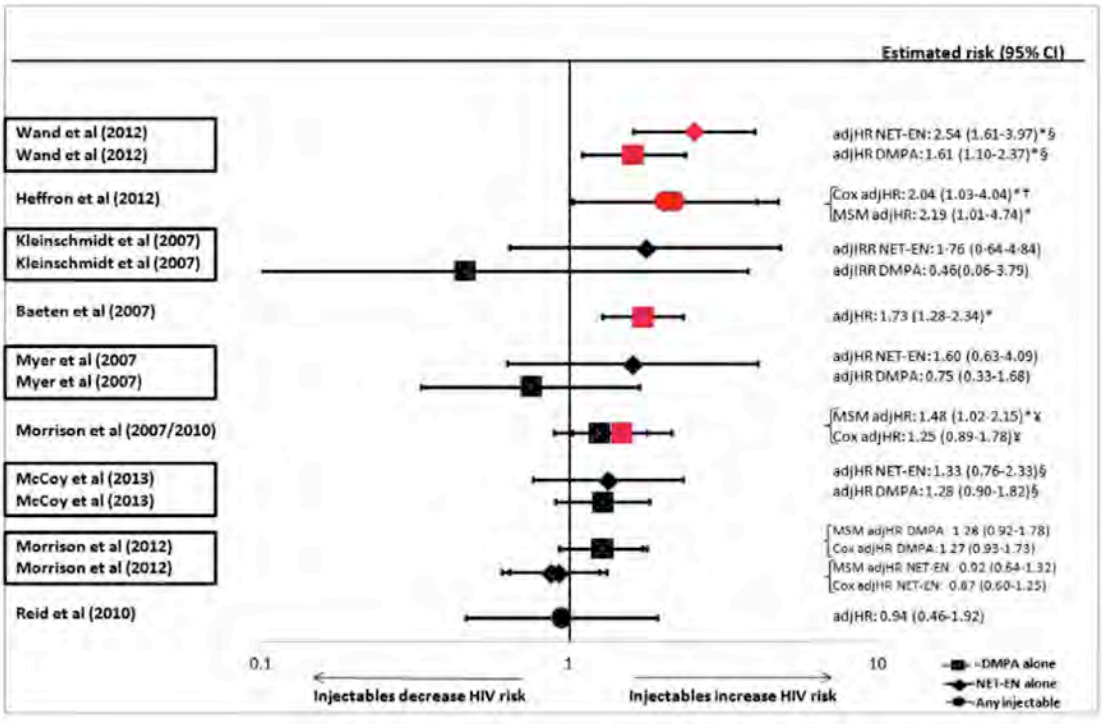
(World Health Organisation, 2014a; World Health Organisation, 2014b). In sub-Saharan Africa, 31 million women do not have access to proper family planning or adequate contraceptive choices (World Health Organisation, 2014a) The importance of contraception for quality of life, freedom of choice, health and economic benefits (Ralph et al., 2015), drives the need to provide safe, cheap and effective contraception options for women. As such, many different progestins have been derived over the past 50 years with equally as many delivery systems (Stanczyk et al., 2013; Sitruk-Ware et al., 2013). However, as previously stated, different progestins administered at different doses via different delivery systems, have different unintended side effects (Hapgood et al., 2004). Of particular concern is the observational evidence over the past 20 years, focusing on the association between hormonal contraception and HIV-1 risk.

The observational studies have produced conflicting observations. Some studies report no association between hormonal contraception and HIV-1 risk (Kleinschmidt et al., 2007; Myer et al., 2007; Reid et al., 2010; Morrison et al., 2012; Lutalo et al., 2013), while other studies report significant associations between hormonal contraception and HIV-1 risk (Leclerc et al., 2008; Morrison et al., 2010; Heffron et al., 2011; Wand & Ramjee, 2012; McCoy et al., 2013). Furthermore, some studies have shown that age and HSV-2 status may have a significant role in HIV-1 susceptibility in women using DMPA (Morrison et al., 2012; Leclerc et al., 2008; Morrison et al., 2010), while others did not (Heffron et al., 2011; McCoy et al., 2013; Wand & Ramjee, 2012). Of the studies that were able to differentiate the different types of injectable contraceptives used, Myer et al. (2007) found that women on COCs, DMPA and NET-EN had similar incidence ratios of HIV-1 infection compared to women not on hormonal contraceptives (Myer et al., 2007). Similar observations were also reported in a study by Lutalo et al. (2013), which found no significant increased risk in HIV-1 acquisition or transmission for DMPA users or COC users compared to no hormonal contraceptive users (Lutalo et al., 2013). In contrast, several other studies have observed an increased risk of HIV-1 infection with the injectable contraceptive MPA, (Leclerc et al., 2008; Morrison et al., 2010). Heffron et al. (2011) found that injectable contraceptives, in particular DMPA, was associated with a two fold increased risk of HIV-1 acquisition. Additionally, the analysis suggested that the rate of transmission from female to male increased two fold if the women were using the injectable contraceptives, compared to women who did not. In this study, the authors found that age and HSV-2 status did not affect the significance of this association (Heffron et al., 2011).

Taken together, the above data highlights the difficulty in addressing the associative risk of hormonal contraception and HIV-1 acquisition. Furthermore, the observational studies to date do not appear to be persuasive enough to definitely address whether hormonal contraceptives affect HIV-1 acquisition in women. Moreover, the studies to date are not detailed enough to address the differences between the type of injectable contraceptives (DMPA and NET-EN), different oral contraceptives (such as progestin-only like levonorgestrel, etonorgestrel, or combined oral contraceptives), or other contraceptive methods (intrauterine devices). Indeed, most often the observation studies were secondary analyses of data obtained to answer other questions, with few that were randomised control trials, specifically designed to

address the question. In addition, most of these prospective studies differed in study design, follow up times, population size and type, sexual behaviour reporting, analytical methods used, rate of discontinuation or switching of contraceptive method and adherence (Smit & Beksinska, 2013; Ralph et al., 2015; Morrison et al., 2015; Haddad et al., 2014; Polis et al., 2014). In response to these inherent differences between the studies, as well as to provide insight into contraceptive choice and HIV-1 risk, recent studies have sought to perform systematic review (Polis et al., 2014) or meta-analyses of all the observational data over the past twenty years (Ralph et al., 2015; Morrison et al., 2015).

A panel of WHO experts comprising of epidemiologists, clinicians and basic research scientists, rigorously assessed all observational studies performed prior to January 2014 using GRADE criteria (Polis et al., 2014). The authors identified 9 key studies that were “informative, but with important limitations”. Of these nine studies, four studies found that DMPA significantly increased HIV-1 risk (Fig. 1.4). One out of five studies that stratified for NET-EN use, found that NET-EN increased HIV-1 acquisition in women (Fig. 1.4). One out of eight studies that reported COC use, reported a significant increased risk with COC use (Polis et al., 2014). Based on this analysis, the WHO recommendation on contraceptive choice for 2014, places no restriction on contraceptive usage, while noting that condoms usage should be highly encouraged for women in high risk areas using long-acting progestins (specifically DMPA) (World Health Organisation, 2014b).



<u>Figure 1.4:</u> The distribution of the adjusted hazard ratios for DMPA and NET-EN use in 9 studies identified as “informative but with important limitations” in the Polis et al. (2014) systematic review.
Red indicates a significant association (p = 0.05). Error bars show 95% confidence intervals (CIs).

Since the Polis et al. (2014) systematic review, several important observational studies have been published. A large prospective study found no association between HIV-1 acquisition and contraceptive

use, as well as no association in contraceptive use and transmission from male to female (Wall et al., 2014b; Wall et al., 2015) or female to male (Wall et al., 2014a; Wall et al., 2015). Crook et al. (2014) analysed hormonal contraception and HIV-1 risk from data acquired from the MDP3-1 microbicide trials. The authors found, after correcting for sexual behaviour, socio-demographic variables, and laboratory findings (HSV-2 status, *Neisseria gonorrhoea*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, syphilis and bacterial vaginosis [BV]), DMPA use associated with a significant increase in risk of HIV-1 acquisition compared to non HC users, while NET-EN and COCs were not. Interestingly in this cohort study, HSV-2 status, age, vaginal discharge, chlamydia and BV were all independently associated with an increased risk in HIV-1 (Crook et al., 2014). Additionally, a recent study by Noguchi et al., (2015) analysing a cohort from the VOICE trials, found that DMPA use was associated with a significant increase in risk in HIV-1 acquisition compared to NET-EN users. However, the study could not estimate the risk associated with either method compared to non-use, due to a lack of a non-hormonal contraception group (Noguchi et al., 2015).

In addition to these new studies, two meta analyses have been performed, whereby key studies were identified, the data accessed and analysed cumulatively (Ralph et al., 2015; Morrison et al., 2015). In Morrison et al. (2015) the authors reviewed and examined individual participant data from several studies. This was performed in order to eliminate some of the methodological limitations of aggregated reviews. This meta analysis sought to address three questions; first if hormonal contraceptive (HC) method increased HIV-1 risk compared to no HC users, second if HSV-2 co-infection had a role in the increase in infection and lastly if there were differences in risk between the different contraceptive types (DMPA, NET-EN and COC users) (Morrison et al., 2015). The grouped data set from 18 studies consisting of 36,973 women of which 26% were on DMPA, 16% on COC and 9% on NET-EN. Of this grouped cohort, 14% of the women discontinued HC between visits, while 13% of the women switched methods multiple times throughout the study (Morrison et al., 2015). Using a two-stage random effects meta-analysis correcting for multiple covariates (age, condom use, region, marital status) the authors found that there was no association with COC and HIV-1 acquisition (aHR 1.03; 95% CI 0.88 - 1.2). DMPA usage had a significant association (aHR 1.51; 95% CI 1.24 - 1.8), while NET-EN usage was weakly associated (aHR 1.24; 95% CI 0.84 - 1.82). The authors found that between-study heterogeneity (that is the differences in study design) was mild (Morrison et al., 2015). In a direct comparisons model, the authors found that DMPA was associated with an increased risk of HIV-1 acquisition, compared to both COC and NET-EN use. In addition, the authors found some evidence to suggest an increased risk of HIV-1 acquisition in NET-EN users compared to COC users (however, this association was not significant). The authors found no association with HSV-2 infection or age on HIV-1 acquisition and hormonal contraception in this data set. Interestingly, the data found stronger associations with DMPA use and increased HIV-1 acquisition in East Africa compared to Southern Africa, or South Africa (Morrison et al., 2015). This could be due to the population type (at risk population versus general population) utilised within the different trials.

Ralph et al. (2015) employed a different strategy in analysing the observational and randomised controlled trials (RCT) data. In this meta-analysis, the authors identified 12 studies and employed inverse probability of treatment weighted marginal structural models in order to obtain pooled hazard ratios that will give insight into the average effect of the treatment on the population (Ralph et al., 2015). Using a series of *a priori* secondary analyses, the authors wanted to identify any studies that disproportionally affected the results, as well to identify if there were population differences in risk profile by stratifying the data sets, as well as to assess whether condom reporting influenced the risk profile obtained (Ralph et al., 2015). Pooled estimates from 10 studies indicated that DMPA increased the risk of HIV-1 acquisition (pooled HR 1.4 95% CI 1.16 - 1.69). Interestingly when correcting for population, there was a lower risk associated with DMPA use and HIV-1 acquisition in the general population (pooled HR 1.31; 95% CI 1.1 - 1.57). In 2 studies where the populations included commercial sex workers and sero-discordant partnerships (that is, the at risk population), the pooled HR increased to 1.73 (CI 1.28 - 2.34). However the authors note that the high heterogeneity between these population groups contraindicated pooled estimates. Additionally the meta-analysis indicated that there was no increase in risk comparing no contraception to COC users (pooled HR 1.0; 95% CI 0.86 - 1.16), or comparing no contraception to NET-EN users (pooled HR 1.1; CI 0.88 - 1.37) (Ralph et al., 2015). The authors found that no one study significantly affected the results obtained. The authors inferred from the data that within the general population, complete withdrawal of DMPA was not warranted. However, the authors strongly support the modelling of regional or geographic and sub-population differences so that context-specific contraceptive policy can be deployed (Ralph et al., 2015). Collectively, a picture is emerging from clinical, studies that the injectable contraceptive, MPA, is likely to increase HIV-1 acquisition in women.

Taken together, the meta-analyses highlight the inherent difficulties and limitations in observational analysis and random control trials. Both Morrison et al. (2015) and Ralph et al. (2015), report that the data is prone to bias and cannot be used to determine whether the association between HIV-1 risk and HC use is causal. Another important factor is that no study has assessed acute HIV-1 infections. This would strengthen the confidence in the timing of exposure to HC and subsequent acquisition of HIV in women, and provide a more causal link in the association (Ralph et al., 2015). That is, that exposure to HIV-1 and subsequent sero-conversion corresponded with HC use. Condom usage may be the greatest confounder in these data sets. If underreporting exists in one group compared to the other, the risk could be over-reported or under-reported (Ralph et al., 2015). In addition it has been reported that women on hormonal contraceptives are less likely to report condom usage than women on no contraceptives (Myer, 2012), which may be a contribution to the observed risk in contraceptive usage. However in the meta-analysis by Ralph et al. (2015) condom use did not greatly affect their outcome (Ralph et al., 2015). In both situations (either under or over-reporting of condom use), one may make policy decisions based on mis-reported risk assessment. Moreover, the data suggests that there could be both geographic (Morrison et al., 2015) and population specific (Ralph et al., 2015) risk factors associated with hormonal contraceptive use. Thus, policy change for the general population may be unwarranted (Jones, 2014; Ralph et al., 2015). Indeed, many are

cautious over general policy change due the significant benefits hormonal contraception have on the health and wellbeing of women (Jain, 2012; Westhoff & Winikoff, 2014; Ralph et al., 2015; Morrison et al., 2015). Jain reported in 2012 that if one were to change from DMPA to OC or no contraception, the increase in unwanted pregnancies and increase in mother and child mortality and morbidity rates outweigh the number of HIV-1 infections prevented in women. Of importance, Jain weighted the analysis so that NET-EN had the same profile as oral contraceptives, thus one could assume that the effect would be the same if moving from DMPA to NET-EN in this scenario. However, Jain (2012) notes, that there are highly variable population effects (different fertility rates in different countries, maternal mortality rates and different proportions of contraceptive use) that could affect the cost-benefit ratios. In addition, the author could not account for factors such as unsafe abortions, neonatal mortality and effects of pregnancy on HIV-1 susceptibility in the analysis performed, which may change the cost-benefit ratios presented in this study. As such, the author proposes region specific assessments, rather than a general policy shift to remove DMPA from the general pool of contraceptives, and promotes individual level planning (Jain, 2012). In line with this, Butler et al. (2013) modelled the effects of removing injectable contraceptives from different countries and its subsequent effect on maternal and HIV-1 related deaths. In this modelling analysis, the authors found that if injectable contraceptives increased HIV-1 incidence with an odds ratio (OR) of 1.2 or 2.19, removing it from the pool of contraceptives would result in 5000 to 27000 (respectively) fewer deaths in South Africa per annum (Butler et al., 2013). However, this was not true for other African and Asian countries, which showed either moderate protect effects or a significant increase in maternal and HIV-1 related deaths per year (Butler et al., 2013). Additionally, the data suggested that injectable contraceptives with an OR of 1.2 or 2.19 would result in 13 000 to 59 000 new HIV-1 infections per year in South Africa alone (Butler et al., 2013). Thus these analyses together with the recent Morrison et al. (2015) and Ralph et al. (2015) meta-analyses are a cause for concern, especially in a South African context, and strongly support the reassessment of DMPA as a suitable contraceptive choice.

Collectively, the data suggest that DMPA more than NET-EN and COCs may increase HIV-1 acquisition in women. HSV-2 status and age were also seen to increase risk in some studies (Morrison et al., 2012; Leclerc et al., 2008; Day et al., 2014; Ralph et al., 2015; Morrison et al., 2015), but not others (Kleinschmidt et al., 2007; Myer et al., 2007; Lutalo et al., 2013). Since the current data from observational studies are not considered persuasive enough to recommend using methods of contraception other than DMPA (World Health Organisation, 2012), more research is urgently required. One approach is to design a large-scale randomised clinical trial. Indeed, some suggest this may provide the clearer picture and provide a more direct analysis over the role of HC and HIV-1 acquisition (Morrison et al., 2015; Morrison & Nanda, 2011). However, many argue that equipoise needed in a trial may be breached in light of the current observational studies, and argue that the funds needed, resources utilised and time taken, may be better utilised in broadening contraception availability and providing regional and population specific policies on contraception use (Jain, 2012; Westhoff et al., 2007; Hapgood, 2013; Ralph et al., 2013; Ralph et al., 2015). Indeed, the ethical considerations and methodological approach to a trial are also complex and

difficult. Another approach is to understand the basic biological mechanisms underlying the differences in HIV-1 acquisition risk with different types of contraceptives. Many state that more basic research is urgently needed, as the exact mechanisms are not known. The advantage of basic research on hormonal contraception and HIV-1 risk, is that time, dose and direct effects may be measured and assessed in appropriate model systems (Hapgood et al., 2013; Michel et al., 2015).

1.3.2. Associated risks, disease progression and susceptibility profiles

In addition to assessing HIV-1 acquisition rates and hormonal contraception use in women, studies have also assessed the effects of hormonal contraception on HIV-1 RNA and DNA shedding, disease progression, inflammatory profiles and susceptibility to other infectious diseases, all of which could contribute to an increased risk in HIV-1 infection. It has been reported in some studies, that hormonal contraceptives (both injectable and combined oral contraceptives) increase HIV-1 DNA shedding in the cervix and vagina (Moss et al., 1991; Mostad et al., 1997; Elovitz & Wang, 2004), while two cohort study found no significant differences in HIV-1 RNA shedding in the cervix or plasma compared to women not on contraception (Richardson et al., 2007; Day et al., 2014). Interestingly, the Mostad et al. 1997 study found that both DMPA and combined oral contraceptive use not only increased HIV-1 DNA shedding, but that this increase was correlated to an increase in CD4+ T cell count in these HIV-1 positive women (Mostad et al., 1997). In a study by Lavreys et al. (2004), women on DMPA had a significant increase in viral set points or viral loads (Log HIV-1 RNA copies/ml) from time of HIV-1 infection to the end of the study, compared to women not on contraceptives. In addition, the authors found that use of DMPA at the time of HIV-1 infection, was associated with higher levels HIV-1 replication. There was no difference in viral set point, or HIV-1 replication levels in women on oral contraceptives compared to women on no contraception (Lavreys et al., 2004). This finding was similar to the Heffron et al. 2012 study, where women who become infected and on injectable contraceptives, had increased genital HIV-1 RNA levels but not plasma HIV-RNA levels, (Heffron et al., 2013). Heffron et al. (2013) suggested that hormonal contraceptives may act to increase HIV-1 infectiousness through direct effects on genital mucosal HIV-1 replication (Heffron et al., 2013).

Several studies to date have found that DMPA use does not increase the rate of disease progression in HIV-1 positive women (Richardson et al., 2007) with one study reporting rates of disease progression significantly lower in women on injectable contraceptives compared to women not on contraception (Heffron et al., 2013). Contrary to these findings, one study has reported that HIV-1 positive women on DMPA or oral contraceptives, but not on antiretroviral therapy, were associated with an increased likelihood of becoming eligible for ART and meeting the authors disease progression outcome (death or becoming eligible for ART) compared to women using an intrauterine device (IUD) (Stringer et al., 2009). Westrich et al. (2014) found that there was no difference in progression to cervical disease when comparing DMPA, NET-EN and COCs compared to no contraception users in HIV-1 positive women (Westreich et al., 2014). Borgdorff et al. (2015) found that OC use was associated with an increase in human papillomavirus (HPV)

and *Chlamydia trachomatis* prevalence, while DMPA use as associated with an increase in HSV-2 prevalence in women (Borgdorff et al., 2015). DMPA usage has also been associated with an increased acquisition of cervical chlamydial and gonococcal infections (Morrison et al., 2004), increased HSV-1 cervical shedding (Mostad et al., 1997) and an increase in cervical ectopy (Morrison et al., 2004; Bright et al., 2011). However, in women who are HIV-1 positive, no differences were observed between STI incidence and hormonal contraception (Overton et al., 2008).

The above data suggest that DMPA and oral contraceptives may have differential HIV-1 infection effects, with more differences observed in susceptibility to HIV-1 than to HIV-1 disease progression. The data also suggest that there may be compartment specific effects on HIV-1 DNA viral shedding as well as differential effects on HIV-1 DNA and RNA shedding within the same compartment, suggesting further complexity on the effects of HC in disease progression. During acute phase infections, DMPA but not oral contraceptives, may have a more pronounced effect on HIV-1 viral loads and CD4+ T cell counts, while this may not be evident in chronically infected women. This data also highlights the lack of information about women on the injectable contraceptive, NET-EN, as well as the lack of information on the type of oral contraceptive associated with increased HIV-1 DNA viral shedding.

It is thus of importance that these apparent differences be further investigated so that appropriate contraceptive choices may be made by women in high risk areas. As the observational data have demonstrated, the inherent confounders may limit observations made. Thus animal studies, *ex vivo* and biochemical studies may provide much needed insight into the differential actions of hormonal contraceptives, the difference in HIV-1 susceptibility and the differential mechanisms thereof.

1.3.3. Animal, *ex vivo* and biochemical studies

As outlined below, evidence suggests that hormonal contraceptives may affect epithelial thickness, barrier integrity, the inflammatory profile, cellular function and gene expression, however greater clarity is needed to determine which hormonal contraceptives have a greater risk profile associated with HIV-1 acquisition. Biochemical studies in cell lines and primary cells as well as animal studies have provided significant insight into the effects on cellular immune function, epithelial integrity and susceptibility to infections of different progestin contraceptives.

1.3.3.1. Epithelial thickness and mucosal barrier integrity

Animal models have proven to be useful in studying the effects of contraceptives and HIV risk, to reduce the confounders present in observational studies (McNicholl et al., 2014). In a seminal study by Trunova et al. (2006), the authors reported a strong association between hormonal contraception and SHIV-1 shedding in cervical secretions in rhesus macaques infected with HIV-1 through the vaginal route, with Rhesus Macaques treated with DMPA having higher viral loads than the no treatment control group (Trunova et al., 2006). Additionally the study found that the genetic complexity of the replicating virus was greater in DMPA-

treated macaques, and that independent of the tropism of the virus (X4 or R5) DMPA treated macaques had higher viral loads (Trunova et al., 2006). A similar finding was reported by Jiang et al. (2009) where pigtail macaques treated with DMPA had higher mean plasma viral loads after the acute phase of infection, and a higher incidence of AIDS-like symptoms compared to the DMPA naive group (Jiang et al., 2009). The natural female hormone, Progesterone (P4), has been shown in macaques to increase SIV-1 susceptibility in macaques by 7.7 fold by thinning the vaginal epithelium (Marx et al., 1996). In a study comparing rhesus macaques with either P4 or estrogen (E2) implants, the authors found that five out of the 6 macaques with P4 implants became infected upon several rounds of SIV-1 intra-vaginal challenges, while the E2 group remained protected (Smith et al., 2000). Interestingly, when the authors infected the macaques at the sub-epithelium layer, the authors found that all the E2 treated macaques became infected, suggesting that E2 blocked infection at the vaginal epithelium or lumen (Smith et al., 2000). Since natural P4 showed similar effects to the hormonal contraceptives, several macaque studies have assessed SHIV-1 risk during the different phases of the menstrual cycles using pigtail macaques, and found that there was an increased susceptibility to SHIV-1 in the luteal phase (high P4) (McNicholl et al., 2014; Vishwanathan et al., 2011; Kersh et al., 2014).

A recent study by Radzio et al. (2014) examined the effects of menstrual cycle and DMPA on SHIV-1 viral shedding, epithelial thickness and density of intraepithelial CD3+ cells. The authors found that with increasing doses of DMPA, vaginal epithelial thickness decreased by up to 80% compared to the follicular phase. Epithelial thickness was reduced in the luteal phase, however it was not significantly reduced compared to the follicular phase. In addition, low doses of DMPA (3 mg) decreased epithelial thickness by 50% for 3 weeks, before returning to similar levels in the follicular phase by week 6. This data suggests that DMPA is more efficacious than the natural ligand P4 at reducing epithelial thickness. DMPA treatment also significantly increased the amounts of CD3+ cells in the vaginal epithelium compared to both the luteal and follicular phase in untreated macaques. Using the low dose of DMPA, which the authors suggest produces similar effects of 150 mg dose in humans; they challenged the macaques with SHIV-1. While the authors found that DMPA treated macaques had higher odds of virus being detected in the serum, there was no significant difference in viral shedding compared to the untreated control group (Radzio et al., 2014). The data from this study highlights the importance of dose, and sampling times. The authors only assessed viral shedding, and not acquisition, thus the data suggests that DMPA may not affect viral RNA shedding in plasma or vaginal secretions after acute infection but it does not provide any information on the likelihood to become infected. Interestingly, DMPA treated macaques exhibited different effects compared to P4 (in the luteal phase) in macaques, suggesting that MPA, more than the natural hormone P4, could change immune parameters and vaginal epithelial thickness to predispose an individual to HIV-1 infection (Radzio et al., 2014).

To elucidate some of the mechanistic underpinnings of how mucosal barrier function could play a role in mediating HIV-1 infection, Nazli et al. (2010) showed that exposure to HIV-1, for 2 - 4 hours, significantly

disrupted the trans-epithelial resistance in primary genital and intestinal epithelial cells. Furthermore in primary genital epithelial cells, the authors found that exposure to HIV-1 increased the permeability of the cells and increased the expression of pro-inflammatory cytokines (Nazli et al., 2010). Further to this, a recent study from Ferreria et al. (2014) suggests that MPA could further exacerbate the risk of HIV-1 infection, by increasing uptake (via endocytosis) of the virus in primary genital epithelial cells. In addition, the authors found that MPA-treated epithelial cells exhibited increased transcytosis of the virus from the apical exposed surface to the basolateral layer, and that when co-cultured with T-cells, MPA-treated conditions had greater infection levels measured in the T-cells than that of the control, P4 or estrogen treated cells (Ferreira et al., 2014). A study in the vaginal epithelial cell line (VK2/E6E7) found that treatment with high concentrations of MPA (388 μM) increased syndecan expression in these cells, resulting in a greater transfer of HIV-1 to T cells compared to the vehicle control. In addition, using the endometrial epithelial HEC-1-A cell line to mimic the epithelial monolayer in the endocervix, MPA treatment (77 and 388 μM) decreased the epithelial integrity (as measured by a decrease in barrier resistance), after 4 days treatment (Irvin & Herold, 2015). The authors also found that this decrease in barrier integrity corresponded to an increase in HIV-1 transmission as measured by an increase in p24 expression levels in the JT-CCR5 indicator T cell line in the basolateral chamber (Irvin & Herold, 2015). The authors suggest that syndecans facilitate the transfer of the virus through the epithelial barrier of the vagina, and could be one mechanism of viral entry in the female genital tract, while HIV-1 could disrupt the endothelial barrier to increase HIV-1 transmission in the cervix, similar to that observed by Nazli et al. (2010).

The data suggest that MPA, more so than P4, significantly reduces epithelial barrier thickness in animal models (Radzio et al., 2014; Marx et al., 1996). It has also been suggested in several macaque studies that the luteal phase may be the most susceptible to SHIV-1 infection (Marx et al., 1996; McNicholl et al., 2014; Vishwanathan et al., 2011; Kersh et al., 2014). As such, the role of progestin contraceptives, which mimic the luteal phase, in HIV-1 acquisition is concerning (Radzio et al., 2014; Kersh et al., 2014; McNicholl et al., 2014). Supporting this is the evidence to suggest that MPA treatment may increase both viral load and genetic diversity of the virus in macaques (Trunova et al., 2006), as well as increasing viral transport mechanisms through the epithelium and into the submucosa, increasing rates of transmission in cell line studies (Ferreira et al., 2014; Irvin & Herold, 2015). Taken together, the data highlights that more research on the effects of different progestins compared to endogenous P4 on barrier integrity, HIV-1 susceptibility and transmission are needed.

1.3.3.2. Mucosal environment, inflammatory profiles and disease susceptibility

It has been observed in several studies that the effects of the menstrual cycle could affect disease susceptibility by altering immune cell populations and subsequent immune responses (reviewed in (Wira et al., 2010; Wira et al., 2014; Wira et al., 2015) and may be in part mediated by the differential levels of P4 and E2 in the follicular and luteal phase. Some studies have observed that natural killer cells, which are important in cell mediated immunity in clearing virus infected cells (Yovel et al., 2001), have higher activity

levels and cell numbers in the follicular phase (high E2, low P4) compared to the luteal phase (high P4, low E2) (Souza et al., 2001; Yovel et al., 2001). Other studies have reported that P4 decreases cell mediated immunity by increasing the population of T regulatory (T reg) cells (which are tolerogenic) in the luteal phase (Weinberg et al., 2011), as well as decreasing the production of Th-1 cytokines (Enomoto et al., 2007; Kaushic et al., 2003; Gillgrass et al., 2003), altering the ability to mount an immune response upon pathogen challenge. It has also been established that the menstrual cycle affects the secretion of immunoglobulins (IgA, IgM and IgG) within the female genital tract (FGT), with the highest amounts produced 2 - 3 days before ovulation, in the follicular phase (Wira & Sullivan, 1985; Kutteh et al., 1996; Franklin & Kutteh, 1999). Furthermore it has been found that the luteal phase has been associated with an increase in HIV-1 RNA viral shedding (Benki et al., 2004), an increase in CXCR4 and CCR5 co-receptor expression (Cabrera-Muñoz et al., 2012; Wira et al., 2005), and a decrease in antimicrobial peptide production (Wira et al., 2015), while pregnancy has been associated with an increase in susceptibility to HIV-1 (Borgdorff et al., 2015; Morrison et al., 2014). Thus it has been proposed, that high progesterone may increase disease susceptibility, including the use of progestogens (Kaushic et al., 2011; Wira et al., 2015).

In order to address the question of how progestogens may alter the genital mucosa in women, several studies have investigated the changes in immune function in the mucosal environment within the female genital tract of animals subjected to progestogen treatment. One study in rhesus macaques suggests that DMPA increases the availability of select HIV-1 target cells and increases the expression of the intergrin $\alpha 4\beta 7$ receptor in these memory CD4+ T cells and dendritic cells (DCs). These cells, with high $\alpha 4\beta 7$ expression are reported to be highly susceptible to HIV-1 infection (Goode et al., 2014). Additionally the authors observed in the endocervix of rhesus macaques, that DMPA treatment altered the expression of Th-1 and Th-2 cytokines, increasing interferon (IFN) – γ, chemokine (C-C motif) ligand (CCL) 4, CCL21 protein expression and decreasing IL6 and IL8 compared to untreated controls (Goode et al., 2014). Interestingly in this study, the authors found that treatment with estrogen (E2) selectively repressed select cytokine expression in the endocervix, while only DMPA had effects on cytokine levels in the plasma (Goode et al., 2014). The authors also reported that the levels of HIV-1 target co-receptor CCR5 were elevated in several cell types upon treatment with DMPA compared to the untreated control (Goode et al., 2014). Importantly, this study suggests that E2 and the synthetic progestin MPA exert differential effects on target cells in a compartment specific manner, and that it is a combination of these factors that alter the susceptibility to infections (Goode et al., 2014).

In mice studies HSV-2 infection was increased by 100 fold in DMPA treated mice while P4 treatment lead to a 10 fold increase. In addition, mice in the estrus phase (high E2), were fully protected against HSV-2 infection (Kaushic et al., 2003). The authors found that several cell types as well as IgA and IgG levels were altered in response to DMPA treatment (Kaushic et al., 2003). Another study found that prolonged DMPA treatment in mice, compared to short treatment, altered the response to effective immunisation to HSV-2,

with long term treatment reducing the ability to mount an effective response to HSV-2 treatment (Gillgrass et al., 2003). The authors found that DMPA decreased the induction of a protective immune response, with a reduction in IFN-ɣ production (Gillgrass et al., 2003). Following this, Miguel et al. 2012, found that DMPA treatment in mice impaired the initial expansion of virus-specific and memory precursor T cells, and dampened the expression select co-stimulatory molecules by dendritic cells necessary to clear the viral infection. The authors also found that these effects were site specific, with most of the observed effects occurring at the mucosal site of infection (Miguel et al., 2012).

Some of these observations on immune function in animal models have been corroborated in prospective cohort studies, when studying the associative risks of HIV-1 acquisition and contraceptive use. Morrison et al. (2014) assessed if certain biomarkers of cervical inflammation in women on contraception, not on contraception or pregnant, were associated with an increased risk of HIV-1 infection. When comparing women who became infected to those that remained uninfected, the authors found that women who became infected had increased levels of regulated upon activation, normal T cell expressed and secreted (RANTES) and beta-defensin (BD)-2 and decreased levels of secretory leukocyte proteinase inhibitor (SLPI). When stratifying for contraceptive use, the authors found that there were distinct patterns of inflammatory markers upregulated in women on COCs compared to women on DMPA. Additionally, the authors found that DMPA use was associated with an increase in RANTES and a decrease in BD-2 levels. While the authors could not stratify between DMPA users who became infected to those that remained uninfected, they suggest that increased RANTES and decreased BD-2 expression levels could explain why they observed an increase in infection in women on DMPA compared to no contraception (Morrison et al., 2012; Morrison et al., 2014). Similarly, Guthrie et al. (2015) assessed the effects of hormonal contraception on changes in anti-viral peptides associated with HIV-1 infection in the female genital tract. The authors found that women on DMPA had higher levels of human neutrophil peptide (HNP)-1-3, human cathelicidin (LL-37) and lactoferrin, compared to women not on contraception. In addition, the authors found an increase in SLPI in COC users compared to women not on contraception, and an increased expression levels in LL-37 and lactoferrin in implant users. HNP-1-3 and LL-37 have been shown to increase HIV-1 replication *in vitro*, by recruiting CD4+ target cells, thus the authors suggest that women on DMPA may have an increased risk in HIV-1 acquisition due to increased levels of HNP-1-3 and LL-37 (Guthrie et al., 2015). Ngcapu et al. (2015) measured secretory protein levels found in the CVL of women who were on injectable contraceptives and found that these women had significantly lower levels of several cytokines (eotaxin, MCP-1, MDC, IL-15), a growth factor (PDGF-AA) and a metalloproteinase (TIMP-2) compared to women not on hormonal contraception (Ngcapu et al., 2015). The authors suggest that women on injectable contraceptives may have a immunosuppressive profile compared to women not on contraception (Ngcapu et al., 2015). However in this study DMPA and NET-EN users were not separated.

In an *ex vivo* study by Chandra et al. (2013), DMPA use significantly increased the number of select activation markers (HLA-DR and CCR5) and receptors (CD45, CD3, CD68) in leukocytes obtained from the

vaginal mucosa 12 weeks post administration, compared to the same women when in the follicular phase and luteal phase. In addition DMPA treatment increased the density of vaginal immune cells (CD3+, CD8+, CCR5+ and HLA-DR+ cells) compared to the luteal phase, and significantly compared to the follicular phase. In this study, the authors found a decrease in vaginal epithelial thickness and a reduction in the number of cell layers upon DMPA treatment compared to both the follicular and luteal phase (Chandra et al., 2013), similar to that observed in macaques (Radzio et al., 2014). In a study by Prakash et al. (2002), CCR5 expression was significantly increased in T cells (both CD4+ and CD8+) isolated from cytobrush samples of women on oral contraceptives compared to women not on contraceptives (Prakash et al., 2002). This study suggests that COCs may increase the risk in HIV-1 acquisition (Prakash et al., 2002). However, since both studies only compare one type of contraception to no contraception, it is not possible to assess if there may be differences between DMPA, COC or other types of contraception (NET-EN, implants, copper IUD). Additionally, in a longitudinal study assessing the effects of DMPA use after one year, DMPA users had a non-significant reduction in vaginal epithelium, and a significant reduction in CD3+ T cells infiltrating the epithelium over the time course evaluated (Mitchell et al., 2014). Interestingly, in this study, CCR5 expression was maintained over time in all groups, unlike that observed by Prakash et al. (2002) and in a macaque study (Goode et al., 2014).

Collectively both animal and observational studies in women, suggest that progestins have significant effects on immune cell function and cell type expression. However the extent to which different progestins at different doses over time affect immune function is still to be determined. Importantly, it must be noted that these studies differ significantly in their time points where they assayed the effects of DMPA on key host responses and functions. In macaques, some studies were assayed at 3 - 5 weeks post injection with DMPA (Goode et al., 2014; Trunova et al., 2006), while for the observational studies in women, samples were taken from 12 weeks post injection (Chandra et al., 2013) to 3 - 12 months (Morrison et al., 2014). Furthermore the differences between the immune response and composition of the FGT in animal models compared to human studies must be taken into consideration when investigating the effects of progestins on immune function. As discussed by McNicholl et al. (2014), rhesus macaques are seasonal breeders with a keratinised lower FGT, as such the effects of progestins may be different in these models compared to women (McNicholl et al., 2014).

Thus, the literature presents an incomplete assessment on the changes in mucosal immune parameters in women and in animal studies upon treatment with DMPA. Since DMPA treatment has peak serum concentrations a few days after injection, before plateauing for 2 - 3 months, it will be of importance to assess changes in HIV-1 susceptibility at the different concentrations throughout the period of DMPA treatment in women, rather than assessing at the end point only. The effects of different concentrations is more feasible to investigate in *ex vivo* and cell line models, where one does not have to repeatedly sample women or animals over a short period of time. In addition, no reported animal studies have compared the different progestins used in contraceptives for the effects on susceptibility to HIV-1 acquisition, nor have

any reports compared changes in humoral and cellular immune parameters thereof. Few of these observational studies in women have compared the effects of different contraceptives in HIV-1 susceptibility (Morrison et al., 2014; Guthrie et al., 2015). However, inclusion of too many types of contraceptives in clinical studies could compromise the power of the study, as observed in the Guthrie et al. (2015) study where the small group of women on oral contraceptives or the implant (Jadelle) prevented the authors from making associations between changes in cationic peptides involved in anti-viral activity and contraceptive use (Guthrie et al., 2015). Lastly, the observational studies are unable to provide a conclusive correlation between changes in immune parameters by progestin contraceptives and HIV-1 acquisition, nor evidence for direct effects on target cells (Morrison et al., 2014; Guthrie et al., 2015) making *ex vivo* studies attractive models in which to assay direct effects of these progestins on various immunological parameters and HIV-1 infection.

1.3.3.3. Differential effects of progestogens on cellular immune function and gene expression

Given that MPA, unlike other progestins, acts as a relatively potent full to partial agonist for the GR, MPA is likely to exert much more potent and efficacious effects on gene promoters involved in immune function than P4 or NET via the GR (Koubovec et al., 2004; Koubovec et al., 2005; Africander et al., 2011). Hapgood et al. (2004) proposed in 2004 that due to the differential activity of MPA versus NET via the GR, MPA is likely to exert different effects on immune function and hence modulate susceptibility to infections like HIV-1 (Hapgood et al., 2004). As mentioned previously, progestins exert their effects via steroid receptors. Koubovec et al. (2004) investigated the effects of MPA, P4 and the synthetic glucocorticoid DEX on GR binding and transcriptional responses. Using L929sA cells (a mouse fibroblast cell line), the authors found that MPA like DEX, repressed TNF-α induced IL6 protein secretion and IL6 and IL8 transcriptional activity, and increased GRE-driven promoter-reporter construct in a dose dependent manner, and that these effects were modulated in part by the GR. Interestingly, both MPA and P4 also appeared to act as partial GR agonists in the transrepression experiments, while P4 had weaker effects on transactivation. The authors also found that MPA induced recruitment of the GR to the nucleus similar to DEX (Koubovec et al., 2004). Following this, the authors investigated the ligand-selective profiles of the different progestins towards the GR. Using HEK293 cells, the authors performed competitive binding assays and found that MPA had a higher RBA for the GR than P4 and NET (Ki of 10.8 nM for MPA compared to 270 and 215 nM for P4 and NET-A respectively). In addition, the authors found that MPA had greater transactivation agonist potency than NET-A or P4, similar to DEX. Similarly MPA, like DEX had greater agonist potency for transrepression than NET-A or P4. The authors also found that MPA induced the phosphorylation of the GR at serine residue 211 (a marker for GR activation) to a much greater extent compared to NET-A and P4 (Koubovec et al., 2005). These two studies show that MPA, unlike NET-A and P4 differentially regulates select GR-target genes via the GR in a concentration dependent manner. This was confirmed in a study by Avenant et al. (2010) investigating the effects of MPA and NET-A on GR phosphorylation at serine (S) 226

and S211, GR half-life and on GR transcriptional responses. The authors found that MPA induced GR S226 and S211 phosphorylation, while NET had minimal effects on phosphorylation. Additionally, MPA induced GR turnover, while NET did not. These results show that MPA, unlike NET, has glucocorticoid-like properties and is able to activate the GR to elicit downstream transcriptional effects (Avenant et al., 2010).

Furthermore, it has been established that the effects of the progestins on GR-target genes are dependent on the concentration of the progestin and the nature of the promoter in target genes. Ronacher et al. (2009) found that there were ligand-selective differences in potency and efficacy for each promoter, as well as for a particular progestin between the promoters assayed. The authors found that in COS-1 cells, MPA behaved as a partial agonist for transactivation, compared to DEX, and a full agonist for transrepression. NET had no activity on any of the constructs tested. In addition, the study found that MPA was more efficacious in transrepression on an AP-1 promoter than an NFκB promoter. In order to further distinguish between the different transcriptional activities exerted by the different ligands, the authors investigated co-factor recruitment. Using a mammalian two-hybrid assay in U2OS cells, the authors found that the co-activators tested, interacted with the GR in a ligand specific manner. MPA increased recruitment of glucocorticoid receptor interacting protein 1 (GRIP-1), steroid receptor co-activator 1 (SRC-1) A, nuclear receptor co-repressor (NCoR)-receptor interacting domain (RID) and silencing mediator for retinoid or thyroid-hormone receptor (SMRT)-RID, while NET weakly recruited these co-activators and co-repressors. The results suggest that NET and MPA have differential effects on co-factor recruitment and supports the idea of differential gene regulation by these ligands on host genes (Ronacher et al., 2009).

Differential gene regulation effects by MPA and NET have been reported in several cell types on endogenous genes. Africander et al. (2011) investigated the relative effects of 1 µM P4, MPA and NET-A on cytokine gene expression in the female genital tract, using two epithelial cell lines (vaginal: Vk2/E6E7 and ectocervical: Ect1/E6E7). The authors found after 24 hours of incubation, that P4 increased TNF-α induced IL6 mRNA expression levels in both cell lines, while MPA and NET had no effect. TNF-α induced IL8 mRNA expression was significantly unregulated by P4 and MPA, but not NET-A in ectocervical cells, while the ligands had no effect on IL8 mRNA expression in vaginal cells. Interestingly, MPA significantly decreased TNF-α induced RANTES mRNA expression levels in ectocervical cells, while P4 significantly increased expression. In vaginal cells, MPA had no effect on RANTES levels, while P4 increased expression. In both cell lines, NET-A had no effect on RANTES mRNA expression levels (Africander et al., 2011). Hadley et al. (2011) found that MPA but not P4 or NET-A, significantly increased glucocorticoid interacting leucine zipper (GILZ) mRNA expression in a lung epithelial cell line (A549), and that MPA significantly recruited the GR to the promoter region of GILZ (Hadley et al., 2011). Irvin and Herold (2015) also observed similar pro-inflammatory effects (in the absence of TNF-α) in the vaginal cell line Vk2/E6/E7 by MPA. The authors found that MPA at high concentrations, dose dependently increased TNF-α, GM-CSF, IL6, IL8, CCL3 and CCL4 mRNA expression levels after five days, and dose dependently increased TNF- α, granulocyte macrophage - colony stimulating factor (GM-CSF), IL6, IL8, CCL3, CCL4 and CCL5 protein

expression levels after 5 days. Interestingly, some cytokines were more sensitive to MPA treatment, with CCL5 expression increasing at lower concentrations of MPA, while increases in CCL4 and CCL3 protein levels were only apparent at the highest concentration of MPA (Irvin & Herold, 2015). Govender et al. (2014) investigated the differential effects, molecular mechanisms of action and the steroid receptor involvement in gene expression by the synthetic progestins MPA and NET compared to the endogenous hormone, P4 in an endocervical cell line model (End1/E6E7). The authors found that 100 nM MPA, unlike NET-A and P4, increased mRNA expression of the anti-inflammatory GILZ and IκBα genes in End1/E6E7 and HeLa cells after 24 hours. Similarly, MPA unlike NET-A, decreased basal mRNA expression of the pro-inflammatory IL6, IL8 and RANTES genes in both cell lines. In these cell lines, MPA behaved as a full to partial GR agonist compared to DEX. In addition, the authors found that after 24 hours of treatment, MPA like DEX, repressed IL6, IL8, RANTES and up regulated GILZ mRNA expression dose dependently, while NET-A like P4 had no effect on gene transcription at any concentration. The authors also found that these responses were mediated via the GR, and that MPA, like DEX recruited the GR to the promoter elements of the genes tested (Govender et al., 2014). Further identifying the role of the progestins in the local mucosa, Louw-du Toit et al. (2014) investigated the regulation of the pro-inflammatory cytokine, interleukin (IL)-12, and the anti-inflammatory cytokine IL-10, by MPA and P4, in Ect1/E6E7 cell lines as a model of the female ectocervical environment. The authors found that MPA and P4 significantly increased the TNF-α induced mRNA expression of IL12p40 and IL-12p35 in a dose dependent manner, and to similar extents. In addition, both P4 and MPA decreased IL10 mRNA expression dose-dependently. Using two different types of siRNA targeting the GR, the authors established that these effects on gene regulation were GR dependent. In addition, the authors found that these GR-dependent effects by MPA and P4 recruited different co-factors to mediate the responses observed (Louw-du Toit et al., 2014a).

These results highlight that the different progestogens, MPA, NET and P4, have differential effects on immune gene expression at different concentrations in different cellular systems. Furthermore, some studies have found that MPA and to a lesser extent, P4, mediate some of their gene specific effects via the GR. Taken together, dose, length of treatment time and cellular compartment have important and significant impact on gene regulation exerted by MPA via the GR. Thus dose, model and time must be carefully considered in the experimental design in order to fully address the effects of progestins on HIV-1 susceptibility.

Few studies have identified the effects of progestins in primary cell models. Tomasicchio et al. (2013) found that MPA, unlike NET and P4, exhibited differential regulation of apoptotic genes and dose-dependently enhanced HIV-1 mediated apoptosis in primary human CD4+ T cells via a GR-dependent mechanism (Tomasicchio et al., 2013). Huijbregts et al. (2013) found that MPA, to a greater extent than P4, suppressed *ex vivo* the production of key regulators of cellular and humoral immunity involved in orchestrating the immune response to invading pathogens such as HIV-1 in peripheral blood mononuclear cells (PBMCs) and vaginal mononuclear cells (VMMCs). In addition the authors found that some of the responses were

mediated most likely via the GR, using the antagonist RU486 (Huijbregts et al., 2013). In addition, the authors found that MPA like DEX increased CCR5 and CXCR4 protein expression in CD4+ and CD8+ T cells (Huijbregts et al., 2013). Hughes et al. (2008) found that plasmacytoid dendritic cells (pDCs) were present in both cervical tissue and in systemic PBMCs. pDCs are key immune sentinels in the anti-viral response, producing large amounts of interferon (IFN)-ɣ that initiate the anti-viral response. When the authors assessed the ability of these cells to produce IFN-ɣ in the presence of the contraceptive MPA and the natural ligand P4, the authors found that DMPA treatment drastically reduced IFN-ɣ expression in pDCs (Hughes et al., 2008). This study suggests that DMPA is able to exert important physiological effects in cell types important in both the local mucosa and in the systemic environment. This was confirmed in Huijbregts et al. (2014) where the authors found that DMPA, but not LNG or NET suppressed IFN-ɣ expression in pDCs (Huijbregts et al., 2014). Further to this, the authors found that MPA, but not NET or LNG, inhibited cytokine production in activated T cells. In addition, MPA but not NET or LNG inhibited the IFN-α and TNF-α induction in pDCs upon exposure to inactivated HIV-1 or CpG (a ligand for the anti-viral response), suggesting that MPA may disrupt the response to infections (Huijbregts et al., 2014). Kleynhans et al. (2011) reported that women using MPA had decreased cytokine protein expression profiles compared to women not on MPA in BCG-stimulated peripheral blood mononuclear cells (PBMCs). In addition, the authors suggest that their findings suggest that MPA affects the systemic immune environment and not just the local mucosal environment (Kleynhans et al., 2011). Following this study, Kleynhans et al. (2013) found that MPA significantly decreased the expression of select cytokines, and that following challenge with BCG in C57BL/6 mice, mice treated with MPA at doses similar to serum concentration in women had higher bacterial loads in their lungs (Kleynhans et al., 2013).

Taken together, these studies show that MPA has significant effects on key immune modulators in both systemic (Huijbregts et al., 2013; Huijbregts et al., 2014; Tomasicchio et al., 2013; Hughes et al., 2008) and local immune environments (Africander et al., 2011; Louw-du Toit et al., 2014b; Govender et al., 2014; Kleynhans et al., 2011; Kleynhans et al., 2013), and has been shown to increase the susceptibility to infections in both mice models (Kleynhans et al., 2011; Kleynhans et al., 2013) and human PBMCs (Huijbregts et al., 2014; Hughes et al., 2008) by modulating or delaying the immune response. However, the extent to which effects of MPA compared to P4, NET and LNG modulate host immune system as well as the direct consequences thereof on HIV-1 infection is yet to be fully elucidated.

1.4. Aims of this study

The aims of this study are to understand the molecular mechanisms underpinning the differential effects by progestogens on select immune gene function in relevant models for systemic and local immunity. In addition, this project aims to elucidate whether different progestogens have differential effects on HIV-1 infection in these relevant biological systems. As mentioned, both local mucosal and systemic immune systems are pivotal in HIV-1 infection, with some progestogens shown to affect immune parameters in both systems. Utilising peripheral blood mononuclear cells from healthy HIV-1 negative individuals representing key targets for HIV-1 infection and replication, as well as *ex vivo* primary cervical explants from women who have undergone hysterectomies for benign reasons representing a key site for transmission, we aim to elucidate mechanisms of differential gene regulation exerted by the progestogens in the different compartments important in HIV-1 infection, and the effects of these progestogens on HIV-1 infection.

This study has focused on three contraceptives used in South Africa, namely the injectable contraceptives MPA and NET, and the progestin used in combined oral contraceptives and implants, LNG. In order to make a persuasive argument to support the reassessment of select progestins used in contraception for women, control ligands are needed to elucidate whether the effects observed by each progestin are different or similar to the natural ligand P4. In addition, since the literature has shown that MPA is a partial glucocorticoid (Bamberger et al., 1999; Koubovec et al., 2004; Koubovec et al., 2005; Ronacher et al., 2009), the synthetic glucocorticoid, dexamethasone (DEX) will be used to infer glucocorticoid effects in these systems. To date, there is little literature comparing progestogens used in contraceptives on immune function in different compartments. This is important, because as Jain (2012) reported, removing a contraceptive from the usable pool, may increase mother and child mortality and morbidity rates (Jain, 2012). As such, it is necessary to compare progestogens so that one can make informed choices over which progestin contraceptive to utilise in areas where HIV-1 acquisition is high.

In order to assess the effects of the progestogens and glucocorticoids on immune function in peripheral blood mononuclear cells (PBMCs) and ex vivo cervical explants, the project focused primarily on 4 key immunomodulatory genes namely, GILZ, IL6, IL8 and RANTES. GILZ is an anti-inflammatory protein that inhibits the activation of pro-inflammatory transcription factors, nuclear factor κB (NFκB) (Di Marco et al., 2007) and activator protein 1 (AP-1) (Eddleston et al., 2007). GILZ is constitutively expressed in many human tissues, including T cells, epithelial cells and mesenchymal cells, and its expression is rapidly up-regulated in response to GCs (Ayroldi & Riccardi, 2009). It has been found that GILZ mediates anti-inflammatory responses similar to that of the anti-inflammatory cytokine IL10 (Berrebi, 2002) and has been found to play a role in the regulation of T helper cell differentiation and dendritic cell function (Ayroldi & Riccardi, 2009). IL6 is an important cytokine involved in mediating T-cell activation, growth and differentiation, and is secreted mainly by dendritic cells and macrophages in response to the activation of pattern recognition receptors (Murphy et al., 2012). Additionally, IL6 is a key cytokine involved in cytotoxic

immunity involved in immune responses directed against virus-infected cells (Borish & Steinke, 2003). It has been observed in two studies that IL6 expression has been associated with an increased risk in HIV-1 susceptibility in women (Roberts et al., 2012; Masson et al., 2014), while one study has indicated that decreased IL6 expression levels were moderately associated with HIV-1 acquisition (Lehman et al., 2014). It has also been found that IL6 protein expression is induced by HIV-1 replication, several days after infection in monocytes and that this correlated with increased viral replication in monocytes (Birx et al., 1990). IL8 is a chemokine that is highly expressed in mononuclear, epithelial, endothelial, macrophages and monocytes (Murphy et al., 2012) and T cells in response to virus-infected cells (Borish & Steinke, 2003). IL8 is a chemotactic factor that recruits neutrophils, basophils and T cells to the sites of infection (Murphy et al., 2012). Additionally, IL8 induces neutrophils to migrate out into the surrounding infected tissue from the blood, making it an important local immunity protein (Murphy et al., 2012). Additionally it has been observed that an increase in IL8 protein levels decreases HIV-1 infection in PBMCs and ectocervical explants (Rollenhagen & Asin, 2010). RANTES (CCL5) is a CC chemokine that is involved in the recruitment of mononuclear cells to sites of chronic inflammation (Charo & Ransohoff, 2006), and is produced predominantly by T cells and endothelial cells (Murphy et al., 2012). RANTES is a chemokine and a ligand for the HIV co-receptor, CCR5 (Cocchi et al., 1995). RANTES protein expression has been shown to directly promote the development of IFN-γ producing Th-1 lymphocytes (Borish & Steinke, 2003), while a decline in RANTES protein levels has been associated with an increase in HIV-1 disease progression (Aukrust et al., 1998). Additionally, RANTES expression has been shown to interfere with the spread of HIV-1 by competitive binding to CCR5, a receptor integral to HIV-1 entry (Cocchi et al., 1995). Some reports have stated that increasing levels of RANTES may lead to an increased outcompeting for the CCR5 co-receptor, thereby reducing HIV-1 infection (Lane et al., 1999). Consequently, it has been found that an increase in RANTES gene expression has been associated with decreasing HIV-1 replication and infection in peripheral blood monocytes (PBMs) (Lane et al., 1999). Interestingly, it has also been found that high protein levels of RANTES has been associated with HIV-1 seroconversion in women and an increased risk in HIV-1 acquisition in women using DMPA (Morrison et al., 2014). In conjunction with mRNA expression studies investigating these genes, select protein expression studies using commercial cytokine Luminex panels will be employed to investigate the effects of these progestogens on key cytokines and chemokines in the inflammatory response.

Additionally, previous research on the biological effects of progestins and P4 has been hampered by a lack of understanding of the significance doses have in determination of physiologically relevant responses (Hapgood et al., 2013; Hapgood, 2013). Unfortunately there is also a lack of information about serum concentrations of progestins in contraception users, which show high inter-individual variability, as well as concentrations in tissues at target sites, which may not mimic serum concentrations (Halpern et al., 2014; Hapgood, 2013). As such, this study aims to elucidate whether the observed effects of MPA, NET and P4 are relevant to the physiological doses of these ligands (Africander et al., 2011; Stanczyk et al., 2013) by dose response analysis to determine potencies and efficacies (Hapgood et al., 2013). It is known that

endogenous P4 serum concentrations vary substantially during the menstrual cycle in premenopausal women and are low during the follicular phase (0.65 nM), rising to about 80 nM during the luteal phase, with higher concentrations of about 600 nM during pregnancy (Africander et al., 2011). MPA serum concentrations are reported to be in the range 2.5 to 65 nM a few days after injection in injectable users (Ortiz et al., 1977; Mishell, 1996; Hiroi et al., 1975; Mathrubutham & Fotherby, 1981) and to plateau at about 2.6 nM for about three months (Africander et al., 2011), while serum concentrations of NET-EN are reported to be in the range 1.5–59 nM (Fotherby, 1981; Africander et al., 2011; Stanczyk et al., 2013). LNG at a 50 µg dose, administered orally, reaches a max serum concentration of 6.4 nM within an hour, before it declines (Sitruk-Ware, 2008). LNG administered in intrauterine devices, reach a serum concentration of 1.6 nM after 6 and 12 months post insertion (Kuhl, 2011), while implant users have peak serum concentration levels of 2.47 nM for the first month before declining to 0.89 nM after 3 years (Sivin et al., 2002). Therefore understanding the effects of the progestogens at relevant concentrations will provide insight into the effects that may occur in vivo, and will inform better contraceptive choice by examining the doses at which contraceptives are administered.

A key question remains as to how do different progestins affect expression of key genes involved in immune function in primary cells relevant to HIV-1 pathogenesis. The Hapgood group proposed in 2004 that due to the differential activity of MPA versus NET via the GR, MPA is likely to exert different effects on immune function and hence modulate susceptibility to infections like HIV-1 (Hapgood et al., 2004). In this study, the aim is to investigate GR involvement in mediating the effects of the progestogens on immune gene modulation in peripheral blood mononuclear cells utilising both antagonist and small interfering RNA (siRNA) techniques.

Lastly, more evidence is needed that compares the effects of the different progestogens on HIV-1 infection in relevant models. Observational (Morrison et al., 2015; Ralph et al., 2015), animal (Trunova et al., 2006; Kaushic et al., 2003) cell line data and some primary PBMCs (Tomasicchio et al., 2013; Huijbregts et al., 2013; Kleynhans et al., 2011; Kleynhans et al., 2013) data has not as yet been persuasive enough to inform policy change in contraceptive use. In addition, these studies have been limited in their choice of progestogens and have not provided clear differential and comparative effects of these progestins on HIV-1 acquisition. As such, the current study seeks to compare the differential effects of HIV-1 infection by the progestogens in both PBMCs and primary cervical explants. Using laboratory strains of X4 and R5 viruses ($HIV\text{-}1_{BaL\_Renilla}$ and $HIV\text{-}1_{pNL4.3}$ respectively) the project aims to understand effects on HIV-1 replication by MPA, NET, LNG and P4 in primary ex vivo explants and primary PBMCs ($HIV\text{-}1_{BaL\_Renilla}$ only). In addition this study aims to understand how these progestins modulate immune gene function over time, and if HIV-1 infection alters the expression of these genes to favour HIV-1 replication in these systems. As has previously been mentioned, inflammation is thought to play a major role in initial infection (Roberts et al., 2012; Masson et al., 2014). As such the effects of progestogens over time on key pro-inflammatory genes

(IL6, IL8 and RANTES) and the anti-inflammatory gene (GILZ) may provide some molecular insight into the relative risks of progestogen use over time, and how this may affect susceptibility to HIV-1 infection.

It is essential to provide clarity around the associative risks of progestin contraceptives on HIV-1 acquisition obtained from observational, animal and biochemical data. As such this study seeks to provide such evidence using relevant models (PBMCs and primary cervical explants) showing that MPA, unlike other progestins and P4 differentially and dose dependently, modulates immune function genes and differentially modulates HIV-1 infection in these systems. In addition the data seeks to provide clarity over the apparent discrepancies in the observational data, whereby MPA is seen to increase HIV-1 acquisition in some cohorts, while not in others (Ralph et al., 2015). It is imperative that there be more effort into providing evidence on contraceptive use and HIV-1 risk. It is important and necessary when one considers that if the observational data is correct, a woman in a high risk area for HIV-1 infection may have a 1.4 increased risk in HIV-1 acquisition (Hapgood, 2013; Ralph et al., 2015). Thus, urgency exists to make informed choices on contraceptives use in high risk areas, and that choice of progestin used in contraception is vital. It may be five years before any data from the ECHO trial (Rees, 2014) is released, in the interim, studies such as this, as well as studies in animal, observational and biochemical data are needed to help make more informed choices now (Jain, 2012; Hapgood, 2013; Morrison et al., 2015; Ralph et al., 2015).

Chapter 2

Methods and Materials

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2.1 Biosafety

Work undertaken in this project was approved by the Science Faculty Biological Safety Committee, The University of Cape Town, South Africa (approval number: BSC021_2014), which included the use of Hazardous Biological Agents and/or Genetically Modified Organisms.

Cell line work was performed under P2 conditions, while primary cell work including PBMC and primary cervical explant experiments and experiments using HIV-1 infectious molecular clones, were performed under P2+ conditions in the Department of Molecular and Cell Biology (The University of Cape Town, South Africa).

2.2. Test compounds, reagents and antibodies

(11b,16a)-9-fluoro-11,17,21- trihydroxy-16-methylpregna-1,4-diene-3,20-dione (dexamethasone; DEX, D4902), 17-hydroxycorticosterone (cortisol; CORT, H0888), 6α-methyl-17α-hydroxy-progesterone acetate (medroxyprogesterone acetate; MPA, D4902), 4-pregnene-3,20-dione (progesterone; P4, P0130), 117α-ethynyl-19-nortestosterone 17β- acetate (norethisterone acetate; NET-A, N6127), 17α-ethynyl-19-nortestosterone (norethisterone; NET, N4128), 13β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one (levonorgestrel; LNG, N2260), and 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one (Mifepristone; RU486, M8046) were purchased from Sigma-Aldrich, South Africa. *In vivo*, both NET-EN and NET-A under go hydrolysis and are converted to NET and its metabolites (Stanczyk & Roy, 1990).

Phytohaemoagglutinin (PHA, L1668) and cycloheximide (CHX, C7698) were purchased from Sigma-Aldrich (South Africa) and interleukin 2 (IL2) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, M5655) was purchased from Sigma-Aldrich, South Africa.

Antibodies to glucocorticoid receptor (GR) (H-300, sc-8992), androgen receptor (AR) (441, sc-7305), mineralocorticoid receptor (MR) (H-300, sc-11412), estrogen receptor alpha (ERα) (MC-20, sc-542), IκBα (C-21, sc-371) and GAPDH (0411; sc-47724) were obtained from Santa Cruz Biotechnology, USA. Antibodies to the progesterone receptor (PR) (NCL-LPGR-312) were purchased from Leica Biosystems (Novocastra, United Kingdom). Secondary antibodies for primary detection were purchased from Santa Cruz Biotechnology, USA, and include anti-mouse (sc-2005) and anti-rabbit (sc-2313). Antibodies for CD4+, (CD4-FITC, 555346) CD14+ (CD14-APC, 345787), IL-6 (IL6-PE, 340527), GILZ (GILZ-PE, 12-4033) for flow cytometry were purchased from Becton Dickinson (BD) Sciences, USA. Isotype controls, anti-mouse IgG (APC, 555751) and anti-rat IgG (PE, 12-4321), were also purchased from BD Sciences, USA.

2.2.1. Incubation with test compounds

All test compounds were made at a stock concentration of 10^{-2} M in absolute ethanol (EtOH; Merck, South Africa), with concentrations confirmed using the respective extinction co-efficient for each test compound in ethanol, where available, and if not the extinction co-efficient of the compound in methanol was used. For dose response experiments, stock concentrations were serially diluted 1:10 in absolute EtOH from 10^{-2} M to 10^{-6} M. All test compounds and vehicle control (absolute EtOH), were added to cells or explant tissue samples at a final concentration of 0.1% (*v/v*), unless stated otherwise. Test compounds were incubated with cells or explants for a variety of time points depending on the experimental question.

2.3. Plasmids

The human MR expression vector, pRShMR, was a kind gift from Ronald Evans (Howard Hughes Medical Institute, La Jolla, USA) and previously described (Arriza et al., 1987) while the human AR expression vector, pSVARo (Brinkmann et al., 1999) was obtained from Frank Claessens (University of Leuven, Leuven, Belgium). The expression vector for the human PR isoform B, pSG5hPR-B (Kastner et al., 1990), was obtained from Stoney Simons Jr (NIH, Bethesda, USA) and the wild type HA-tagged human GR (pCMV-HA-human GR) was a kind gift from Prof. M. J. Garabedian (New York School of Medicine, New York). The expression vector for the human ERα, pSG5-hERα (Denger et al., 2001) was a gift from F. Gannon (European Molecular Biology Laboratory, Germany). An X4 tropic infectious molecular clone (IMC), pNL4-3, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Malcolm Martin (Adachi et al., 1986), and named HIV-$1_{pNL4.3}$ in this study. An R5 infectious molecular clone, HIV-1_{BaL}, that had a luciferase gene inserted adjacent to the *env* gene in the HIV-1 NL4-3 backbone known as NL–LucR.T2A–BaL.ecto, was kindly gifted to us by Dr. Christina Ochsenbauer (Edmonds et al., 2010). For the purposes of this study, the clone was named HIV-$1_{BaL\_Renilla.}$

2.3.1. Transformation of bacterial cells and plasmid preparation

Plasmids were transformed into *Escherichia coli* DH5α cells by heat shock transformations according to Sambrook and Russell, (2006) (Sambrook & Russell, 2006). Ten nanograms of plasmid DNA was incubated with 100 µl of competent *E. coli* cells. The mixture was incubated on ice for 30 min, followed by 2 min incubation at 42°C and 2 min incubation on ice (Sambrook & Russell, 2006). Immediately after transformation, cells were mixed with 900 µl Luria broth (LB) (1% (*w/v*) tryptone, 0.5% (*w/v*) yeast extract, 0.5% (*w/v*) NaCl) and incubated for 1 h at 37°C while shaking at 200 rpm. Cells were subsequently plated out on LB agar plates (1% (*w/v*) tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar) with 100 µg/ml final concentration of ampicillin (Sigma-Aldrich, South Africa) and allowed to grow overnight at 37°C. The following day, 5 ml LB starter cultures with ampicillin (100 µg/mL, final concentration) were inoculated with single colonies for 8 h at 37°C with shaking. For glycerol stocks 500 µl 70% (*v/v*) glycerol was mixed with

500 µl of the cell suspension and stored at -80°C. For plasmid preparations, 200 µl of the starter culture was inoculated into 200 ml LB with ampicillin (final concentration of 100 µg/mL) and incubated at 37°C with shaking for 16 hours. The next day the plasmid DNA was purified from the transformed cells, using the PUREYIELD plasmid maxiprep kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. The yield, purity and integrity of the plasmid prep were determined using a spectrophotometer (NanoDrop®, ND-1000 Spectrophotometer, NanoDrop Technologies), followed by gel electrophoresis. Additionally, confirmation of plasmid type was tested by restriction enzyme digestion (Fermentas, Thermo Scientific, USA) followed by agarose gel electrophoresis. One microgram of purified plasmid DNA was digested with restriction enzymes (Fermentas, Thermo Scientific, USA) for 10 minutes, using the FastDigest™ system with a universal buffer with sample application dye, at 37°C, with a corresponding undigested control. Five hundred nanograms of the undigested and digested DNA was electrophoresed on a 0.8% 1 X TRIS acetate EDTA (TAE) agarose gel (see Appendix D), with 10 µg/ml ethidium bromide (Sigma-Aldrich, South Africa) for 1 hr at 60 V. The gel was visualised at 256 nM on a Syngene, G:Box (Vacutec, England) and images acquired using GeneSnap from synGene, version 7.08 (SynGene, England), where plasmid DNA integrity (supercoiled) and confirmation of the plasmid type was determined. HIV-1_{pNL4-3} and HIV-$1_{BaL\text{-}Renilla}$ plasmids were transformed into *E. coli* and cultured as described above with the addition of carbenicillin (100 µg/ml final concentration; C1389, Sigma-Aldrich, South Africa).

2.4. Cell Culture

2.4.1. COS-1, HEK293T and TZM-bl cell lines

COS-1 (African green monkey kidney fibroblast) cells and human embryonic kidney cells (HEK293T) were purchased from America Type Culture Collection (ATCC, USA). Human cervical TZM-bl cells, were procured from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. (ARP, NIH, USA). The TZM-bl cell line is a HeLa cell line that has been modified to stably express CD4 and CCR5 receptors and contains separate integrated copies of the luciferase and β-galactosidase genes under the control of the HIV-1 promoter (LTR). Cells were cultured in 75 cm^2 flasks (Greiner Bio-one International, Austria) in Dulbecco's modified Eagle's medium [(DMEM) (Sigma-Aldrich, South Africa) supplemented with 1 mM sodium pyruvate (58636, Sigma-Aldrich, South Africa), 44 mM sodium bicarbonate (Sigma-Aldrich, South Africa), 10% (*v/v*) foetal bovine serum (Thermo Scientific, South Africa) 100 IU/ml penicillin and 100 mg/ml streptomycin (P4333, Sigma-Aldrich, South Africa); full DMEM]. All cells were maintained at 37°C in a water jacket incubator (90% humidity and 5% CO_2). Cells were passaged twice a week, with 0.25% (*w/v*) trypsin/ 0.1% (*w/v*) EDTA in PBS (Highveld Biological, South Africa). Trypsinisation was terminated with neutralisation medium (full DMEM). The cell lines were regularly tested for mycoplasma infection by means of Hoechst staining (Freshney, 1987), and only mycoplasma-negative cells were used in experiments.

2.4.2. PBMC Isolation

Buffy packs were obtained from healthy donors who were negative for HIV-1, syphilis and hepatitis B and C from the Western Province Blood Transfusion Services. Ethics for this study was approved by the Human Research Ethics Committee at The University of Cape Town (approval number: HREC 210/2011). PBMCs were isolated using Histopaque (H1077 Hybri-MaxTM; Sigma-Aldrich, South Africa) density centrifugation with Leucosep tubes (Greiner Bio-One, Germany) according to the manufacturer's instructions (Thorsby & Bratlie, 1970). Briefly, 15 ml Histopaque was loaded onto a Leucosep tube and centrifuged (ThermoScientific, swing-bucket centrifuge) for 1 minute at 2500 rpm at room temperature. Whole blood was diluted 1/3 with 50% Roswell Park Memorial Institute medium (RPMI) 1640 (BE12-7012, Lonza, Switzerland), with no supplementation, and 50% phosphate buffered saline (PBS; Sigma-Aldrich, South Africa) and 30 ml of the diluted blood was placed onto the 50 ml Leucosep tube with Histopaque. The sample was centrifuged at 2500 rpm for 15 minutes at room temperature, with the brakes set to 3. After centrifugation, the peripheral blood mononuclear cells separate into a clean white buffy coat, separate from the red blood cells (now present in the Histopaque). The buffy coat was removed with a Pasteur pipette, and washed twice with PBS supplemented with 1% (*v/v*) charcoal-stripped foetal calf serum (c-s FCS) (Thermo Scientific, South Africa). After each wash the samples were centrifuged for 5 minutes at 1200 rpm. During the second wash, 10 µl of the PBMC sample in PBS was added to 90 µl trypan blue (Lonza, Switzerland) and counted on a Haemocytometer to determine cell number and viability. PBMCs were subsequently cultured in high glucose (4.5 g/ml) RPMI 1640 (Lonza, Switzerland) with 10% (*v/v*) charcoal-stripped foetal calf serum (c-s FCS) (Thermo Scientific, South Africa), 2 mM L-glutamine (G7513, Sigma-Aldrich, South Africa), 100 IU/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich, South Africa) at 37°C in a water-jacket incubator (90% humidity and 5% CO_2).

2.4.2.1 Time course and dose response experiments

20 million PBMCs were seeded into sterile 50 ml V-bottomed tubes (Griener, Germany) at a concentration of 2 million/ml in full RPMI, and stimulated with either saturating conditions of ligands or with increasing doses and the tubes placed upright in an incubator, with the lids loosely attached for the duration of the experiment.

For the shorter time course experiments, samples were harvested at 4, 24 and 48 hours. Samples were centrifuged for 5 minutes at room temperature at 400 X *g*, after which the supernatant was discarded, and the pellet was resuspended in 400 µl TriReagent® (Sigma-Aldrich, South Africa) for subsequent RNA isolation (see section 2.5 for RNA isolation method). For the dose response experiments samples were harvested 48 hours after stimulation and 1 ml of the supernatant from each condition was harvested after centrifugation and stored in 2 ml cryovials (Nunc, Germany) at -80°C for subsequent secreted protein analysis (see section 2.8). The pellet was processed for RNA isolation as mentioned above.

For the longer time course experiments, 20 million PBMCs were seeded into sterile 50 ml V-bottomed tubes (Griener, Germany) at a concentration of 2 million/ml in full RPMI containing IL2 at a final concentration of 30 U/ml. PBMCs were stimulated with 100 nM of ligand and tubes were placed upright in an incubator, with the lids loosely attached for the duration of the experiment. Samples were harvested at days 2, 3, 4, 5, 6 and 7 post stimulation, with a half media exchange (containing 100 nM of the appropriate ligand) at days 3 and 6. At each day of harvesting, PBMCs were resuspended through gentle agitation where 300 µl of each sample was removed and used in an MTT viability assay. The remainder of each sample was divided into two new 50 ml tubes, and centrifuged at room temperature for 5 minutes at 400 X *g*. The supernatant from both conditions was discarded and one tube was processed for RNA as described above, while the other tube was processed for protein isolation. For the protein isolation, the pellet was gently resuspended in 1 ml ice cold 1 X PBS and the cell suspension was placed into a new sterile 1.5 ml microfuge tube (Eppendorf, USA) and centrifuged at room temperature for 5 minutes at 400 X *g.* The supernatant was gently removed and the pellet was stored at -80°C until protein isolation (see section 2.6.1).

For the MTT viability assay, 100 µl of the PBMCs was aliquoted into a 96 well plate in triplicate. 5 mg/ml MTT was added to the PBMCs to a final concentration of 0.5 mg/ml MTT. PBMCs were incubated for 2 hours at 37°C in a water jacket incubator. The plate was centrifuged for 5 minutes at 400 X *g*, the supernatant removed and 100 µl of 0.1 N HCl in isopropanol was added to each well and incubated for 10 minutes to ensure complete cellular lysis. The plate was read on a spectrophotometer (Thermo Scientific, USA) at 520 nm and 695 nm (secondary background absorbance). Cellular viability was determined by normalising each sample OD/ml reading to the average vehicle control MTT (OD/ml).

2.4.2.2. Inhibitor and RNA interference experiments

In order to determine GR involvement in ligand-dependent gene regulation, a selective PR/GR antagonist, RU486 and selective GR siRNA knockdown strategies were employed. For antagonist experiments, 20 million PBMCs were seeded at a density of 2 million cells/ml in 50 ml tubes (Greiner, Germany) in full RPMI. PBMCs were treated with vehicle (EtOH), 100 nM DEX or 100 nM MPA in the absence or presence of 1 µM RU486. PBMCs were incubated for 48 hours at 37°C in a water jacket incubator, before harvesting in 400 µl TriReagent®, and stored at -80°C for RNA isolation.

For siRNA knockdown of the GR in PBMCs, 100 million PBMCs were seeded in sterile 50 ml V-bottomed tubes (Griener, Germany) at a concentration of 2 million/ml in 50 ml tubes (Greiner, Germany) and rested overnight at 37°C. The following day, PBMCs were washed once in 1 X PBS and counted, and 15 million cells per condition were used. PBMCs were pelleted and resuspended in 100 µl P3 buffer (Amaxa™ P3 Primary Cell 4D-Nucleofector™ X Kit L, Lonza, Switzerland). Three hundred nanomolar GR5 (GR specific siRNA, Qiagen, Netherlands), or non-silencing control (NSC, Qiagen, Netherlands) was added per condition. Each mix was subsequently aliquoted into an electroporation cuvette (Amaxa™ P3 Primary Cell 4D-Nucleofector™ X Kit L, Lonza, Switzerland) and all air bubbles were disrupted with gentle tapping.

Nucleofection was performed on a Nucelofector 4-D (Lonza, Switzerland) using the Monocyte, human (code EA100) program (Nucleofection occurred at the SUN Immunology Research Group, University of Stellenbosch, South Africa). After nucleofection, 500 µl full RPMI was added to each cuvette. Using Pasteur pipettes, the nucleofected PBMCs were gently transferred to 50 ml tubes (Griener, Germany) and a further 4.5 ml full RPMI was added to each condition. PBMCs were rested for 1 hour, before transporting back to the Molecular and Cell Biology Department, at the University of Cape Town, South Africa. Upon arrival, nucleofected PBMCs were rested overnight at 37°C in a water jacket incubator. The following day, nucleofected PBMCs were stimulated with 100 nM ligands or vehicle for 48 hours at 37°C. In order to confirm GR knockdown, PBMC control samples were harvested 24 hours later and pellets stored at -80°C for protein isolation. After 48 hours, PBMCs were harvested in 400 µl TriReagent and stored at -80°C for subsequent RNA isolation. Initial pilot experiments by members in the Hapgood laboratory, found that samples harvested 48 hours post nucleofection had the greatest knockdown visible, compared to 24 hours post infection, as determined by western blot. As such, in these experiments, confirmation of knockdown was achieved by harvesting two controls (NSC and GR5) to be analysed via western blot at 48 hours post nucleofection, but 24 hours post stimulation with ligands.

2.4.2.3 Cycloheximide experiments

Twenty million PBMCs were seeded into 50 ml culture tubes (Griener, Germany) at a concentration of 2 million cells/ml in full RPMI. Optimisation experiments were performed to elucidate the concentration of cycloheximide needed to inhibit *de novo* protein synthesis, while maintaining cell viability (as assessed by western blot) over 48 hours (data not shown). PBMCs were incubated in the absence and presence of 10 µg/ml cycloheximide (Sigma-Aldrich, South Africa), a *de novo* protein synthesis inhibitor, for 15 minutes at 37°C before the addition of 100 nM DEX, 100 nM MPA or vehicle (EtOH) for 48 hours at 37°C. 10 million PBMCs were harvested in 400 µl TriReagent® (Sigma-Aldrich, South Africa) for subsequent RNA isolation, and the remaining PBMCs were processed as previously described for subsequent protein isolation, and stored at -80°C.

2.4.3. Primary Cervical Explants

Female Genital Tissue (FGT) was obtained from HIV-1 negative, normal Pap smear, pre-menopausal women undergoing hysterectomies for benign reasons, after informed consent. Ethics was obtained from the Human Research Ethics Committee (The University of Cape Town, South Africa) for the duration of this study (HREC 210/2011). Fresh tissue was supplied from two sites in the Western Cape, South Africa, namely Groote Schuur Hospital (under Professor Zephne Van Der Spuy) and Tygerberg Hospital (under Dr. Hennie Botha). Patient consent was obtained before the operation and blood samples and Pap smear information were obtained. Blood samples were sent to the National Laboratory Health services (NHLS, Groote Schuur Hospital, South Africa) to determine HIV-1 and HSV 2 status by serum antibody testing. Additionally, serum luteinising hormone (LH), follicle stimulating hormone (FSH), progesterone (P4) and

estrogen (E2) levels, were obtained to determine phase of menstrual cycle. Menstrual cycle was determined using the guidelines given by the NHLS (South Africa). Most women were older pre-menopausal donors, with most not on any form of contraception at the time of surgery. All of the women were HIV-1 negative and HPV negative (as determined by a normal pap smear), with the majority of the women being HSV-1 positive. Table 2.1 below indicates the average age, HSV-1, HSV-2, HIV-1 status and phase of menstrual cycle. For further donor information, appendix C contains individual donor information including which experiment each piece of tissue was used in.

Table 2.1. The donor information collected during this study.

n = 35	Patient Profile
Average age (in years)	44.1 ± 4.3
Phase of menstrual cycle:	
Follicular	45.7%
Luteal	34.3%
Perimenopausal	14.7%%
Ovulatory	5.3%
Serum ELISA antibody detection:	
HSV-1 Positive	94%
HSV-2 Positive	34.3%

Explant tissue containing the ectocervix and/or the endocervix was processed under P2+ conditions, between 1 to 3 hours post procedure (see Figure 2.1), and processed according to methodology reported in the literature (Greenhead et al., 2000; Fletcher et al., 2006; Richardson-Harman et al., 2009; Fletcher et al., 2005), with a few modifications. The fresh tissue was processed with the epithelial cell layer facing upwards, in the lid of a sterile 15 cm^2 dish with warm full RPMI just covering the tissue, and using a stainless steel punch, 3 mm^3 pieces of tissue were obtained. Pieces were randomly chosen and each tissue explant was placed into 96 well round bottomed plate (Cell Star, Greiner, Germany) using sterile forceps (soaked in 70% EtOH). The explants in these experiments were not polarised or placed onto any gel-foam raft for support. Explants were cultured in 200 µl RPMI (Lonza, Switzerland) supplemented with 10% (*v/v*) charcoal-stripped foetal calf serum (c-s FCS) (Thermo Scientific, USA), 2 mM L-glutamine (Sigma-Aldrich, South Africa), 10 µg/ml Fungizone (Sigma-Aldrich, South Africa), 100 IU/ml penicillin and 100 mg/ml

streptomycin (Sigma-Aldrich, South Africa) and incubated at 37°C in a water jacket incubator (90% humidity and 5% CO_2). For each piece of tissue processed, one 3 X 3 mm piece was immediately placed into a 2 ml cryovial (Nunc, Germany) with 200 µl All-Protect® (Qiagen, Netherlands) at -20°C for future protein isolation. Pilot experiments indicated that 80% cell viability was lost if tissue was frozen and thawed before use (data not shown), as such all experiments in this study was performed on fresh tissue.

After 24 hours in culture, migratory cells such as dendritic cells and Langerhan cells, spontaneously migrate out of the tissue explant, and remain within the 96-well chamber throughout the duration of the experiment. Additionally, it has been observed within the literature, as well as within the duration of this study, that the epithelial sheath detaches from the stroma after 36 – 48 hours after processing (Anderson et al., 2010). However, it was not observed for all tissue explants within an experiment, and the detachment of the epithelial sheath from the stroma appeared to be random.

Three replicates per treatment were used for all cervical explant experiments to ensure reproducibility within an experiment. In addition a cellular viability assay (MTT assay), where possible, was performed upon processing to ensure that the tissue was viable at the time of the experiment. Briefly, 5 mg/ml MTT in phenol free RPMI (Sigma-Aldrich, South Africa) was added to the tissue explant at 10% (*v/v*) final culture volume. The sample was incubated for 2 hours at 37°C in a water jacket incubator. The tissue was removed using sterile forceps, excess liquid was removed (by gently dabbing the explant onto tissue paper) and the explant tissue was weighed to determine the mass (mg). The tissue explant was subsequently incubated overnight in 200 µl of 0.1 N HCl (Merck, South Africa) in isopropanol (Merck, South Africa) in a cryovial (Nunc, Germany). 100 µl of the solution was measured on a 96-well microtitre plate on a spectrophotometer (Thermo Scientific, USA) at 570 nm and 695 nm (background absorbance). Viability was assessed by calculating the OD/mg of tissue. This process was repeated for experiments with long incubation times (more than 48 hours), to ensure tissue viability was maintained for the duration of the experiment (see appendix A Fig. A.5.1).

2.4.2.1. Progestin treatment and dose response experiments

After processing, ectocervical or endocervical tissue explants were treated in triplicate with 100 nM of the ligands (CORT, DEX, MPA, P4, NET or LNG) or with increasing concentrations of MPA with a vehicle control for each experiment (0.1% (*v/v*) EtOH), for 48 hours. This time point was chosen based on the optimisation experiments performed in PBMCs where MPA exerted maximal repressive effects on select mRNA gene expression after 48 hours post incubation (see chapter 3, Figure 3.1). After 48 hours, the supernatant was harvested, into a new round bottomed 96-well plate, and centrifuged for 5 minutes at 400 X *g.* The supernatant was removed and stored in a flat bottomed 96-well plate, sealed, and stored at -80°C for future use. For subsequent RNA isolation, individual explant pieces were placed into new 2 ml cryovial tubes (Nunc, Germany), where 600 µl TriReagent® (Sigma-Aldrich, South Africa) was added into each tube. Additionally 200 µl TriReagent® was added into each well and incubated for 5 minutes at room

temperature. Thereafter, the lysate was removed and added to into each corresponding cryovial tube, to ensure that migratory cells present in the well were harvested for RNA. Samples were stored at -80°C until processed for RNA.

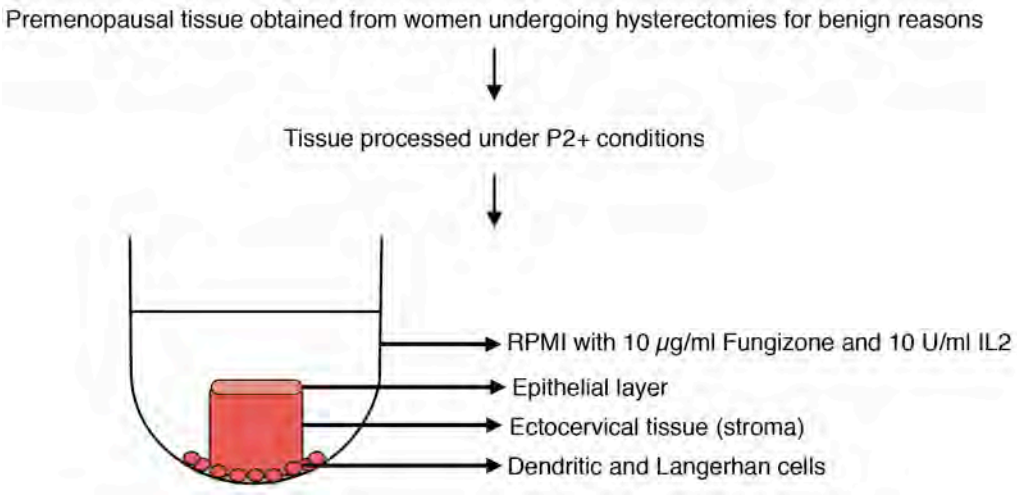

Figure 2.1: Diagram representing the culturing of a piece of ectocervical tissue in a 96-well for experimental purposes. Premenopausal tissue was processed under P2+ conditions using a sterile punch to produce 3 X 3 mm pieces of tissue that contained both the upper epithelial layer and the underlying stroma. Each tissue piece was placed into a single well of a 96-well plate and cultured in 200 µl of media (full RPMI with Fungizone and IL2). While the diagram depicts the tissue explant with the epithelial side up, this was not mimicked in culture experiments, where pieces were randomly placed into each well.

2.5. Infectious Molecular Clones (IMCs)

Initial viral stocks were prepared according to Pear et al. (1993) with a few modifications (Pear et al., 1993). HEK293T cells were seeded at a density of 4 X 10^5 cells/well in a 10 cm^2-well plate in high glucose (1 g/ml) full DMEM at 37°C in a water jacket incubator (90% humidity and 5% CO_2). The next day, media was changed to full phenol red-free DMEM and the cells were transfected with 20 µg HIV-$1_{pNL4.3}$, HIV-$1_{BaL\text{-}Renilla}$ or a control (DMEM) using X-tremeGENE 9 DNA transfection reagent (Roche Applied Science, South Africa) according to the manufacturer's specifications. Cells were incubated for 60 hours at 37°C, the medium was passed through a 0.22 µM filter and charcoal- stripped (c-s) FCS (Thermo Scientific, USA) was added to a final concentration of 40%. The viral stocks were aliquoted and stored at -80°C until use.

Virus titres were determined using the TZM-bl assay as described in Edmonds et al. (2010), with a few modifications (Edmonds et al., 2010). Briefly, TZM-bl cells were seeded at a concentration of 1 X 10^5 cells/ml into a 96-well plate in full DMEM. The next day, virus was serially diluted 1 in 5 in full phenol-red free DMEM (Sigma-Aldrich, South Africa) for 8 dilutions, with a no virus control. The media was aspirated from the TZM-bl cells, and cells were washed once with PBS. Thereafter 200 µl of the serially diluted virus in full phenol-red free DMEM was added. Cells were harvested 72 hours later with 120 µl Bright-Glo luciferase lysis buffer (Promega, USA). To ensure complete cellular lysis, cells were incubated in the dark for 5 minutes before the supernatant was removed and added into a while 96-well luminometer plate

(Griener, Germany). Fluorescence was determined on a luminometer (Modulus Microplane, Promega, USA), where relative light units were measured for each well. Each dilution was performed in quadruplicate. The titre of the virus stock was determined using the Reed Muench method and expressed as log infectious units (IU)/ml (Reed & Muench, 1938). Initial viral titres between 100 – 500 IU/ml were routinely produced for both viruses.

Higher titre viral stocks were prepared according to methods learned at Imperial College, London in 2011 (from Professor Robin Shattock's laboratory) with a few modifications. In order to produce high titre viral stocks for all subsequent virus experiments, 50 million PBMCs at a concentration of 2 million cells/ml in full RPMI were activated 2 ways to ensure maximum cellular activation. PBMCs were stimulated with either 5 µg/ml (final concentration) Phytohaemoagglutinin (PHA) and 30 U/ml (final concentration) interleukin-2 (IL2) or with or 0.5 µg/ml PHA and 30 U/ml IL2 in 50 ml tubes (Griener, Germany) and incubated upright at 37°C in a water-jacket incubator. After 3 days, cells from each condition were washed 4 times with 1 X PBS. Cell numbers were subsequently determined and PBMCs from each condition were pooled equally (20 million cells form each activation condition). Pooled PBMCs were centrifuged for 5 minutes at 1200 rpm and 20 ml of full RPMI with 30 U/ml (final concentration) IL2 was added so that PBMCs were at a concentration of 2 million cells/ml. One hundred infectious units per ml seed virus was used to infect activated PBMCs for 2 hours, before washing 4 times with 1 X PBS and replacing media. PBMCs were incubated for 7 days at 37°C in a water jacket incubator. The supernatant containing the virus was collected into a new 50 ml tube and centrifuged for 5 minutes at 1200 rpm at room temperature. The supernatant (~20 ml) was subsequently collected and passed through a 0.22 µM filter disk into a new 50 ml tube where c-s FCS (Thermo Scientific, USA) was added to a final concentration of 40%. The viral stocks were aliquoted into 2 ml cryovials (Nunc, Germany) and stored at -80°C until use. Viral titres were determined as previously described using the TZM-bl assay, where viral titres between 1000 – 2500 IU/ml were routinely produced. A control group of PBMCs (RPMI only) was included for every batch of virus produced and mock infected with the DMEM control obtained from the initial HEK293T virus preparation and processed in the same manner described above.

2.5.1. Infection experiments in PBMCs

One hundred million PBMCs at a concentration of 2 million cells/ml were activated with either 5 or 0.5 µg/ml PHA for 24 hours in T75 culture flask (Griener, Germany), as previously described above. After activation, PBMCs were, aliquoted into 50 ml tubes (Griener, Germany) and washed once with 1 X PBS (Sigma-Aldrich, South Africa) supplemented with 1% cs-FCS (Thermo Scientific, USA). Cell number was determined by adding 10 µl of the PBMC in PBS solution to 90 µl trypan blue solution (Lonza, Belgium) in a 1.5 ml microfuge tube (Eppendorf, USA). Thereafter, 10 µl of the solution was added to a haemocytometer, and cell number calculated by counting the trypan blue negative cells under a light microscope at a 10X objective lens magnification. PBMCs were centrifuged at room temperature for 5 minutes at 400 *X g* and re-

suspended in full RMPI with a final concentration of 30 U/ml IL2. In order to pool the activated PBMCs, 40 million cells from each activation condition was added into a new T75 flask (Griener, Germany), where full RMPI with 30 U/ml IL2 was added to a final concentration of 1 million cells/ml.

For each condition, 5 million cells in 5 ml media was incubated in 12 ml culture tubes (Griener, Germany), upright with loose lids, with the appropriate ligand, for 48 hours at 37°C in a water jacket incubator. For these experiments, 100 nM concentrations of ligands were used, with the dilution such that the final concentration of ethanol was 0.01%. This was performed as optimisation experiments indicated a slight ethanol effect at 0.1% (*v/v*) on renilla luciferase readings compared to a no treatment control. After 48 hours, PBMCs were infected with 10 IU/ml HIV-$1_{BaL\text{-}Renilla}$ or with a mock infection control (RPMI only) for 2 hours at 37°C. PBMCs were washed 4 times with 1 X PBS supplemented with 1% cs-FCS. Thereafter, full RPMI with IL2, containing ligands at the indicated concentrations was added PBMCs were incubated for a further 7 days at 37°C, with half media exchange at day 3 and day 5 post infection. Briefly, 2.5 ml media was removed, and replaced with 2.5 ml full RPMI with IL2 and the appropriate concentration (100 nM) of ligand for each condition. PBMCs were harvested at day 7 post-infection for renilla luciferase expression, cell viability using MTT and RNA isolation. Optimisation experiments were performed where it was observed that reproducible and measurable levels of renilla (RLU) were detected at day 7 post infection compared to day 5 post infection (see appendix E, Fig. E.1.2 B and C). In addition it was found that cell viability decreased over this time course, but differences between day 5 and 7 post infection were negligible (see appendix E, Fig. E.1.2 A). Thus in these experiments, day 7 post infection was chosen as the harvest time point. Additionally, pilot infection experiments were performed in the absence and presence of IL2. However, low levels were detected at day 7 post infection in the absence of IL2 (see appendix E, Fig. E.1.1). Thus all the experiments were performed in the presence of IL2.

PBMCs from each condition were re-suspended by gentle agitation; where after 1200 µl was removed for the renilla luciferase and MTT viability assay. For detection of renilla luciferase expression, 200 µl of the PBMCs was aliquoted into round bottomed 96 well plates (Griener, Germany). Each condition was aliquoted out in triplicate, and the 96 well plate centrifuged at room temperature for 5 minutes at 400 X *g* to pellet the cells. The supernatant was removed and discarded, and 100 µl of 1 X reporter lysis buffer with renilla luciferin (Promega, USA) was added to each well, and incubated in the dark for 10 minutes at room temperature to ensure complete cellular lysis (this was confirmed by visually examining under a light microscope). Samples were added to a white luminometer plate (Qiagen, Netherlands) and read on a luminometer (Microplate Modulus, Promega, USA) to determine the relative light units (RLU). For the MTT viability assay, 200 µl of the PBMCs were aliquoted into a round-bottomed 96 well plate in triplicate where 5 mg/ml MTT was added to the PBMCs to a final concentration of 0.5 mg/ml MTT into each well. PBMCs were incubated for 2 hours at 37°C in a water jacket incubator. The plate was centrifuged for at room temperature for 5 minutes at 400 X *g*, the supernatant removed and 100 µl of 0.1 N HCl in isopropanol was added to each well and incubated for 10 minutes to ensure complete cellular lysis. The plate was read on a

spectrophotometer (Thermo Scientific, USA) at 520 nm and 695 nm (background absorbance). Infection was calculated by dividing the RLU obtained for each sample by the average MTT (OD/ml) for that sample group. This was performed to account for the changes in cellular viability over time exerted by the different ligands on the PBMCs. Once these values were obtained, relative infection was calculated by setting vehicle control (EtOH) to 100% infection.

The remainder of the PBMCs in each tube was centrifuged at room temperature for 5 minutes at 400 X *g*, the supernatant removed, and 400 µl TriReagent® (Sigma-Aldrich, South Africa) was added. Samples were stored at -80°C until RNA isolation was performed.

2.5.2. Infection experiments in primary cervical explants

Primary ectocervical tissue was processed as described in section 2.3.3 and incubated in 200 µl full RMPI with 10 U/ml IL2 (final concentration) with 100 nM final concentration of ligands (section 2.1.1) in a round bottomed 96 well plate (Griener, Germany). The infection experiments were performed according to Arakelyan et al. (2013), with a few modifications (Arakelyan et al., 2013). Samples were incubated for 48 hours, before being exposed in a non-polarised manner with either HIV-$1_{BaL\text{-}Renilla}$ or HIV-$1_{pNL4.3}$ at 500 IU/ml at 37°C in a water jacket incubator. Briefly, each tissue explant, was removed using sterile forceps, and added to a 24 well plate (Griener, Germany). Each triplicate group was added to one well in the 24-well plate. HIV-$1_{BaL\text{-}Renilla}$ or HIV-$1_{pNL4.3}$ with a mock infected group (RPMI only) was added to a final concentration of 500 IU/ml in a final volume of 500 µl, with the remainder of the volume made up with the media, containing ligands that the explants were incubated in for the 48 hours previously. To ensure reproducibility between infection experiments, virus stocks were diluted to the same concentration (in full RMPI) so that the amount of media added was constant (and thus the same concentration of cytokine milieu present per infection). Explants were washed 4 X with 1 X PBS (with 1% (*v/v)* cs-FCS) at room temperature in the 48 well plate and each explant was subsequently added back to the 96-well plate using sterile forceps (dipped in 70% EtOH between each treatment group, and used after EtOH was evaporated). A final wash with 1 X PBS (with 1% (*v/v)* cs-FCS) was performed in the 96-well plate. Thereafter, 200 µl per well of full media with the appropriate concentration of ligand and IL2 was added to each well and explants were incubated for a further 10 days post infection. Half of the media (100 µl) was removed at days 3, 5 and 7 post infection, and replaced with 100 µl of new media containing IL2 and 100 nM final concentration of the appropriate ligand for each treatment group. Media harvested at days 0, 3, 5, 7 and 10 post infection was aliquoted into a flat bottomed 96 well plate (Griener, Germany) and incubated with 5% (*v/v*) Empigen® (B13 Detergent, 45165, Sigma-Aldrich, South Africa) in 1 X PBS (D8537, Sigma-Aldrich, South Africa) for an hour at room temperature, to ensure viral inactivation. Thereafter, the supernatants were sealed with plate sealers and stored at -80°C. Culture supernatants were subsequently assayed for HIV-1 $p24_{Gag}$ release using a sensitive p24 ELISA assay (Innotest, Innogenetics, Belgium). Briefly, culture samples inactivated with a 5% (*v/v*) Empigen® (Sigma-Aldrich, South Africa) solution, were further diluted 1:10 with a buffer supplied by the ELISA kit (Matrix Buffer, Innogenetics), before proceeding with the ELISA. Samples were

read on a spectrophotometer (Thermo Scientific, USA) at 420 nM and 595 nM (background absorbance). p24 concentrations were calculated using the equation generated by the standard curve, and relative infection was calculated by setting vehicle control at day 3 post infection (EtOH) to 100%. After 10 days post infection, the tissue explants were harvested in 800 µl TriReagent® in 2 ml cryovials and stored at -80°C for subsequent RNA isolation. Where possible, an MTT assay (described in section 2.3.3) was performed on a single piece of tissue (not infected with HIV-1) to determine cellular viability at the end of the experiment (see appendix A, Fig A.6.1).

Optimisation experiments found that infecting the explants in a larger volume and concentration of HIV-1 yielded more reproducible results (see appendix E. Fig E.2.2 B). Additionally, due to the high amount of starting virus needed to productively infect the cervical explants tissue, it was not possible to individually infect each tissue sample with the viral titres (1000 – 2000 IU/ml) produced throughout this study. Thus the approach as described above was taken for the virus experiments to reduce variability as far as possible.

2.6. RNA Isolation, cDNA synthesis and PCR

2.6.1. RNA isolation

PBMCs harvested in 400 µl TriReagent® (T9424, Sigma-Aldrich, South Africa), were processed for RNA according to the manufacturer's instructions. Briefly, PBMCs in TriReagent® were incubated at room temperature for 5 minutes, before centrifugation at 12 000 *X g* (4°C) to pellet cellular debris. The supernatant was transferred into a new microfuge tube. Two hundred microlitres of chloroform per 1 ml TriReagent® was added to each sample and vortexed for 30 seconds. Samples were centrifuged for 15 minutes at 20 000 *X g* at 4°C. The aqueous phase was carefully removed and transferred into a new microfuge tube. Where less than 10 million cells/ml were used per incubation, 1 µl of RNAse-free glycogen (Roche Applied Science, South Africa) was used to precipitate the RNA from the aqueous phase to better identify the RNA pellets. Five hundred microlitres isopropanol (Merck, South Africa) per 1 ml of TriReagent® used (200 µl), was added to the samples and gently mixed by inversion. Samples were incubated for 10 minutes at room temperature to precipitate the RNA. Samples were subsequently centrifuged at 20 000 *X g* for 10 minutes at 4°C to pellet the RNA. The supernatant was removed, and the pellet was washed once in 70% EtOH (in DEPC-treated H_2O). Samples were centrifuged at 20 000 *X g* for 5 minutes at 4°C and the supernatant was carefully removed to ensure that the majority of the EtOH had been removed. Samples were air dried for 5 minutes and the pellets were resuspended in 10 µl RNase-free DEPC treated H_2O and stored at -80°C.

Primary cervical explants in 800 µl TriReagent® in 2 ml cryovial tubes (Nunc, Germany) were homogenised using a hand-held homogeniser (TissueRuptor®, Qiagen, Netherlands) with disposable probes (TissueRuptor Probes, Qiagen, Netherlands). Samples were homogenised for 20 second pulses, for up to 1 minute on ice. Homogenates were transferred to new microfuge tubes and incubated for 5 minutes at room

temperature and centrifuged at 12 000 *X g* for 10 minutes at 4°C. The remainder of the procedure was followed as described above for PBMC RNA isolation, including the addition of RNase-free glycogen for maximal RNA pellet visibility. Several RNA isolation kits were used in the initial optimisation experiments for RNA isolation (Qiagen Allprep RNA/DNA/Protein, Qiagen RNeasy Nano Kit, and Machery Nagel XS-RNA isolation kit). However, no kit produced sufficient RNA yields for subsequent downstream applications (see appendix E).

RNA was assessed for quantity and quality on a spectrophotometer (Nanodrop, Thermo Scientific). RNA was assessed for integrity using denaturing formaldehyde agarose gel electrophoresis (Sambrook & Russell, 2006). Sample loading buffer (see Appendix D) was added at a ratio of 3:1 (*v/v*) to 250 ng RNA for tissue samples and 500 ng RNA for PBMC samples. The samples were analysed by gel electrophoresis on a 1% agarose formaldehyde gel (see Appendix D) and electrophoresed for 25 minutes at 65 V in 1 X Morpholinopropanesulfonic acid (MOPS) buffer (see Appendix D) (Sambrook & Russell, 2006) and visualised on a Syngene, G:Box (Vacutec, England) and images acquired using GeneSnap from synGene, version 7.08 (SynGene, England).

2.6.2. cDNA synthesis

Total RNA was reverse-transcribed using the Transcriptor First Strand Synthesis cDNA kit (Roche Applied Science, South Africa) according to the manufacturer's instructions. Briefly, 250 ng (primary explant samples) or 500 ng (PBMC samples) RNA was reverse-transcribed using anchored oligo (dT) priming in a final volume of 10 µl. cDNA samples were stored at -80°C until use in subsequent real time qPCR reactions.

2.6.3. Conventional PCR

Conventional PCR was performed using GoTaq DNA polymerase (Promega, USA, M3001) with the steroid receptor specific primers (Table 2.1) according to the manufacturer's specifications. The cycling parameters for 40 cycles are shown in Table 2.1. PCR samples were electrophoresed on a 1.5% agarose 1 X TAE gel (See Appendix D) at 70 V for 1.5 hours and visualised on a Syngene, G:Box (Vacutec, England) and images acquired using GeneSnap from synGene, version 7.08 (SynGene, England).

2.6.4. Real Time quantitative PCR

Real-time quantitative PCR (qPCR) was performed using the Bioline SensiMix™ SYBR® no ROX kit (QT650-05, Bioline USA) on a RotorGene 3000 (Qiagen, Netherlands) real time qPCR machine, according to manufacturer's instructions. Each 20 µl reaction in a 0.1 ml strip-tube (SSI, USA), contained 1 X Bioline SensiMix™(Bioline, Germany), forward and reverse primers (see Table 2.2 for primer sequences and concentrations), RNase free H_2O and 1 µl template cDNA. The qPCR profiles was established by Verhoog et al. (2011) for IL6, IL8, RANTES and GAPDH, while the steroid receptor primers were optimised for this study and described in Tomasicchio et al. (2013). Each profile contained a standard initial denaturation at

95°C for 5 minutes followed by 40 cycles of 95°C for 8 sec, annealing for 10 sec and 72°C for 8 sec, a final elongation step at 72°C. A melt curve analysis was performed after the endpoint (1°C increase/5 sec from 65°C to 95°C). Annealing temperatures can be found in Table 2.2. Data was acquired using the RotorGene Software (version 1.7) with melting curve analysis to confirm the amplicon generated in each sample. In addition, real time qPCR samples were electrophoresed on a 2% agarose (1 X TAE) gel at 70 V for 1.5 hours and visualised on a Syngene, G: Box (Vacutec, England) and images acquired using GeneSnap from synGene, version 7.08 (SynGene, England), to confirm product sizes. Standard curves for efficiency determination were performed for each primer set, with pooled cDNA. Pooled cDNA was serially diluted 1 in 2, five times, and the dilutions used in triplicate reactions for subsequent standard curve determination. Standard curves that had efficiencies (E) between 1.9 - 2.1, slopes (M) between -3.4 and -3.8 and R^2 values between 0.95 and 1 were deemed acceptable. Relative transcript levels were determined using the "Fit Points" method described by Pfaffl et al. 2001 and were normalised to relative GAPDH transcript levels (Pfaffl, 2001).

Primers for the steroid receptors were designed using the Primer-BLAST function on the NCBI BLAST platform (www,ncbi.nlm.nih.gov/BLAST). Primers were designed to be intron spanning, to produce product sizes between 150 – 300 bp, have low self and inter-complementarity and to have an annealing (TA) temperature between 50-65°C. Forward and reverse primers were designed towards the following sequences obtained from the NCBI database: GR: NM_000176.2, AR: NM_000044.2, ER: NM_000125.3, MR: NM_000901.4 and PR: NM_001202474.1. All other primers used in this study were also intron spanning, and confirmed with the Primer-BLAST platform using sequences obtained from the NCBI database (IL6: NM_000600.3, GILZ: NM_198057.2, IL8: NM_000584.3, RANTES: NM_002985.2 and GAPDH: NM_002046.5).

Table 2.2: Primer sequences and specifications used in qPCR

Primer Name	Sequence	Exon position F	Exon Position R	Final Conc.	TA	Product size	Reference
GILZ	Quantitect Primer QT00091035	2	3	1 X	60°C	69	Qiagen, Netherlands
IL6	F: 5' - TCTCCACAAGCGCCTTCG - 3' R: 5' - CTCAGGGCTGAGATGCCG - 3'	1,2	2	250 nM	60°C	193	(Wolf et al., 2002)
IL8	F: 5' - TGCCAAGGAGTGCTAAAG - 3' R: 5' - CTCCACAACCCTCTGCAC - 3'	2	3	250 nM	60°C	197	(Wolf et al., 2002)
RANTES	F: 5' - TACCATGAAGGTCTCCGC - 3' R: 5' - GACAAAGACGACTGCTGG - 3'	1	2,3	250 nM	60°C	199	(Wolf et al., 2002)
GAPDH	F: 5'- TGAACGGGAAGCTCACTGG-3' R: 5'-TGTCAGTTGATAAAACCGCTGCC-3'	8	9	200 nM	60°C	307	(Ishibashi et al., 2003)
GRα	F: 5'- TGCTGTGTTTTGCTCCTGATCTG-3' R: 5'-TGTCAGTTGATAAAACCGCTGCC-3'	9	10	250 nM	53°C	299	This study
PR A and B	F: 5'- GTGCTCAAGGAGGGCCTGCCG-3' R: 5'- TGTGCTGCCCTTCCATTGCCC-3'	2	3	250 nM	65°C	214	This study
MR	F: 5'- GAGCAGTGGAAGGGCAACAC-3' R: 5'- TGGCTGCTCCTCGTGAATCC-3'	4	5	250 nM	60°C	182	This study
ERα	F: 5'- TCGACGCCAGGGTGGCAGAG-3' R: 5'- TGGTGCACTGGTTGGTGGCTGG-3'	7	8	250 nM	60°C	218	This study
AR	F:5'-CAGGAAAGCGACTTCACCGCACC-3' R: 5'ATCAGGCAGGTCTTCTGGGGTGG-3'	3	4	250 nM	60°C	209	This study

2.7. Western Blotting

2.7.1. PBMC Protein Isolation

Protein isolation was performed according to Tomasicchio et al. (2013) with a few modifications. Briefly, PBMCs (8 million) were centrifuged at room temperature for 5 minutes at 400 X *g,* and pellets stored for 30 minutes at -80°C. Pellets were incubated on ice with 24 µl 1 X N-[TRIS (hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) buffer (0.1 M TAPS, pH 9.5) lysis buffer [1 X TAPS buffer with 1 mM (final concentration) PMSF, 5 µg/ml Leupeptin (Roche Applied Science, South Africa), 2 µg/ml Aproptinin (Roche Applied Science, South Africa) and 1 X completeMini protease inhibitor tablet (1 tablet/ 10 ml buffer; Roche Applied Science, South Africa)], was added to each pellet. Samples were mixed by vortexing every 10 minutes for a total incubation time of one hour, before centrifugation at 12 000 X *g* for 15 minutes at 4°C. Supernatant was collected into a new microfuge tube, where 5 X protein sample buffer (100 mM TRIS-HCl pH 6.8, 5% (*w/v*) SDS, 20% (*v/v*) glycerol, 2% β-mercaptoethanol and 0.1% (*w/v*) bromophenol blue), was added to a final concentration of 1 X (*v/v*) to each sample. Samples were incubated at 100°C for 7 minutes,

stored on ice for 1 minute, and the full volume collected by brief centrifugation. Samples were stored at -20°C until use.

2.7.2. Explant Protein Isolation

Individual primary cervical explants were harvested in either 1 X Qiagen buffer RLT (All Prep, Qiagen, Netherlands) or in 800 µl TriReagent® (Sigma-Aldrich, South Africa). The isolation methods were optimised in this study (appendix E, E.2, Table E.2). The following methods produced the best quality protein. Samples were homogenised on ice using a tissue rotor-homogeniser at full speed (TissueRuptor, Qiagen, Netherlands) for 20 seconds (for up to an accumulated time of 1 minute, dependent on tissue).

Samples in Qiagen buffer RLT, were processed for protein isolation according to the manufacturer's instructions using the Qiagen AllPrep isolation kit (Qiagen, Netherlands). Samples were resuspended in 80 µl 1 X reporter lysis buffer (E397A, Promega, USA) with 20 µl 1N NaOH, and incubated at 50°C for 10 minutes. Samples were subsequently vortexed for an hour at 4°C to ensure complete pellet resuspension. After pellet resuspension, samples were analysed for total protein concentration using the Bradford method of protein quantitation. Samples were stored at -20°C until use. Before SDS-PAGE gel electrophoresis, 20 µg total protein per sample was removed and aliquoted into a new 1.5 ml microfuge tube after which 5 X protein sample application buffer was added to a final concentration of 1 X. Samples were subsequently incubated at 100°C for 7 minutes and stored on ice for 1 minute, before use in SDS-PAGE gel electrophoresis.

Samples that had been processed for RNA (section 2.6.1) were subsequently processed for protein extraction, using the phenol fraction of the isolation, according to the manufacture's instructions (TriReagent, Sigma-Aldrich, South Africa). Briefly, protein samples from the TriReagent fraction, were washed 4 times in 0.1 M guanidium hydrochloride (Merck, South Africa) in 95% EtOH, resuspended in 40 µl 1 X reporter lysis buffer (E397A, Promega, USA), and incubated at 50°C for 10 minutes to ensure complete resuspension. Ten microlitres of 5 X protein sample application buffer was added, to a final concentration of 1 X, samples incubated at 100°C for 7 minutes before being stored on ice for 1 minute, and the full volume collected by brief centrifugation. Samples were stored at -20°C until use.

2.7.3. Generation of Positive Controls

For the generation of AR, ERα, MR, GRα and PR B positive controls for western blotting, COS-1 cells were seeded at a density of 1 X 10^5 cells/ml in a 6 well plate in full DMEM for 24 hours at 37°C in a water jacket incubator (90% humidity and 5% CO_2). Cells were transiently transfected with 1 µg of a nuclear receptor expression vector using X-tremeGENE 9 (06365809001, Roche Applied Science, South Africa) according to the manufacturer's instructions. After 24 hours incubation, cells were harvested in 50 µl of 2 X protein sample buffer, and incubated at 100°C for 7 minutes. Samples were subsequently stored on ice for a minute, and the full volume recovered by centrifugation. Positive controls were confirmed by western

blotting using the appropriate nuclear receptor specific primary antibodies. Samples were stored at -20°C until use.

2.7.4. Western Blotting Protocol

Western blotting was performed according to Avenant et al., (2010). Protein samples were separated by SDS-PAGE at 75 V for 20 minutes followed by 120 V for 1 hour at room temperature in 1 X running buffer [25 mM TRIS-HCl, 250 mM glycine and 0,1% (*v/v*) SDS, pH 8,4 (Sambrook & Russell, 2006)], using a Bio-Rad Mini Protean II electrophoresis set (Bio-Rad, South Africa). Proteins were subsequently electroblotted onto Hybond-ECL (AEC Amersham Biosciences, South Africa) nitrocellulose membrane for 1 hour at 4°C in transfer buffer (25 mM TRIS, 200 mM glycine, 10% (*v/v*) methanol) at 180 milliamps. Membranes were subsequently incubated in 4% (*w/v*) ECL advance blocking powder (AEC Amersham Biosciences, South Africa) in 1 X TRIS-buffered saline (50 mM TRIS, 150 mM NaCl; TBS) containing 0.1% (*v/v*) Tween (TBS-Tween) for 1 hour at room temperature, on a shaker at 65 rpm. Primary antibody was diluted (see Table 2.3 for specific dilutions) in 4% (*w/v*) ECL in 1 X TBS-Tween was added overnight at 4°C on a shaker at 65 rpm. Three 5 minutes washes with 1 X TBS-tween were performed to remove non-specific primary antibody binding. Blots were subsequently incubated with the corresponding secondary antibody (see Table 1 for specific pairings and dilutions) for 1 hour in 5% (*w/v*) skim milk powder in 1 X TBS-Tween at room temperature. Washes were repeated as before with 1 X TBS-tween, followed by one 5 minute wash in 1 X TBS. Membranes were incubated in ECL-chemiluminescent substrate (Thermo Scientific, USA) for 1 minute before being visualised using autoradiography, with varying exposure times (see figure legends for length of exposure). Autoradiography and ECL visualisation were performed with Amersham Hyperfilm™ MP high performance autoradiography film. Autoradiograms were scanned for quantification at 600 dpi in grayscale on a flatbed scanner (Hewlett Packard, USA). Autoradiograms were quantified using AlphaEaseFC™ Software, version 3.1.2 (Alpha Innotech, USA).

For consecutive detection steps, membranes were stripped in stripping buffer (100 mM β-mercaptoethanol, 2% (*v/v*) SDS, 62.5 mM TRIS-Cl, pH 6.7, (Sambrook & Russell, 2006)) for 30 min at 65°C, washed 2 X 10 min at room temperature with TBS-Tween and blocked for 1 h in 4% blocking solution at room temperature before repeating antibody incubations as described above.

Table 2.3: Dilutions of antibodies used in western blot analysis

Primary Antibody	Size (kDa)	Dilution	Secondary Antibody
GRα	95	1: 2000	Rabbit (1: 10 000)
PRB/PRA	114/94	1: 500	Mouse (1: 4000)
AR	110	1:1000	Rabbit (1: 10 000)
ERα	68	1:500	Rabbit (1: 10 000)
GAPDH	37	1:15 000	Mouse (1: 4000)
IκBα	55	1: 500	Rabbit (1: 10 000)

2.8. Fluorescence activated cell sorting (FACS) by Flow Cytometry

Flow cytometry was performed as described in Tomasicchio et al. (2013) with a few modifications. Five hundred thousand PBMCs (at a concentration of 2 million/ml) in full RPMI were placed into 5 ml Becton-Dickinson Falcon tubes (BD Scientific, South Africa). PBMCs were subsequently stimulated with ligands or vehicle for 48 hours, and tubes were placed upright, at 37°C in a water jacket incubator. In order to measure secreted cytokines intracellularly, Brifaldin A (Sigma-Aldrich, South Africa), a secretion inhibitor, was added to the PBMCs at a final concentration 10 µg/ml 6 hours prior to processing the samples (Baran et al., 2001). Compensation and antibody titrations were performed by Dr. Michele Tomasicchio in the Hapgood Laboratory (Tomasicchio et al., 2013). After treatment, PBMCs were surface stained with 20 µl/million cells anti-CD4 fluorescein isothiocyanate (FITC) (Becton-Dickinson, USA, 555346), 20 µl/million cells and anti-CD14 allophycocyanin (APC) (Becton-Dickinson, USA, 555399) antibodies per ml PBS, in order to discriminate between cellular populations in PBMCs, at room temperature for 20 minutes in the dark. In addition, cells were incubated with a viability marker, 7-AAD (Becton-Dickinson, USA), and only viable populations were examined for protein expression. After surface staining, PBMCs were permeabilised in 1 X permeabilisation solution (Becton-Dickinson, USA) for 15 minutes at room temperature in the dark, and washed twice with 1 X perm wash solution (Becton-Dickinson, USA). PBMCs were subsequently stained with either 1.25 µl/million cells GILZ-PE (Becton-Dickinson, USA) with a corresponding isotope control (anti-rat IgG-PE) or IL-6 (Becton-Dickinson, USA) with a corresponding isotope control (anti-rat IgG-PE), and incubated for 30 minutes at room temperature in the dark. PBMCs were washed once with 1 X perm wash solution (Becton-Dickinson, USA), and resuspended in 500 µl 1 X

Cell Fix solution (Becton-Dickinson, USA). Samples were acquired using a Becton-Dickinson FACS Calibur flow cytometer (Becton-Dickinson, USA) and analysed using Flo Jo software (version 7.1, Treestar, Inc, Ashland, Ore). Relative fold change in expression levels was calculated by setting vehicle control (EtOH) expression to 1.

2.9. MSD® ELISA and Luminex®

Supernatants obtained from PBMC and explant samples incubated for 48 hours with ligands, were stored at -80°C. PBMC dose response supernatants were individually stored in cryovials and sent to Harvard University (Brigham & Women's Hospital, Boston, USA), to Professor Raina Fichorova's laboratory, where Meso Scale Delivery (MSD®) ELISA was performed. Relative fold expression of IL-8, IL-10, TNFα and VEGF was obtained by setting vehicle control (EtOH) to 1. Supernatants obtained from primary ectocervical explants and PBMCs, were processed for cytokine and chemokine secretion using a Bio-Rad Pro™ Human Cytokine Group 1 panel (27-plex) Luminex (Bio-Rad, Germany), according to the manufacturer's instructions. Data was obtained on a Luminex 200 pro (Bio-Rad, Germany) at the SUN Immunology Research Group, University of Stellenbosch (South Africa) or at the Centre for Proteomics and Genomics Research (CPGR; Western Cape, South Africa), that had been validated and calibrated prior to the analysis. Sample concentrations were determined using the equation generated for each individual cytokine or chemokine. Relative fold change expression was determined by setting vehicle control (EtOH) to 1. Analytes included: IL-1β, IL-1ra, IL2, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12(p70), IL13, IL15, IL17, basic FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNFα and VEGF.

2.10. Statistical Analysis

Results were analysed using GraphPad PRISM (version 6, for MAC) software from GraphPad Software Inc (La Jolla California, USA). Data were tested for normalcy before parametric tests were performed using the D'Agostino-Pearson omnibus normality test for large data sets and the Kolmogorov-Smirnov test with Dallal-Wilkonson-Lille for P value for small data sets ($n < 6$). Where samples were treated with ligands at one time point, a one-way ANOVA, with either a Dunnet's or Tukey's post-test (comparing each group to control), was performed. For experiments that had different treatments at different time points, a two-way ANOVA was performed with a multiple comparison's Tukey's post test (either comparing all groups to each other or to a control). For comparison between two samples, an unpaired student's t-test was performed. Data were expressed as mean ± SEM on histograms or XY scatter charts, with n values represented in each Figure legend. For dose response curves, data was analysed with vehicle control set to 100%, and a non-linear regression model was employed using log agonist vs. Response (variable slope - 4 parameters) curve. When correlation studies were performed, a Pearsons r correlation test was performed. For infection data in the primary cervical explants, repeated measures two-way ANOVA was performed, with post-hoc Tukey tests to assess significance between the treatments at each time point assayed. For data that was

non-parametric, a Wilcoxon singed-rank test was performed, with the hypothetical value set to the vehicle control to determine the effect of a treatment to the control. When comparing two treatment groups to each other a Wilcoxon paired-rank sign test was performed. In order to establish productive infection in the primary ectocervical explant experiments, the relative infection values of the vehicle control over time were analysed for a linear trend using a one-way ANOVA with a post-test for a linear trend (see appendix E, Fig E.2.3 for more details). Where statistical significance of difference was determined relative to a single control statistical significance is denoted by *, **, ***, or **** to indicate $p<0.05$, $p<0.01$, $p<0.001$, or $p<0.0001$ respectively. Where two values are compared to each other, statistical significance of difference is indicated with lines indicating which sample sets were compared.

Chapter 3

MPA, unlike NET, P4 and LNG regulates immunomodulatory gene expression via the GR and increases HIV-1 replication in the majority of primary human PBMCS

Part of this work has been published in a peer review journal (Fig 3.2 and 3.6) (Hapgood et al., 2014) , as well as presented at the HIV R4P Conference in Cape Town South Africa, 2014 (Fig 3.2, 3.6 and 3.10).

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Introduction

The effects of different progestogens in peripheral blood mononuclear cells (PBMCs) are not well understood. In a study by Huijbregts et al. (2013), the authors found that MPA compared to P4 differentially regulated key cytokines and chemokines in a dose dependent manner (Huijbregts et al., 2013). The effects of the progestogens MPA, P4, NET and LNG on their ability to modulate immune gene mRNA and protein expression have not yet been fully elucidated, nor have there been many comparative studies of these progestogens on gene expression. Indeed, while it has been established that MPA behaves as a partial agonist for the glucocorticoid receptor in cell lines (Koubovec et al., 2004; Koubovec et al., 2005; Ronacher et al., 2009; Govender et al., 2014), this has not been established in primary PBMCs.

It has been observed In a Jurkat T cell line, that MPA (250 nM) after 8 hours, but not P4, was able to decrease expression of an IL-2 promoter reporter construct, similar to the synthetic GC agonist, DEX (Bamberger et al., 1999). Furthermore, the study observed that IL1, IL2 and IL6 protein levels were reduced by MPA, but not P4 and that this mechanism was most likely via the GR, as the authors did not detect PR or AR protein expression in normal human lymphocytes (Bamberger et al., 1999). Kontula et al. (1983) using competitive binding assays in human mononuclear leukocytes, observed that MPA had a 42% relative binding affinity for the GR, compared to the natural glucocorticoid, cortisol, that had a relative binding affinity of 25% for the GR (Kontula et al., 1983). Huijbregts et al. (2013) reported that MPA, more than P4 and E2, repressed select cytokine protein expression in a dose dependent manner after 24 hours incubation (Huijbregts et al., 2013). It has also been found that that MPA may dysregulate immune priming in human pDCs that delays the normal immune response and increases HIV-1 infection risk (Hughes et al., 2008; Huijbregts et al., 2014; Michel et al., 2015). In addition Huijbregts et al. (2013) have found that after five days stimulation with MPA and P4, HIV-1 replication in primary human CD8+ and CD4+ cells increased more than the vehicle control (Huijbregts et al., 2013) and that MPA but not P4 increased CCR5 and CXCR4 co-receptor expression in these cell types (Huijbregts et al., 2013). Subsequent to the current study, Sampah et al. (2015) found that MPA increased HIV-1 infection levels in un-stimulated human CD3+CD8+ T cells after three days using an R5 and an X4-tropic HIV-1 pseudovirus. Additionally the authors found that this increase in infection was reduced if CD14+ monocytes were removed from the sample population of cells (Sampah et al., 2015). However, the extent to which these progestogens may affect HIV-1 replication in comparison to NET and LNG has not been explored, nor have the effects of longer term incubations with the progestogens on gene expression been investigated.

The specific aims of this chapter were to:

1.) **Identify whether differential gene regulation occurred (both mRNA expression and protein expression) by the progestogens and glucocorticoids on key immune response genes, IL6, IL8, RANTES and GILZ.**

Using PBMCs isolated from healthy female donors, varying concentrations of DEX, MPA, P4 and NET were used to stimulate the PBMCs, after which RNA was isolated and converted to cDNA and mRNA transcriptional responses determined using real time qPCR. In addition, the effects of these ligands on protein expression and secretion were determined using a combination of flow cytometry, multiplex Luminex and MSD ELISA assays on either cells or supernatants. The immune response genes, IL6, IL8, RANTES and GILZ were chosen based on their previously established robust responses to glucocorticoids and MPA via the GR (Koubovec et al., 2004; Koubovec et al., 2005; Ronacher et al., 2009; Verhoog et al., 2011; Hadley et al., 2011; Africander et al., 2011; Govender et al., 2014). Additionally, these genes were chosen as they have established roles in the regulation of the immune response (Borish & Steinke, 2003; Commins et al., 2010; Ayroldi & Riccardi, 2009; Murphy et al., 2012) as well as some of the genes having implicated roles in the susceptibility to HIV-1 infection (Birx et al., 1990; Rollenhagen & Asin, 2010; Roberts et al., 2012; Lehman et al., 2014; Aukrust et al., 1998; Lane et al., 2001; Lane et al., 1999; Morrison et al., 2014).

2.) **Determine the steroid receptor profile of PBMCs, and if the GR is involved in mediating the differential gene expression by the different progestogens using the selective GR/PR antagonist RU486 and GR specific siRNA knockdown by nucleofection in PBMCs.**

Using PBMCs from healthy female donors, GR involvement in transcriptional effects will be determined using the selective GR/PR antagonist RU486. In order to support a role for the GR, the GR-specific ligand DEX was used as a reference agonist in which to compare the effects. In addition the steroid receptor content of PBMCs was analysed in order to aid in determination of whether the activity of RU486 is via the GR and/or the PR present in PBMCS. siRNA targeted GR knockdown was used as another method to confirm the GR involvement in mediating gene transcription. Using the nucleofection technique, whereby the siRNA is electroporated into PBMCs, mRNA expression analysis was performed, 72 hours post knockdown, and 48 hours post stimulation with DEX, MPA, P4 or NET.

3.) **Determine the effects of progestogens on HIV-1 replication in PBMCs, and the effects of HIV-1 on mRNA expression levels of IL6, IL8, RANTES and GILZ in combination with the different progestogens.**

PBMCs from healthy female donors were stimulated with CORT, DEX, MPA, P4, NET or LNG for 48 hours, before being infected with a luciferase tagged HIV-$1_{BaL\_Renilla}$ IMC for 2 hours. Infection was assessed by

measuring resultant luciferase activity, and normalised to MTT activity within each condition to account for the effects of CORT and DEX on cell viability. In addition, expression of IL6, IL8, RANTES and GILZ mRNA was assessed in the absence and presence of HIV-1.

4.) **Determine the effects of longer term incubation with MPA on gene regulation in PBMCs and what factors may be involved in mediating the effects of this response.**

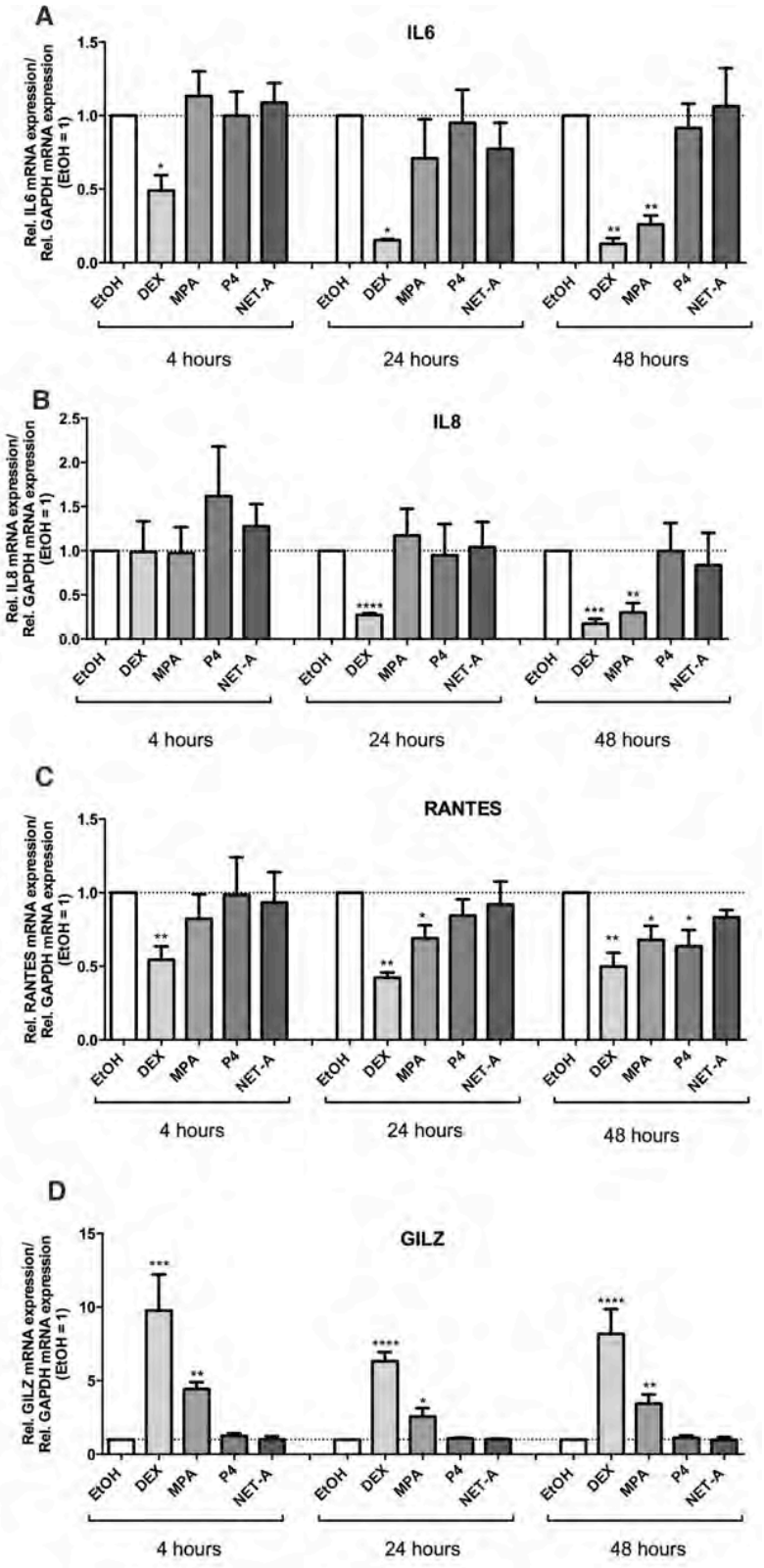
PBMCs were treated with either MPA or DEX with effects on mRNA expression of IL6, IL8, RANTES and GILZ determined over time. In addition, the effects on cellular viability, using the MTT assay and effects on steroid receptor expression were determined using western blots and mRNA expression analysis.

Results

MPA unlike NET-A differentially and dose dependently modulates key immunomodulatory gene expression in primary PBMCs

It was necessary to establish the conditions in order to study the immunomodulatory effects of the progestogens, P4, MPA and NET, and the glucocorticoid, DEX on IL6, IL8, RANTES and GILZ mRNA expression levels. PBMCs were isolated from the blood of healthy females obtained from the Western Province Blood Transfusion Services (WPBTS), South Africa. All blood used in this study was STI free, as verified by the WPBTS, South Africa. As such, a time course experiment was performed with saturating concentrations of ligand, assessing the transcriptional responses at 4, 24 and 48 hours by the ligands. Saturating conditions of ligands were used so that maximal effects on gene transcription could be observed. These concentrations were determined by using 10 X concentrations of their potencies or EC_{50} values reported in binding studies for the GR (Koubovec et al., 2005; Ronacher et al., 2009). For these shorter term incubation experiments, PBMCs were not activated with PHA or incubated in the presence of IL2.

Figure 3.1 shows that of the times investigated, the strongest repressive effects exerted by DEX and MPA on IL6, IL8 and RANTES repression were after 48 hours incubation with the ligands. Interestingly, MPA only exerted significant repressive effects at 48 hours on IL6 and IL8 mRNA expression, while having no significant regulatory effects after 4 or 24 hours post incubation (Figure 3.1 A and B). However MPA exerted repressive effects on RANTES mRNA expression after 24 hours post incubation (Fig. 3.1 C). DEX had significant and greater repressive effects on IL6 and IL8 and RANTES mRNA expression than MPA. However repression was only observed after 24 hours incubation on IL8 expression. The results suggest that there could be different regulatory mechanisms involved in mediating the repressive effects observed between MPA and DEX, and that time is a factor in this. P4 and NET had no effects on IL6 or IL8 mRNA expression at any of the time points assayed (Fig 3.1 A and B). Interestingly, P4 has repressive effects on RANTES mRNA expression at 48 hours (Fig. 3.1 C). Both DEX and MPA increased GILZ mRNA expression, with DEX exerting stronger transcriptional effects than MPA, at all the time points assayed (Fig. 3.1 D), while P4 and NET-A had no effect.



<u>Figure 3.1:</u> DEX and MPA, significantly regulates IL6, IL8, RANTES and GILZ mRNA levels in primary PBMCs in a time dependent manner. PBMCs were stimulated with saturating concentrations of DEX (100 nM), MPA (100 nM), P4 (1 µM), NET-A (10 µM) or vehicle (0,1% EtOH) for 4, 24 or 48 hours. Samples were harvested in TriReagent® and subsequently processed for RNA. 500 ng RNA was reversed transcribed to cDNA (Roche, South Africa), where it was used in subsequent real time qPCR as template to determine differential mRNA expression levels. Relative levels of IL6 (A), IL8 (B), RANTES (C) and GILZ (D) mRNA expression levels were normalised to GAPDH mRNA expression levels. Relative fold change in mRNA expression was determined by setting vehicle treatment (EtOH) to 1. Histograms represent pooled data from four independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a post hoc Dunnett's test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with *, **, ***, **** denoting $p<0.05$, $p<0.01$, $p<0.0001$ and $p<0.001$respectively.

Having established that 48 hours incubation with the progestogens exerted the greatest effects on transcriptional regulation on the genes tested, the next step was to establish the effects of concentration on mRNA expression levels. PBMCs were stimulated with increasing concentrations of DEX, MPA, P4 or NET-A for 48 hours; followed by RNA isolation, reverse transcription and real time qPCR. DEX served as the control for glucocorticoid effects, while P4 the natural ligand served as the control for progestogenic effects.

The results indicate that MPA, like DEX dose dependently decreased mRNA expression of IL6 and IL8 after 48 hours stimulation, while P4 and NET-A did not (Fig. 3.2 A and B). In addition MPA, like DEX dose dependently increased GILZ mRNA expression at 48 hours, while P4 and NET-A did not (Fig. 3.2 D). Only DEX was able to significantly repress RANTES mRNA expression dose dependently under these conditions (Fig. 3.2 C), while MPA appeared to reduce RANTES mRNA expression levels at 100 nM and 1 µM. Interestingly, this dose response indicated that MPA exerted its most significant differential effects at 100 nM and 1 µM, with weak effects at 10 nM (Fig. 3.2). Additionally, it appeared that MPA increased GILZ mRNA expression at 10 nM (Fig. 3.2 D), suggesting that at low concentrations, MPA is more efficacious in promoting transactivation versus transrepression gene effects.

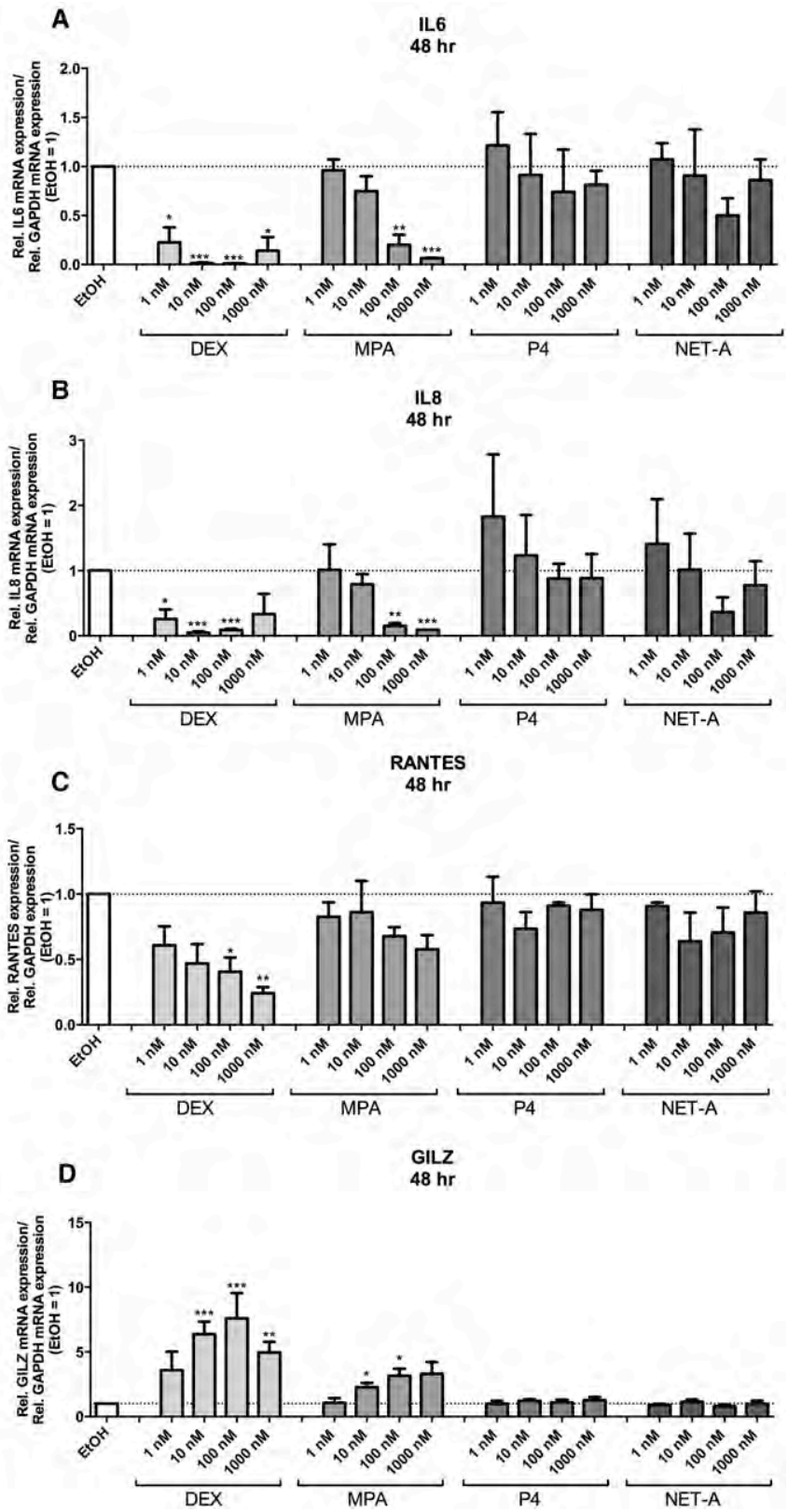


Figure 3.2: DEX and MPA, dose-dependently and significantly regulate IL6, IL8, and GILZ mRNA levels, while only DEX appears to significantly regulate RANTES mRNA expression in primary PBMCs. PBMCs were stimulated with increasing amounts of DEX, MPA, P4, NET-A or vehicle (0,1% EtOH) for 48 hours. Samples were harvested in TriReagent® and subsequently processed for RNA. 500 ng RNA was reversed transcribed to cDNA (Roche, South Africa), where it was used in subsequent real time qPCR as template to determine differential mRNA expression levels. Relative levels of IL6 (A), IL8 (B), RANTES (C) and GILZ (D) mRNA expression levels were normalised to GAPDH mRNA expression levels with vehicle treatment (EtOH) set to 1. Histograms represent pooled data from four independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a post hoc Dunnett's test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with *, **, *** denoting $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

To more accurately determine whether MPA compared to NET, at concentrations similar to serum concentrations, differentially modulate gene expression in a smaller sample set, PBMCs were stimulated with 10 nM DEX, MPA, P4 or NET-A for 48 hours, followed by RNA isolation, reverse transcription and real time qPCR. Similar to the results in the dose response analyses (Fig. 3.3), only DEX was able to exert significant changes in mRNA expression levels, reducing IL6 mRNA expression levels (Fig. 3.3 A) and increase GILZ mRNA expression levels (Fig. 3.3 D). MPA appeared to exert a small repressive effect on IL6 mRNA expression, and had weak transactivation effects on GILZ mRNA expression levels, although significance of these effects could not be established (Fig. 3.3 A and D). Interestingly, while P4 and NET-A had no statistically significant effects on mRNA gene expression levels, the mRNA expression levels appeared to be elevated compared to basal for IL6, IL8 and RANTES mRNA expression levels (Fig. 3.3 A - C). In addition these results highlight the differential sensitivities of the genes to these ligands, with DEX having stronger effects on IL6 and GILZ mRNA expression and weaker effects on IL8 and RANTES mRNA expression levels, and MPA appearing to follow this trend.

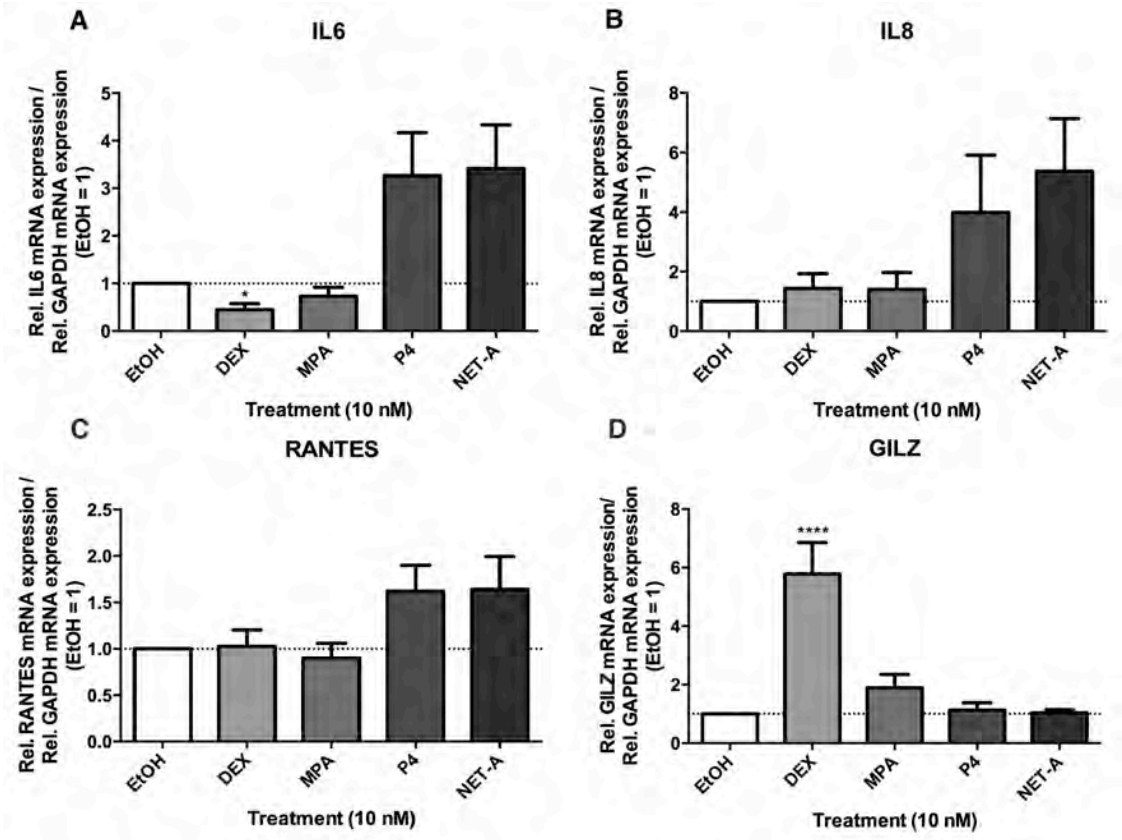


Figure 3.3: Low concentrations DEX and MPA, show weak to no effect on IL6, IL8, RANTES and GILZ mRNA expression levels in primary PBMCs. PBMCs were stimulated with 10 nM DEX, MPA, P4, NET-A or vehicle (0,1% EtOH) for 48 hours. Samples were harvested in TriReagent® and subsequently processed for RNA. 500 ng RNA was reversed transcribed to cDNA (Roche, South Africa), where it was used in subsequent real time qPCR as template to determine the differential expression levels. Relative mRNA expression levels of IL 6 (A), IL 8 (B), RANTES (C) and GILZ (D) were normalised to GAPDH mRNA expression, with vehicle treatment set to 1. Histograms represent pooled data from four independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a Dunnett's post-test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with * and **** denoting $p<0.05$ and $p<0.0001$ respectively.

In order to assess differential gene regulation by MPA and NET-A at a protein expression level, supernatants from the dose response experiments were harvested after 48 hours and measured for differential levels of secreted proteins using a 4-plex MSD ELISA and a 27-plex Luminex assay. Concurrent with the mRNA expression level data (Fig. 3.2 B), relative IL8 protein expression measured by MSD ELISA (Fig. 3.4 A) was significantly repressed by DEX and MPA, while P4 and NET-A had no effect at 100 nM concentrations. In addition, 100 nM MPA, like DEX, significantly repressed IL-1β protein expression (Fig. 3.4 B), while P4 and NET, at the same concentration had no effect. DEX significantly repressed IL-10 protein expression, while MPA appeared to reduce expression of IL-10 at 100 nM (Fig. 3.4 D), while P4 and NET at 100 nM had no effect. DEX appeared to reduce TNF-α protein expression at 100 nM (Fig. 3.4 C), while MPA had no effect. Interestingly P4 and NET had no effect on any of the proteins measured by MSD ELISA (Fig. 3.4 A-D).

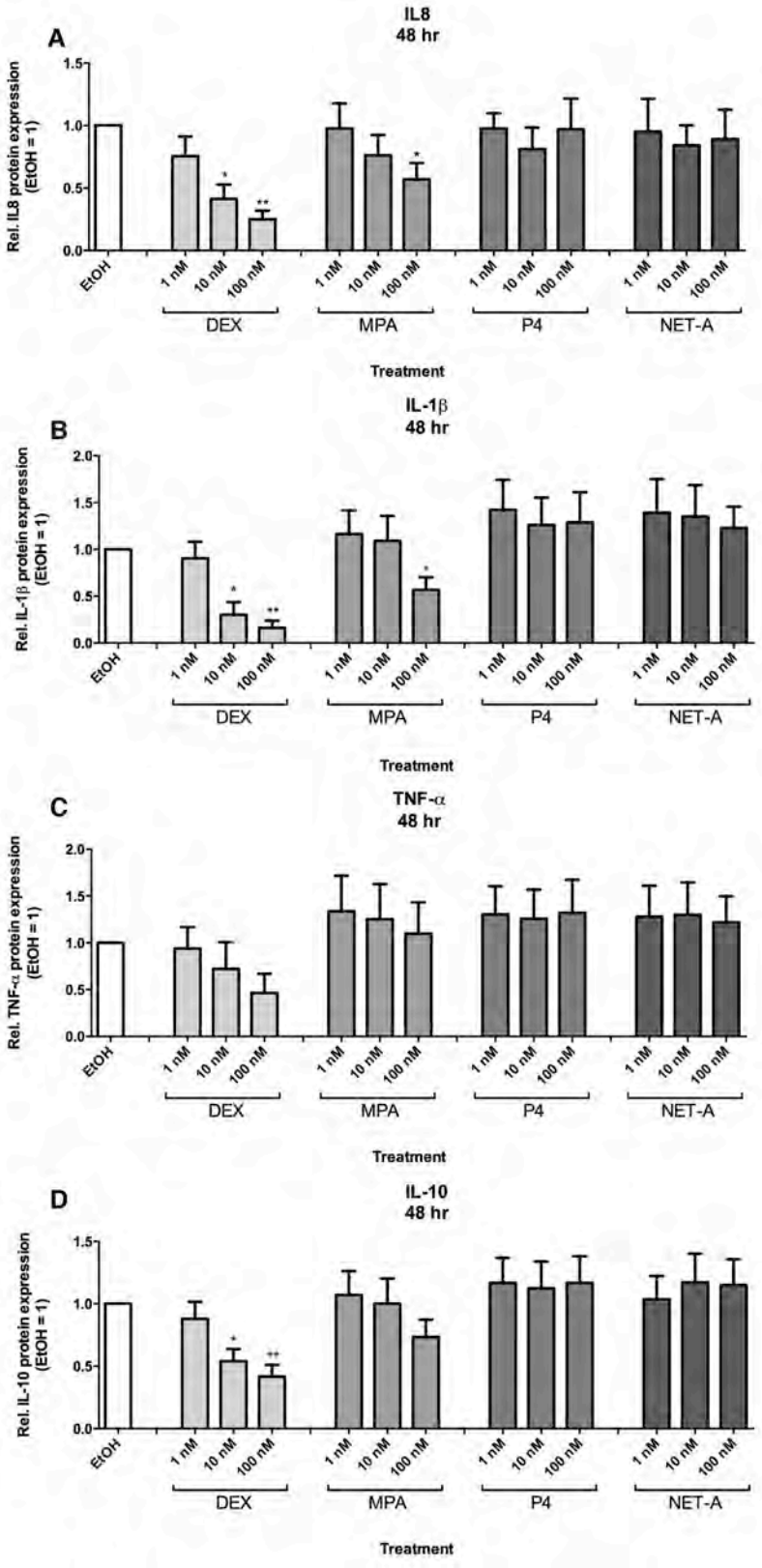
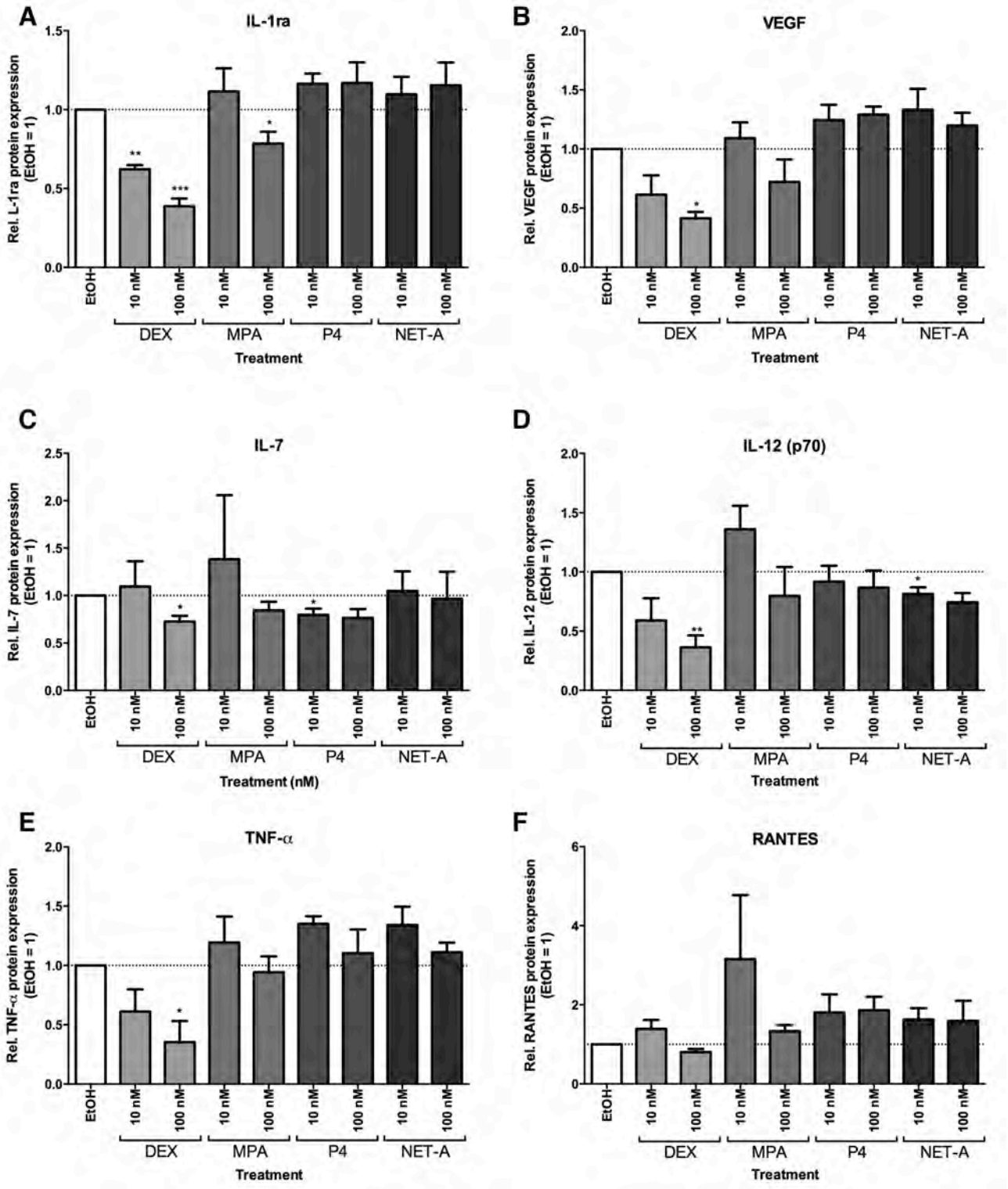


Figure 3.4: DEX dose-dependently regulates IL8, IL-1β, TNF-α and IL-10 basal protein expression levels, while MPA appears to exhibit a similar trend in regulating IL8 and IL-1β expression in primary PBMCs. PBMCs were stimulated with increasing amounts of DEX, MPA, P4, NET-A or vehicle (0,1% EtOH) for 48 hours. Supernatants were harvested and processed for differential protein expression using MSD ELISA (Harvard University, Brigham & Women's Hospital, Boston, USA). Relative fold change protein expression levels of IL8 (A), IL-1β (B), TNF-α (C) and IL-10 (D) were normalised to vehicle control (EtOH) set to 1. Histograms represent pooled data from four independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a post hoc Dunnett's test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with * and ** denoting $p<0.05$ and $p<0.001$ respectively.

Following the MSD ELISA, a Luminex assay on the same supernatant samples was performed, and 27 cytokines expression levels assessed. As with the MSD ELISA, IL-1β expression was significantly reduced upon stimulation with DEX (10 and 100 nM) and MPA (100 nM) (Appendix A, Fig A.1.1). DEX significantly reduced the protein expression levels of IL-1ra, VEGF, IL-17, IL-12 (p70), and TNF-α (Fig. 3.5 A-E), while MPA significantly reduced IL-1ra at 100 nM, and appeared to reduce the apparent expression levels of these proteins at 100 nM (Fig. 3.5 A-E). Unlike the mRNA expression data, RANTES did not appear to be repressed by either DEX or MPA (Fig. 3.5 F). This could be due to selective degradation of sensitive cytokines in the supernatant that had been freeze/thawed twice, however the exact reason is not known. Protein determination for IL6, MIP-1α, MIP-1β and MCP-1 was not possible due to the fluorescent values being out of range (too high) for most samples in the Luminex assay. In addition to the protein expression levels affected by both DEX and MPA, several protein expression levels were significantly reduced by DEX, but not MPA (Appendix A, Fig. A.1.1). These results highlight that DEX and MPA may selectively regulate key host genes in a concentration dependent manner, and are consistent with the literature, that MPA is a partial GR agonist. Additionally the data from Fig. 3.4 and Fig.3.5 suggest that at 100 nM, MPA is more similar to DEX in its effects on protein expression levels than to the other progestogens, P4 and NET, at the same concentration.



<u>Figure 3.5:</u> DEX and MPA dose-dependently regulate select cytokine and chemokine basal protein expression levels in primary PBMCs. PBMCs were stimulated with increasing amounts of DEX, MPA, P4, NET-A or vehicle (0,1% EtOH) for 48 hours. Supernatants were harvested and processed for differential protein expression using the Bio-Rad 27-plex Human cytokine and chemokine panel (Bio-Rad, Germany) on a Bio-plex 200 (Bio-Rad, Germany). Relative protein expression levels of IL-1ra (A), VEGF (B), IL-7 (C), IL-12 (p70) (D), TNF-α (E) and RANTES (F) levels were normalised to vehicle control (EtOH) set to 1. Histograms represent pooled data from four independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a post hoc Dunnett's test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with *, **, *** denoting $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

In order to establish whether the progestins differentially regulated protein expression of IL6 and GILZ in different cellular populations, protein expression analysis using FACS was performed. PBMCs were stimulated with saturating conditions of ligand for 48 hours, before staining cells with surface antibody markers (CD4+ and CD14+) followed by GILZ antibody or IL6 intracellular antibody staining for protein expression (Figure 3.6).

MPA (100 nM), like DEX (100 nM), significantly repressed IL6 protein expression after 48 hours while P4 (1 µM), NET-A (10 µM), NET (10 µM and LNG (10 µM) did not affect basal expression in CD14+ monocytes (Fig. 3.6 A). Interestingly, IL6 expression was not readily detected in CD4+ T cells (data not shown), indicating that under these conditions IL6 is predominantly expressed in CD14+ monocytes.

DEX and MPA significantly increased GILZ protein expression levels in CD4+ T cells, after 48 hours. P4, NET, NET-A and LNG did not affect GILZ protein expression levels (Fig. 3.6 B). GILZ expression was detected in CD14+ monocytes, however no differential expression levels were observed in the cell type (data not shown).

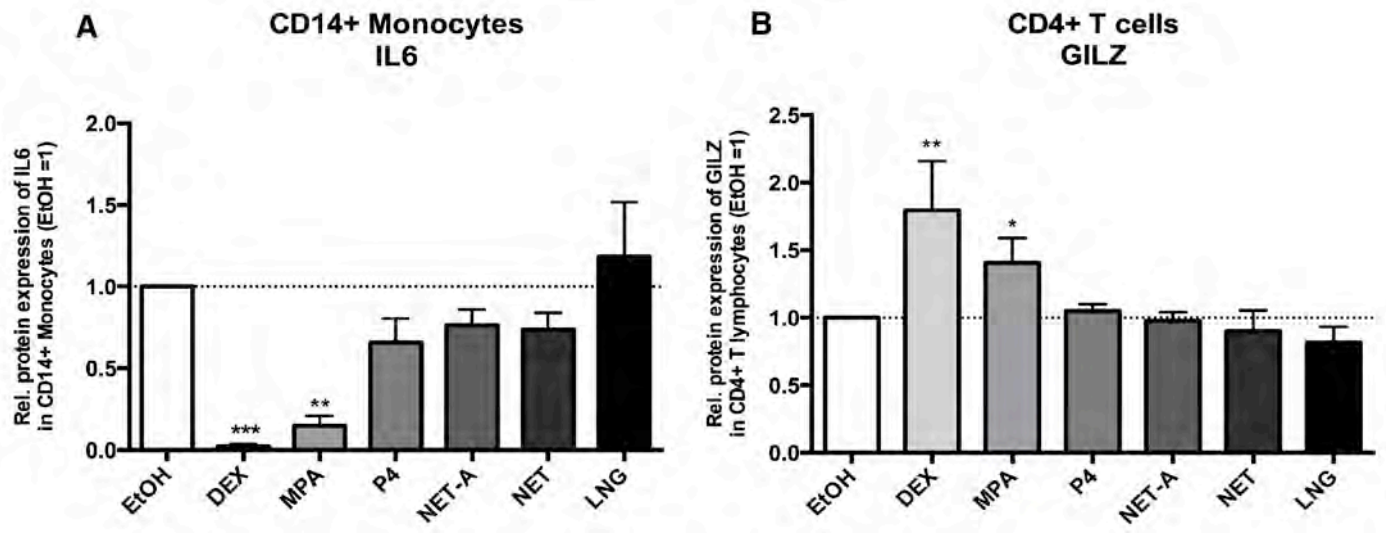


Figure 3.6: DEX and MPA, but not NET-A or P4, significantly decrease IL6 and increase GILZ protein expression in primary human CD14+ Monocytes and CD4+ T cells respectively. Cells were treated with 100 nM DEX, 100 nM MPA, 1 µM P4, 10 µM NET-A, 10 µM NET, 10 µM LNG or vehicle control (ETOH) for 48 hrs. Thereafter cells were stained with surface antibody markers (CD4+ and CD14+) followed by IL6 or GILZ intracellular antibody staining for protein expression. Protein expression was measured using a Becton-Dickinson FACsCalibur flow cytometer and analysed using FlowJo (software version 7.1, Treestar Inc., Ashland, Ore). Gating strategy in Appendix A (Fig.A.2.1 and A.2.2). (A) Relative IL6 protein expression in CD14+ monocytes and (B) relative GILZ expression in CD4+ T cells were normalised to vehicle (EtOH) control set to 1. Histograms represent pooled results from five independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a Dunnet's post-test or with an unpaired student t-test, comparing the sample to the vehicle control, with *, ** and *** denoting $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

MPA and DEX mRNA gene expression effects are mediated via the glucocorticoid receptor (GR) in primary PBMCs

PBMCs appear to express the GR, ER and MR mRNA, with no AR or PR mRNA being detectable (Fig. 3.7 A). Western blotting shows that only the GR protein was detectable in PBMCs (Fig. 3.7 B), suggesting that the effects of DEX and MPA are most likely to be via the GR. Due to the cross-reactivity of the MR antibody used in this study, MR protein expression by western blot could not be determined. There have been a few studies that have detected the PR in PBMCs, however detection was low suggesting that the PR may be weakly expressed in PBMCs (Asin et al., 2008; Cabrera-Muñoz et al., 2012).

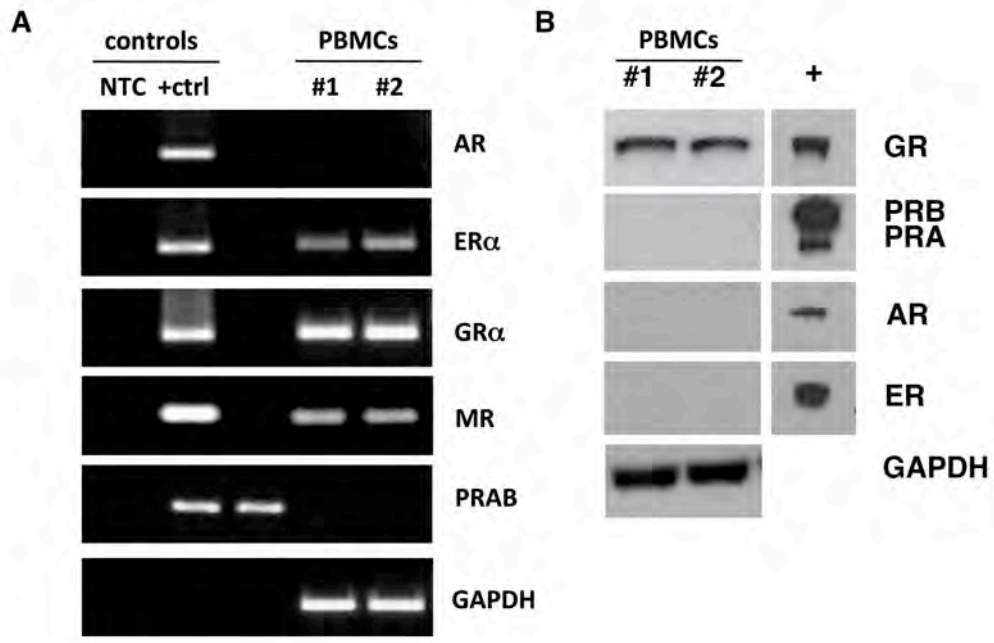


Figure 3.7: The steroid receptor profile shows that ER, GR and MR mRNA are present, while only GR was detectable via western blotting in PBMCs. (A) Conventional PCR of cDNA prepared from human PBMCs using primers specific for the relevant steroid receptor (see Chapter 2). The controls were prepared by PCR amplification of the relative steroid receptor cDNA from plasmid DNA from two female donors (denoted as #1 and #2). GAPDH served as a control for mRNA levels. PRAB has two positive controls, with the first positive control showing results using PRB plasmid DNA and the second representing PRA. MW: molecular weight; NTC: no template control. Products were visualised at 256 nM using a Syngene, G:Box (Vacutec, England) and images acquired using GeneSnap from synGene, version 7.08 (SynGene, England). (B) Protein samples isolated from two donors (#1 and #2) using TAPS buffer, were electrophoresed on an 8% SDS-PAGE gel before being electroblotted onto a nitrocellulose membrane and probed for GRα, PRAB, ERα and AR and GAPDH. Nitrocellulose blots were incubated for 1 minute in ECL-chemiluminescent substrate before being visualised by audio-radiography. Positive controls (donated as +) for each of the steroid receptors were included in each western blot to confirm the product of the donor samples.

To investigate the role of the GR in mediating the DEX and MPA effects on mRNA gene expression, PBMCs were treated with DEX and MPA in the absence and presence of RU486, a selective GR/PR antagonist for 48 hours. Although RU486 can also antagonise the PR, we have recently shown that PBMCs under our conditions express no detectable PR mRNA or protein [(Tomasicchio et al., 2013) and Fig 3.7].

Thus, the RU486 results will support a role for the GR in mediating these anti-inflammatory effects in PBMCs. In Figure 3.8, IL6, IL8 mRNA expression was significantly repressed by DEX and MPA. In the presence of the antagonist RU486, the repressive effects exerted by DEX and MPA was lifted (Fig. 3.8 A and B). Similarly, DEX significantly repressed RANTES mRNA expression, while MPA appeared to reduce basal expression. In the presence of RU486, the repressive effects exerted by DEX were lifted, while RANTES mRNA expression remained the same in the MPA/RU486 treated samples (Fig. 3.8 C). Additionally, both DEX and MPA appeared to induce GILZ mRNA expression levels, an effect which was lost when co-stimulated with RU486 (Fig. 3.8 D). In Fig 3.8 A, DEX and MPA repression on IL6 appears to be more efficacious than on IL8 (Fig. 3.8 B) and RANTES (Fig. 3.8 C), with partial DEX repression still maintained on IL6 by DEX in the presence of RU486, an effect that is lost completely on the other genes.

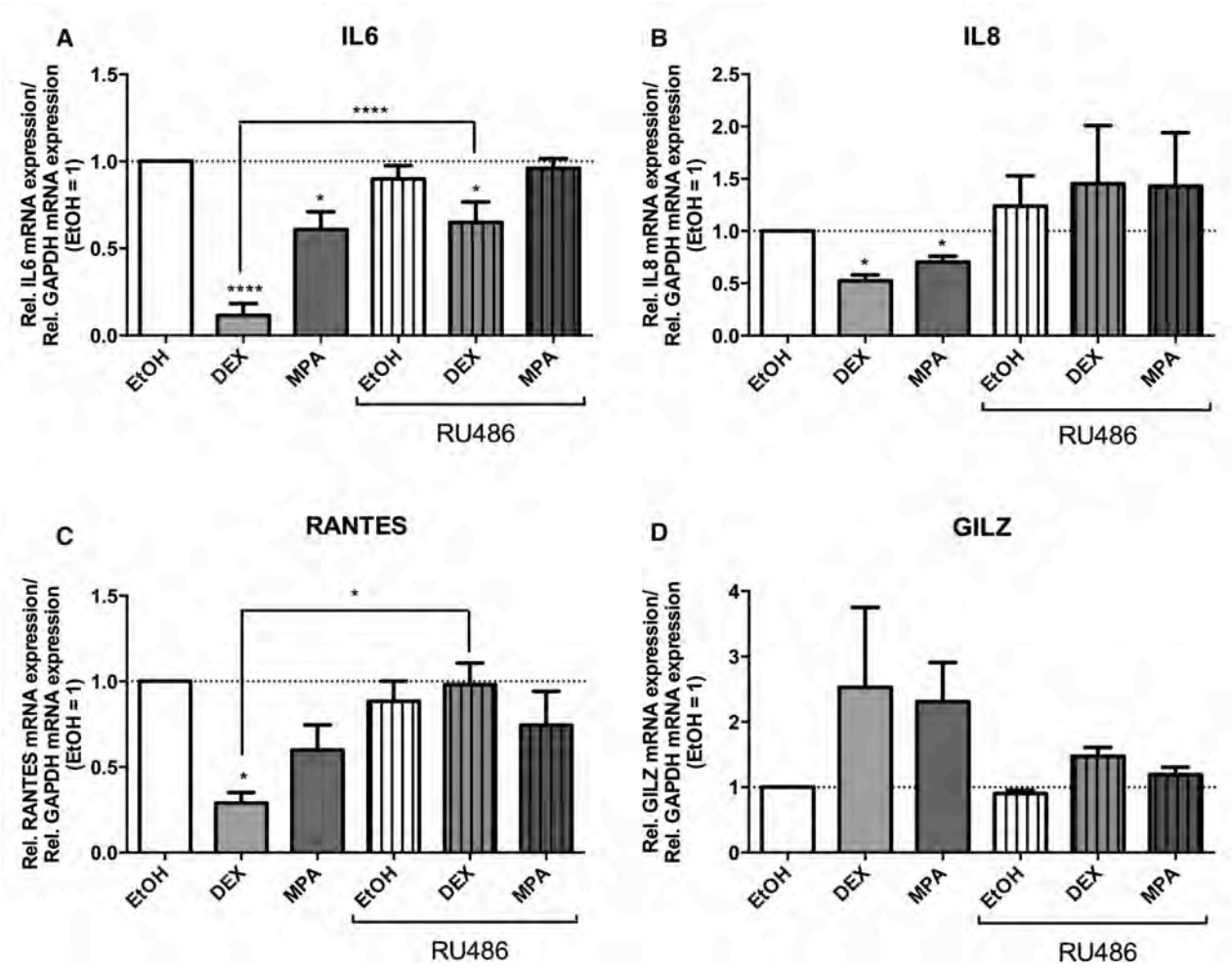


Figure 3.8: DEX- and MPA-mediated regulation of GILZ, IL6, IL8 and RANTES mRNA is reversed upon RU486 treatment. PBMCs were stimulated with 100 nM DEX and MPA in the presence and absence of 1 µM RU486 for 48 hours. Samples were harvested in TriReagent® and harvested for RNA isolation. 500 ng RNA was reversed transcribed to cDNA (Roche, South Africa), and used in subsequent real time qPCR as template to determine differential mRNA expression levels. Relative mRNA expression levels of IL6 (A), IL8 (B), RANTES (C) and GILZ (D) were normalised to GAPDH. Relative fold change in mRNA expression was determined by setting vehicle treatment (EtOH) to 1. Histograms show pooled results from three independent experiments plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with Dunnet's post-test or unpaired student t-tests, with $p < 0.05$ denoted as * and $p < 0.0001$ denoted as ****.

To confirm the effects of the antagonist experiment, PBMCs were electroporated with GR specific siRNA and assessed for GR knockdown and resultant mRNA expression. Under these conditions, the repressive effects of DEX and MPA on IL6, IL8 and RANTES were muted in the NSC conditions (Fig. 3.9 A-C), as compared to the repression observed in Figs. 3.1, 3.2 and 3.8, where 100 nM DEX significantly repressed IL6, IL8 and RANTES and MPA significantly repressed IL6 and IL8 mRNA expression after 48 hours stimulation. However, the apparent repressive effects appeared to be lifted under knockdown conditions (Fig. 3.9 A-C). Indeed, DEX and MPA responses on IL6 increased from a mean of 0.51 fold and 0.75 fold to a mean of 0.72 fold and 2.32 fold respectively (Fig. 3.9 A), suggesting that the GR is involved in mediating the repression of IL6 in PBMCs. A similar trend was observed for IL8 mRNA expression, where DEX and MPA responses were lifted under knockdown conditions. DEX and MPA responses increased from a mean response of 0.37 fold and 0.64 fold response under NSC conditions to a mean response of 0.64 fold and 1.51 fold respectively under knockdown conditions (Fig. 3.9 B). RANTES mRNA expression was significantly repressed by DEX under NSC conditions, and appeared to be less repressed when the GR was knockdown (Fig. 3.9 C). Similarly MPA appeared to weakly repress RANTES under NSC conditions, which was lifted under knockdown conditions (a mean of 0.60 fold under NSC conditions to 0.92 fold under knockdown conditions). GILZ transactivation by DEX and MPA was observed under NSC conditions. However, under knockdown conditions, only DEX treated responses were muted, while MPA transactivation appeared to be maintained (Fig. 3.9 D). In the presence of the knockdown conditions basal levels of IL6 modestly increased, as well as in P4 and NET conditions. This result suggests that the GR may be involved in regulating the basal mRNA expression levels of IL6 (Fig. 3.9 A). A similar trend was observed in the IL8 knockdown conditions, where NET treatment appeared to elevate IL8 mRNA expression levels (Fig. 3.9 B). The apparent pro-inflammation effects may be due to the involvement of the unliganded GR, that may regulate basal transcription of pro-inflammatory genes, similar to what Verhoog et al. (2011) observed in an epithelial cell line (Verhoog et al., 2011).

The partial repression maintained on some of the genes by DEX (IL6, IL8 and RANTES) as well as the maintenance of GILZ transactivation by MPA, may be due to a relatively low knockdown level (40%) achieved under these conditions (Fig. 3.9 E), suggesting that there was sufficient GR under the knockdown conditions to exert repressive or transactivating effects on the select panel of genes tested. These results suggest that GR is involved in modulating DEX and MPA effects on select gene expression. Furthermore, these experiments support the RU486 experiments for a role of the GR in mediating DEX and MPA responses on key target genes involved in HIV-1 infection.

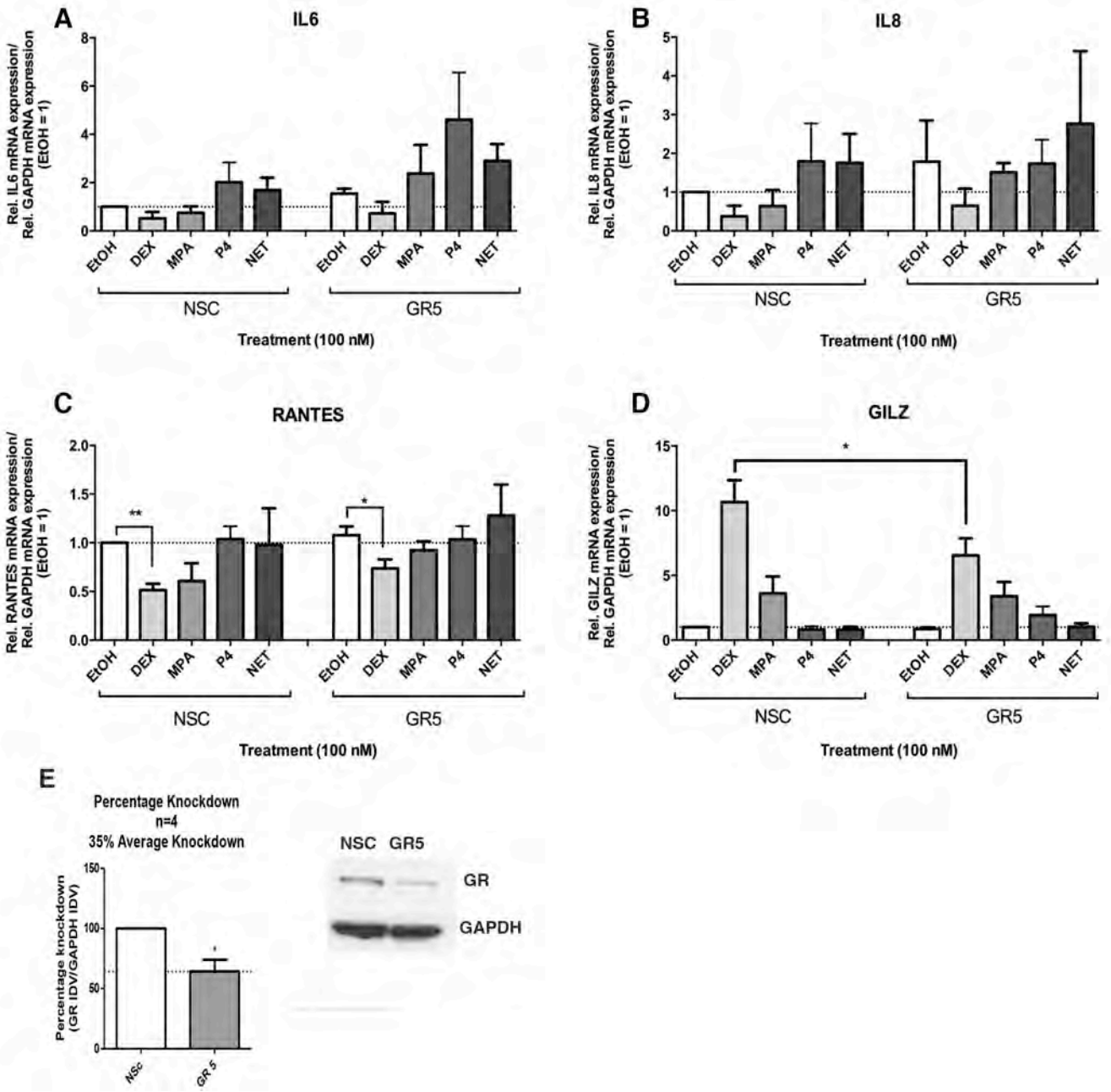
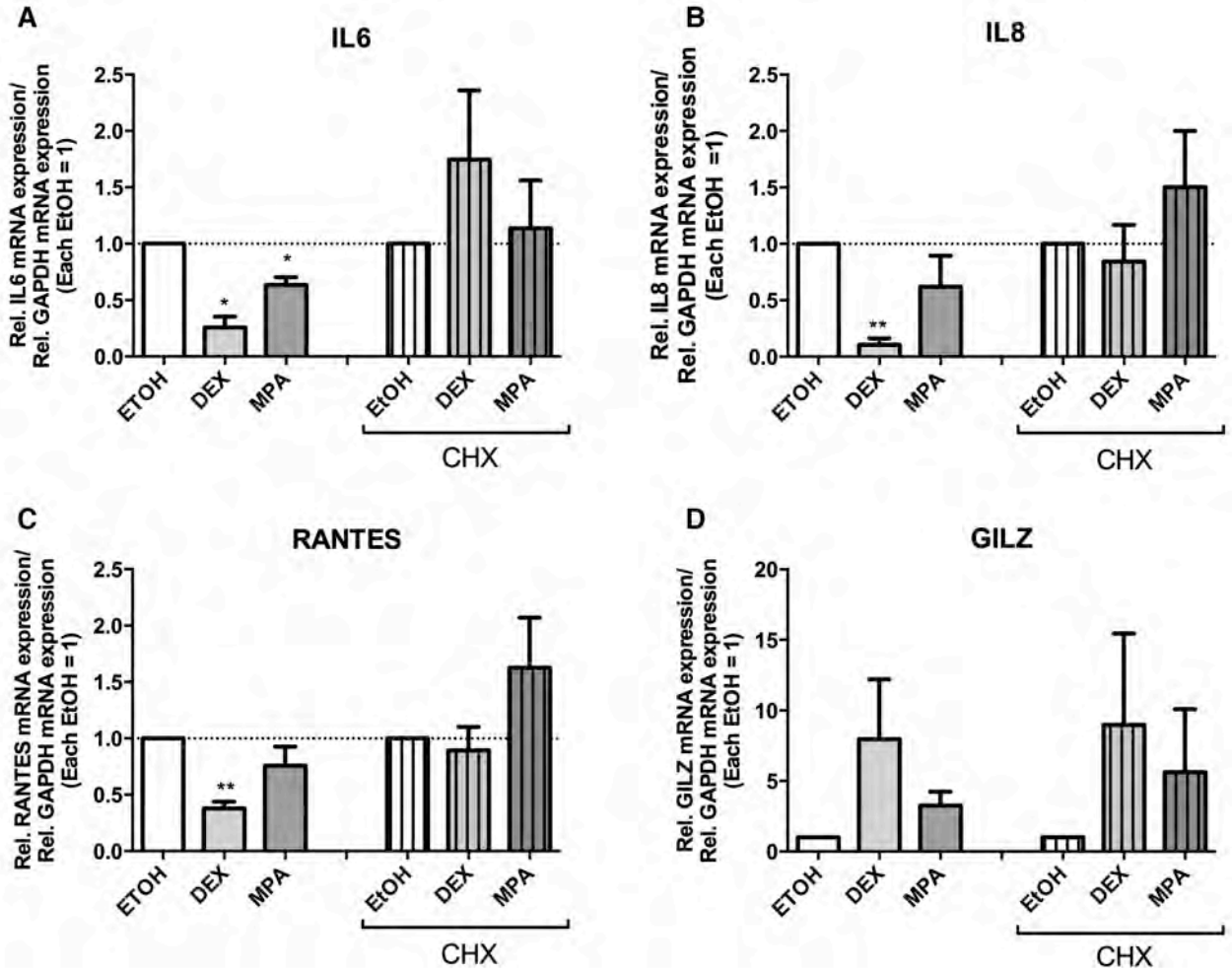


Figure 3.9: DEX- mediated regulation of GILZ mRNA requires the presence of the GR, while MPA-mediated repression of IL6, IL8 and RANTES also appears to require the presence of the GR in primary human PBMCs. PBMCs were electroporated using a Nucleofector (Lonza) with 20 nM GR5 (Qiagen) siRNA or control siRNA and after 24 hours stimulated with 100 nM DEX, MPA, NET or progesterone (P4) for a further 48 hours. Samples were harvested for RNA isolation followed by reverse transcription of 500 ng RNA to cDNA. Relative mRNA expression levels of IL6 (A), IL8 (B), RANTES (C) and GILZ (D) were determined by real time qPCR, normalised to GAPDH. Relative fold change in mRNA expression was determined by setting vehicle treatment (EtOH) to 1. For each experiment, an untreated NSC and GR5 sample was harvested at 48 hrs post nucleofection for protein isolation using TAPS buffer. Samples were electrophoresed on an 8% SDS-PAGE gel before being electroblotted onto a nitrocellulose membrane and probed for GRα and GAPDH. Nitrocellulose blots were incubated for 1 minute in ECL-chemiluminescent substrate before being visualised by audio-radiography. (E) Average knockdown as a percentage of NSC GR was determined by densometric analysis (AlphaEC™, Alpha Innotech, version 3.1.) setting NSC GR levels to 100%. Histograms (A - E) are pooled results from representative of four independent experiments, plotted at mean ± SEM, while the western blot in E is representative of one donor. Statistical significance was determined by a one way ANOVA, followed by post-test unpaired student t-tests with *, ** denoting $p<0.05$ and $p<0.01$ respectively.

In order to establish whether the effects exerted by MPA on mRNA levels required new protein synthesis, cycloheximide (CHX) experiments were performed (Fig. 3.10). CHX prevents *de novo* protein synthesis Thus, if the repression is lost in the presence of CHX, it suggests that new proteins, other than those present, are necessary to produce the effects on the mRNA levels assayed.

Results suggest that in the presence of CHX, DEX and MPA repression of IL6 mRNA is lost after 48 hours (Fig. 3.10 A). Further DEX repression of IL8 and RANTES mRNA expression was also lost in the presence of CHX (Fig. 3.10 B and C). While MPA repression was not significant on IL8 or RANTES mRNA expression after 48 hours in this study, in the presence of CHX, IL8 and RANTES mRNA expression levels appeared to be elevated (Fig. 3.10 B and C). These results suggest that repression by DEX, and MPA may be indirect. In this experiment, GILZ mRNA appeared to be elevated by DEX and MPA (Fig. 3.10 D). Further, in the presence of CHX, GILZ mRNA expression remained elevated with DEX and MPA, suggesting that the increase in GILZ mRNA expression may be through direct effects.

Taken together, the results are consistent with a direct transcriptional effect of the GR in up-regulating GILZ in response to MPA and DEX, but not for the down regulation of IL6, IL8 and RANTES mRNA levels.



<u>Figure 3.10:</u> DEX- mediated regulation of IL6, IL8 and RANTES and MPA-mediated regulation of IL6, appears to require additional protein synthesis to exert the repressive effects via the GR, unlike for GILZ, in primary human PBMCs. PBMCS were treated with 100 nM DEX, MPA or with a vehicle control (0.1% EtOH) in the absence and presence of 10 µg/ml cycloheximide (CHX) for 48 hours. PBMCs were harvested in parallel for RNA isolation or subsequent protein isolation. 250 ng of the RNA samples were reverse transcribed, and used in subsequent real time qPCR. Relative mRNA expression levels of IL6 (A), IL8 (B), RANTES (C) and GILZ (D) were determined by real time qPCR, normalised to GAPDH. Relative fold change in mRNA expression was determined by setting vehicle treatment (EtOH) in each condition (± CHX) to 1. Histograms are pooled results from representative of four independent experiments, plotted at mean ± SEM. Statistical significance was determined by a one way ANOVA, followed by post-test unpaired student t-tests with *, ** denoting $p<0.05$ and $p<0.01$ respectively.

Progestogens and glucocorticoids, differentially modulate immunomodulatory genes in PBMCs and variably modulate HIV-1 replication in primary PBMCs after long term exposure

Having established that MPA, like DEX, regulates select key immune response genes via the GR, in a manner unlike that of NET and LNG and P4, it was next determined whether these ligands affect HIV-1 replication in PBMCs. For this set of experiments, initial pilot experiments suggested that it was necessary to culture activated PBMCs in 30 U/ml IL2 to obtain reproducible and measureable HIV-1 infection levels (appendix E, Fig. E.1.1). Further pilot experiments found that measuring HIV-1 replication levels via luminescence at day 7 post infection was optimal, and that cell viability was not significantly effected with 0.01% EtOH (appendix E, Fig. E.1.2). As such, PBMCs isolated from healthy female donors, were activated for one day and subsequently stimulated with 100 nM of each ligand; cortisol (a natural GR ligand), DEX, MPA, P4, NET and LNG or vehicle control (0.01% EtOH). After 48 hours, samples were infected with 10 IU/ml HIV-$1_{BaL\_Renilla}$ and incubated for a further 7 days. PBMCs were harvested for RNA, luciferase activity and MTT at day 7 post infection. Cell viability as measured by MTT was assessed, in order to normalise the luciferase readings to viable cells. This was necessary as incubations times were long (9 days) and it has been reported that both DEX and MPA increase GR dependent apoptosis in PBMCs (Tomasicchio et al., 2013).

Seven days after infection with HIV-$1_{BaL\_Renilla}$, the level of renilla activity in the PBMCs from 10 different donors varied substantially in all treatment groups (Fig. 3.11 A), suggesting that HIV-1 infection and replication levels are dependent upon the donor. It could be that the relative concentrations and states of activation of different cell types between donors may vary. However, this remains to be confirmed. In 5 out 10 donor PBMCs, CORT treatment increased HIV-1 viral replication, while appeared to be protective (i.e. less infectious) for the other donors. Similarly, 4 out of 10 DEX treated donor PBMCs had in increase in viral replication. Similarly to the glucocorticoids, the progestogens P4 and LNG treated PBMCs increased viral replication in 5 out of the 10 patient PBMC samples. In contrast to this variability, in MPA treated samples, 7 out of 10 donor PBMCs exhibited an increase in HIV-1 replication, while 8 out of 10 NET-treated samples had little to protective effects on HIV-1 replication (Fig 3.11 A). MTT viability analysis indicated that cell viability decreased significantly in CORT and DEX treated samples, while MPA showed a slight reduction in cell viability (Fig. 3.11 B). P4, NET and LNG had no effect on cellular proliferation in the absence or presence of HIV-1. Indeed, the presence of HIV-1 did not appear to further alter viability for any of the ligands tested (Fig. 3.11 B).

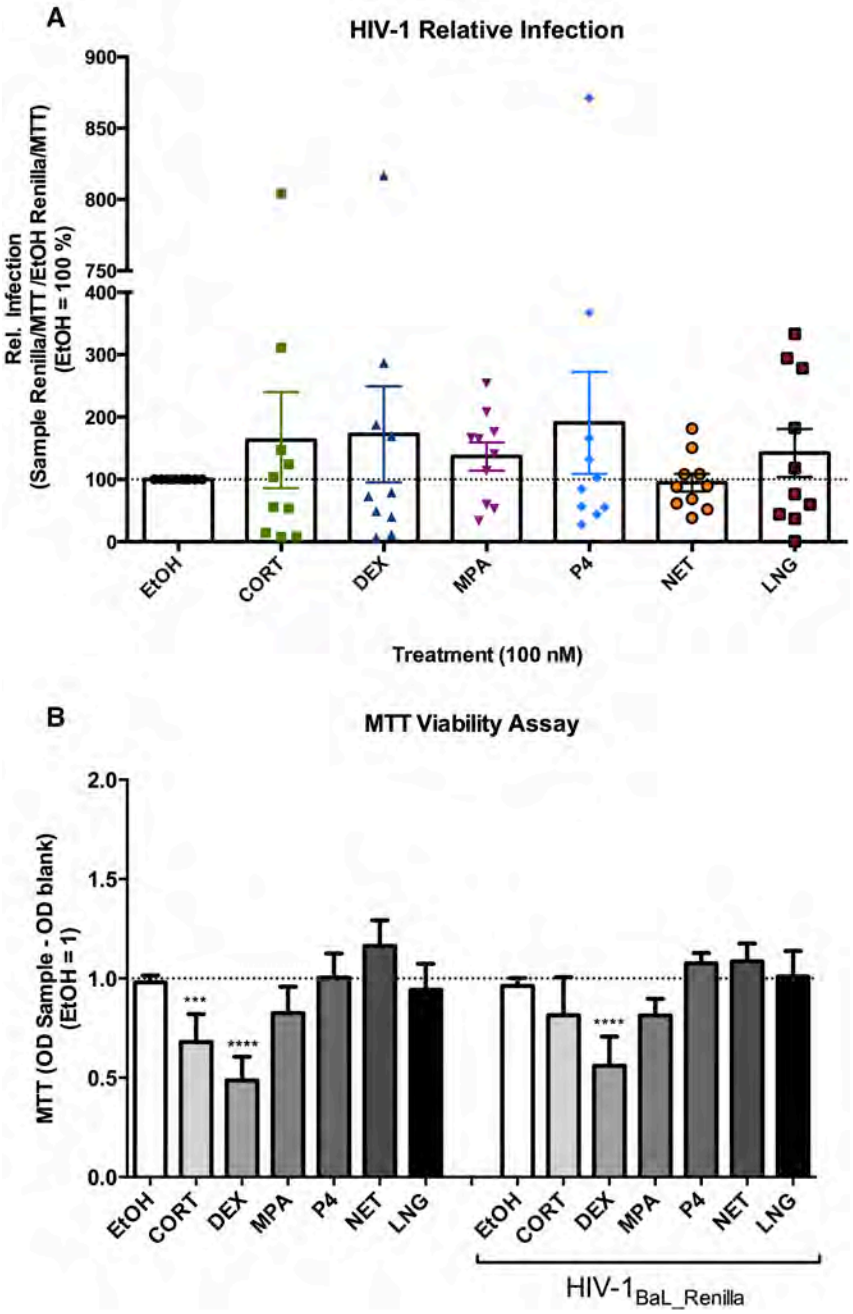


Figure 3.11: HIV-1 infection levels are highly varied within each treatment group, while it appears that CORT, DEX and MPA reduce viability of PBMCs compared to P4, NET or LNG in primary PBMCs. PHA activated PBMCs were pre-treated with 100 nM Cortisol (CORT), DEX, MPA, P4, NET and LNG or vehicle control, (0.01% (*v/v*) EtOH) for 48 hours. Pre-treated PBMCs were infected with 10 IU/ml HIV-$1_{BaL\text{-}Renilla}$ IMC for 2 hours, washed 4 times with 1 X PBS and media with the respective ligands was added. Media was replaced every second day. PBMCs were harvested 7 days post infection and MTT viability and luciferase activity were measured. (A) Renilla luciferase was measured with the Promega Renilla-Glo™ kit using a luminometer (Modulus Microplate, Glomax, Promega) and values were normalised against corresponding MTT values, and normalised to vehicle set to 100% relative infection. (B) MTT activity was detected with a multiplate reader (Thermo Scientific) using a MTT cell viability kit (Sigma.) Values were normalised to vehicle, which was set to 1. Histograms (A-B) show pooled results of ten independent experiments, with ten donors and plotted as mean ± SEM. Statistical analysis was carried out using GraphPad Prism™ software, using a one-way ANOVA with student t-tests. Statistical significance is denoted by *** or **** to indicate P< 0.001, or P< 0.0001, respectively.

When assessing relative mRNA expression of IL6, IL8, RANTES and GILZ after infection, CORT, DEX and MPA treatment did not repress IL6 or RANTES mRNA expression in both the absence and presence of HIV-1 7 days post infection (Fig. 3.12 A and C). Repression of IL8 mRNA expression by CORT, DEX and MPA was maintained in the absence of HIV-1 but lost in the presence of HIV-1 (Fig. 3.12 B). Relative IL6 mRNA expression in the presence of HIV-1 by CORT, DEX and MPA was no longer repressive, as previously observed in this study, while relative IL8 mRNA expression varied in the presence of HIV-1 infection. RANTES mRNA expression levels in CORT, DEX and MPA treated samples in the absence and presence of HIV-1 were no longer repressed (Fig. 3.12 C). In contrast, transactivation of GILZ mRNA expression by CORT, DEX and MPA was maintained after 7 days post infection (Fig. 3.12 D). Interestingly, DEX treatment elevated GILZ mRNA expression to a greater extent in the presence of HIV-1, compared to the no virus control (Fig. 3.12 D). P4, NET and LNG had no significant effect on IL6, IL8, RANTES and GILZ mRNA expression levels, and the presence of HIV-1 did not alter this expression profile (Fig. 3.12 A-D). However it did appear that NET and LNG, more so than P4, appeared to elevate IL6, IL8 and RANTES mRNA expression levels in the absence of HIV-1, while this was not evident in the presences of HIV-1 (Fig. 3.12 A-C). Basal expression mRNA expression levels of IL6, IL8, RANTES and GILZ did not change in the presence of HIV-1 compared to the uninfected control (Figure A.5.1, Appendix A).

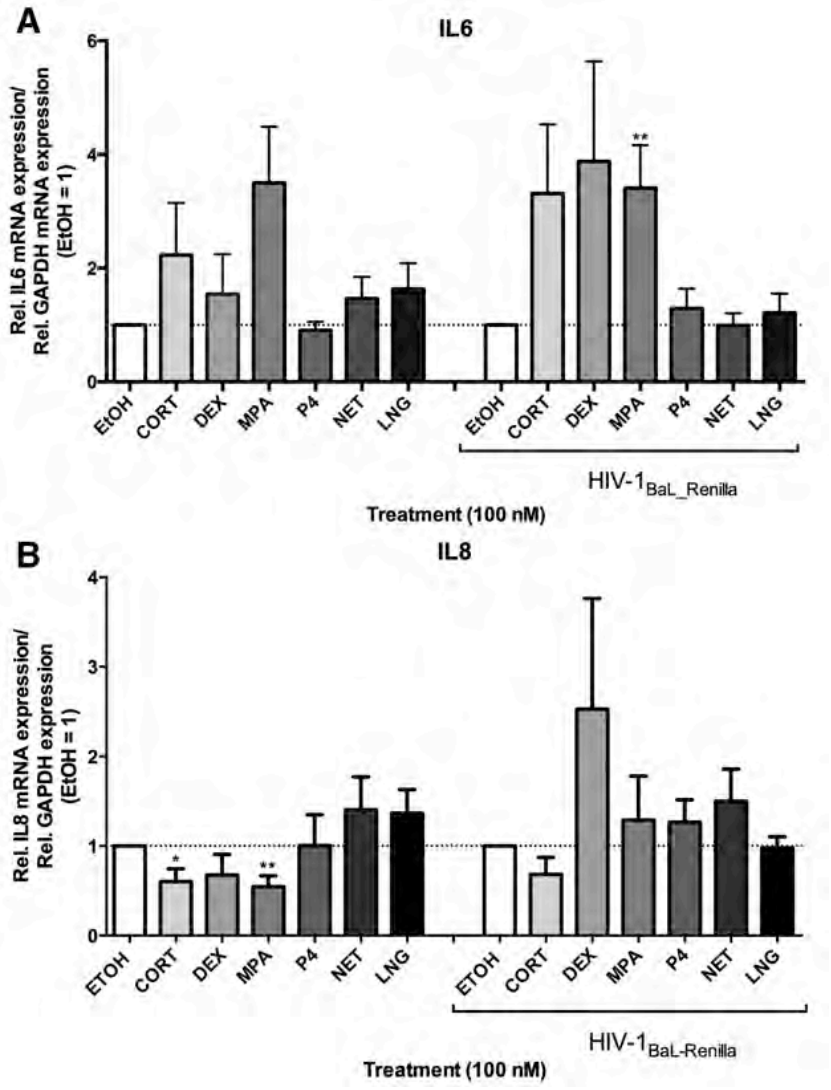
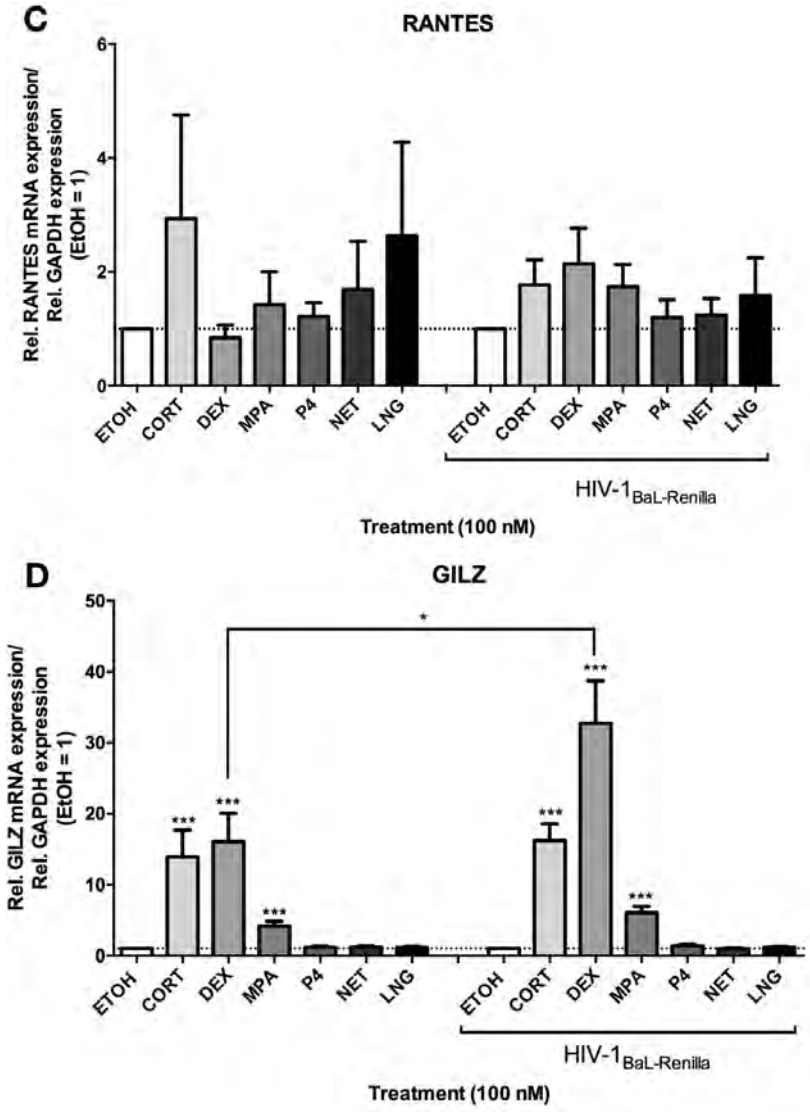


Figure 3.12: mRNA levels of select immunomodulatory genes after 9 days in the presence of ligands and after 7 days in the absence and presence of HIV-$1_{BaL\text{-}Renilla}$. PHA activated PBMCs were pre-treated with 100 nM Cortisol (CORT), DEX, MPA, P4, NET or LNG, with vehicle control (0.01% EtOH), for 48 hours. Pre-treated PBMCs were infected with 10 IU/ml HIV-$1_{BaL\text{-}Renilla}$ IMC for 2 hours, washed extensively and the media replaced. Media with the respective ligands was replaced every second day. PBMCs were harvested at 7 days post infection in TriReagent® and subsequently processed for RNA. 250 ng RNA was reversed transcribed to cDNA (Roche Applied Science, South Africa), where it was used in subsequent real time qPCR as template to determine the differential expression levels. Relative mRNA expression levels of IL6 (A) and IL8 (B) were normalised to GAPDH mRNA expression levels. Relative fold change expression was determined by setting the vehicle treatment (EtOH) to 1. Histograms represent pooled data from ten independent experiments from ten female donors plotted as mean ± SEM. Statistical significance was determined by a non-parametric Wilcoxon signed-rank test with the hypothetical values set to the vehicle control comparing each sample to the vehicle control (EtOH) with * and ** denoting $p<0.05$ and $p<0.01$ respectively.



<u>Figure 3.12 continued:</u> mRNA levels of select immunomodulatory genes after 9 days in the presence of ligands and after 7 days in the absence and presence of HIV-$1_{BaL\text{-}Renilla}$. PHA activated PBMCs were pre-treated with 100 nM Cortisol (CORT), DEX, MPA, P4, NET or LNG, with vehicle control (0.01% EtOH), for 48 hours. Pre-treated PBMCs were infected with 10 IU/ml HIV-$1_{BaL\text{-}Renilla}$ IMC for 2 hours, washed extensively and the media replaced. Media with the respective ligands was replaced every second day. PBMCs were harvested at 7 days post infection in TriReagent® and subsequently processed for RNA. 250 ng RNA was reversed transcribed to cDNA (Roche Applied Science, South Africa), where it was used in subsequent real time qPCR as template to determine the differential expression levels. Relative mRNA expression levels of RANTES (C) and GILZ (D) were normalised to GAPDH mRNA expression levels. Relative fold change expression was determined by setting the vehicle treatment (EtOH) to 1. Histograms represent pooled data from ten independent experiments from ten female donors plotted as mean ± SEM. Statistical significance was determined by a non-parametric Wilcoxon signed-rank test with the hypothetical values set to the vehicle control or a paired Wilcoxon matched-pairs signed rank test comparing each sample to the vehicle control (EtOH) with *, and *** denoting $p<0.05$ and $p<0.001$ respectively.

MPA induced increase in HIV-1 replication correlates with an increase in IL6 mRNA expression level in primary PBMCs

The differential effects of the progestogens on HIV-1 replication and mRNA gene expression after 9 days, suggested that there may be a correlation between effects on expression of immunomodulatory genes and HIV-1 replication Thus the differential effects of the different ligands on HIV-1 replication after 9 days, may be, in part, due to their differential effects on select immunomodulatory gene expression. Upon analysis of

the infection data, it was evident that in the MPA treated samples; three donor samples exhibited a decrease in HIV-1 replication, while seven donor samples exhibited an increase in HIV-1 replication. In addition, the loss of IL6 mRNA repression by MPA (Fig. 3.12) suggested that there may be a relationship between IL6 expression levels and HIV-1 replication. As such, the data was analysed to determine whether there was any correlation between HIV-1 replication and gene expression. When mean relative infection levels were correlated with their matched relative IL6 mRNA expression levels, there was a significant positive association between MPA IL6 mRNA expression and relative MPA infection levels (Pearsons r 0.7826, p value 0.0074; Fig. 3.13 A). That is, low relative infection levels correlated with low relative IL6 mRNA expression levels, while high relative infection levels were associated with high relative IL6 mRNA expression levels after 9 days, as seen in Figure 3.13 B. In addition there was a weak positive correlation between DEX IL6 mRNA expression and HIV-1 replication (Pearsons r 0.6939, p value = 0.0381; see Appendix A, Fig. A.3.1), while CORT, P4, NET and LNG had no correlation between IL6 mRNA expression levels and HIV-1 replication (data not shown).

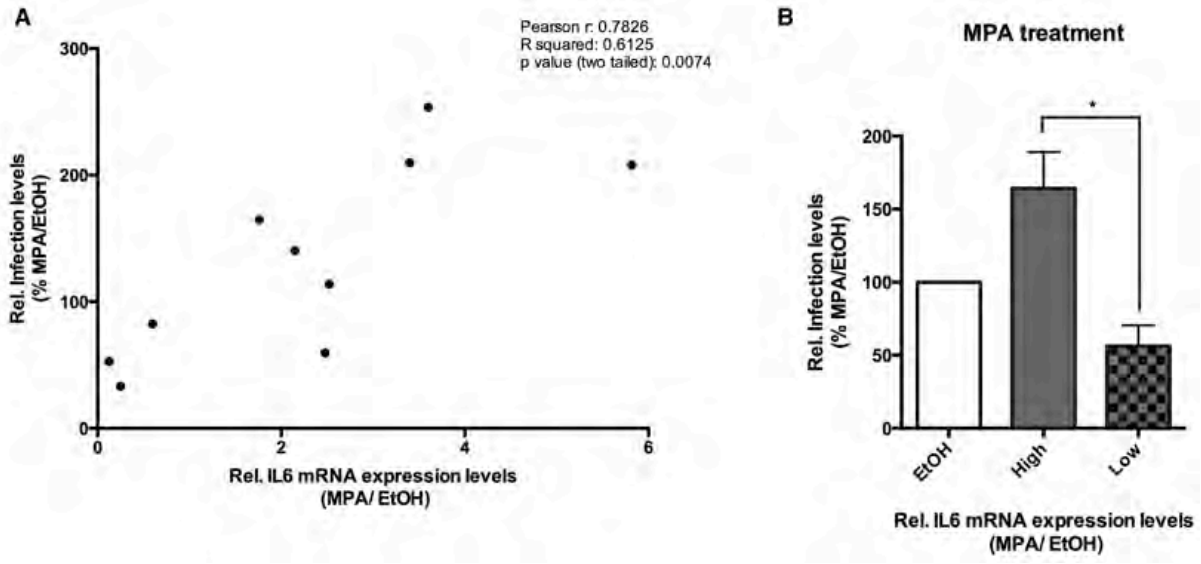


Figure 3.13: Higher MPA infection levels positively correlate with higher IL6 mRNA expression levels. (A) Relative MPA infection levels, normalised to vehicle treatment (EtOH) were plotted against matching relative IL6 mRNA expression levels, normalised to vehicle treatment (EtOH). Relative IL6 mRNA transcript levels were determined by comparing C(t) values to a standard curve derived from a serial dilution of pooled cDNA (from the experimental repeats) with a known concentration. An XY scatter plot represents 10 individuals with mean infection levels on the Y-axis and IL6 relative mRNA expression on the X-axis. A Pearsons correlation analysis (comparing r for X vs. Every Y data set) was performed with a two-tailed post-test t-test analysis. (B) MPA infection levels were grouped according to low relative IL6 mRNA expression levels or high relative IL6 mRNA expression levels. Relative infection data from Fig. 3.10 was used to plot (B), with vehicle control (EtOH) set to 100%. The histogram is representative of 3 (low IL6) and 7 (high IL6) biological repeats plotted as mean ± SEM. Statistical significance was determined by an unpaired student t-tests comparing the two groups, with * denoting $p<0.05$ (actual p value = 0.0279).

Figure 3.14 shows the results for the pooled data of the 7 out of 10 donor samples in which an increase in HIV-1 replication was observed upon stimulation with 100 nM MPA after 9 days. For donors where relative IL6 mRNA expression was elevated relative to the vehicle control (Fig. 3.14 A), HIV-1 replication (Fig. 3.14 B) appears to be elevated in CORT and DEX (4 out of 7 donors) samples, while HIV-1 replication is significantly up-regulated in all MPA treated samples (174% mean relative infection levels). This was

confirmed, with DEX treated samples also exhibiting a positive correlation with relative IL6 mRNA expression levels and HIV-1 replication (Appendix A, Fig. A.3.1), while CORT had no correlative effect. In Fig. 3.14 A, CORT, DEX and MPA samples, IL6 mRNA expression levels are elevated in the uninfected group, and appear to further increase in the HIV-1 infected group, while P4, NET and LNG have no effect on IL6 mRNA gene expression. Both P4 and LNG responses are still varied in this grouping with an increase of HIV-1 replication observed in 3 out of 7 samples. Interestingly, NET treated samples appear to have no change in HIV-1 replication (92% mean relative infection levels) compared to the vehicle control (EtOH).

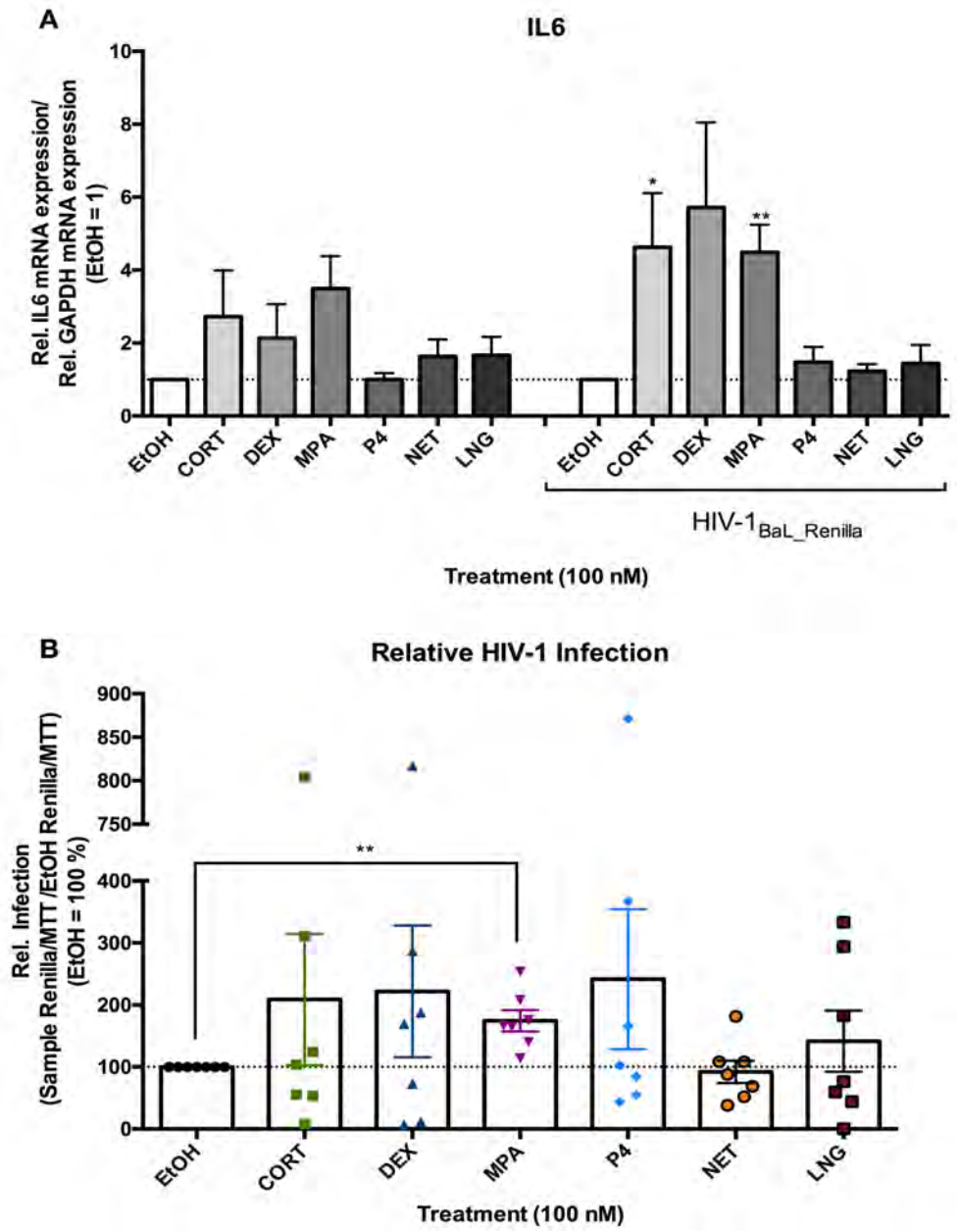


Figure 3.14: MPA only increases HIV-1 infection in primary PBMCs when IL6 mRNA repression by MPA is lost. (A) A loss of IL6 mRNA repression in the presence of HIV-1 correlated with (B) Increased MPA induced relative infection levels. Histograms represent pooled data of seven independent experiments from seven female donors plotted as mean ± SEM. Statistical significance was determined with a non-parametric Wilcoxon signed-rank test with the hypothetical value set to the vehicle control or with a paired Wilcoxon matched-pairs signed rank test comparing each sample to the vehicle control (EtOH) with * and ** denoting $p<0.05$ and $p<0.01$ respectively.

Interestingly when relative IL6 mRNA expression was low in the HIV-1 infected group (Fig. 3.15 A), HIV-1 replication was low in all conditions (Fig. 3.15 B). Indeed, CORT, DEX and MPA appear to be protective in the group compared to P4, NET and LNG that have similar infection levels to the vehicle control. The relative IL6 mRNA expression patterns appear to be similar between HIV-1 uninfected and infected groups. In Fig. 3.15 A, CORT, DEX significantly repressed IL6 mRNA expression levels compared to the vehicle control, while MPA appeared to have no effect on IL6 mRNA expression levels compared to the vehicle control in the presence of HIV-1, which is mimicked in the HIV-1 infected group. Interestingly in the HIV-1 negative control, only DEX repression was maintained similar to the HIV-1 infected group, while this was not true for the CORT and MPA treated samples.

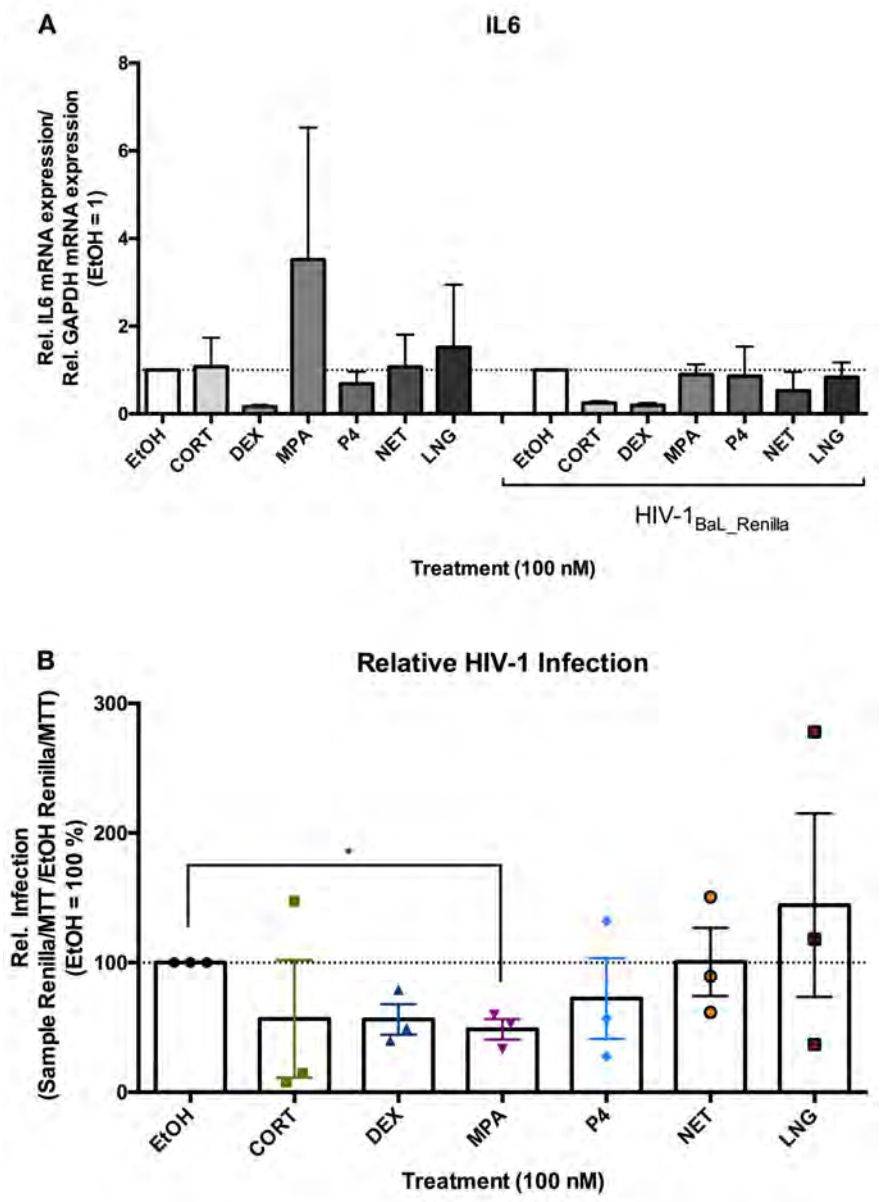


Figure 3.15: DEX and MPA decrease HIV-1 infection when IL6 mRNA repression is maintained in the presence of HIV-1. (A) Maintenance of IL6 mRNA repression by DEX and MPA in the presence of HIV-1 corresponded with (B) a decrease in HIV-1 replication. Histograms represent pooled data from three independent experiments from three female donors plotted as mean ± SEM. Statistical significance was determined with a non-parametric Wilcoxon signed-rank test with the hypothetical value set to the vehicle control or unpaired student t-tests (for the parametric MPA group) comparing each sample to the vehicle control (EtOH) with *,denoting $p<0.05$.

The data suggest that responses to MPA treatment over time may vary, and could be population dependent. In one population (7/10 donors) MPA is no longer able to repress IL6 mRNA gene expression and increase HIV-1 infection, while in the other population (3/10 donors), repression is maintained on IL6 mRNA gene expression and HIV-1 replication is similar to reduced, compared to the control treatment. Additionally, it appears that the population that is able to maintain relative repression of IL6 mRNA levels is smaller than those that lose the ability to repress IL6 mRNA levels. When assessing the effects on gene expression of IL8, RANTES and GILZ between these groups, it was found that IL8 and RANTES mRNA levels were not repressed in the high HIV-1 replication group (Appendix A, Fig. A.4.1), while CORT and DEX appeared to repress IL8 mRNA levels in the group that had low HIV-1 replication levels, MPA had no effect (Appendix A, Fig. A.4.2). Interestingly it appeared that MPA increased RANTES expression in the group that had low HIV-1 viral replication (Appendix A, Fig. A.4.2). GILZ transactivation was maintained in both groups (Appendix A, Fig. A.4.1 and A.4.2), showing that the differential effects on pro-inflammatory genes are unlikely to be due to factors such as loss of GR activity or RNA degradation. Despite the interesting results, IL8, RANTES and GILZ mRNA expression did not correlate with HIV-1 relative infection levels (data not shown).

DEX and MPA repression on IL6, IL8 and RANTES is time dependent

Comparing the 48 hour gene expression data, with DEX and MPA treatment in the absence of HIV-1 and PBMC activation (Fig. 3.1), to the gene expression data after 9 days treatment with DEX or MPA (Fig. 3.12), suggested that there may be a potential switch from repression to no repression of relative IL6 mRNA expression. As such, it was hypothesised that the differential regulation mediated by DEX and MPA on immunomodulatory gene expression may be time dependent. In order to address this question, PBMCs were stimulated with 100 nM DEX or MPA and samples were harvested every day from day 2 to day 7, where viability was assessed by MTT, RNA was harvested for gene expression studies, and protein was harvested for western blot analysis. IL6 mRNA gene expression analysis indicated (Fig. 3.16 A) that DEX repressed mRNA levels at days 2, 3 and 4, but that this repression was lost at days 5, 6 and 7. Similarly MPA significantly repressed IL6 mRNA levels at day 2, with expression levels reduced at days 3 and 4, while IL6 mRNA levels at days 5, 6 and 7 were elevated. DEX and MPA exerted similar regulatory patterns on relative IL8 mRNA levels (Fig. 3.16 B). However, unlike the relative IL6 mRNA levels, IL8 mRNA levels were variable throughout the time course, maintaining reduced or similar mRNA levels compared to the control treatment at days 5, 6 and 7. RANTES mRNA levels were modulated by DEX and MPA in a similar manner to IL8 mRNA levels (Fig. 3.16 C), with DEX having stronger repressive effects on mRNA expression at days 2, 3 and 4 compared to MPA that only exhibited significant repressive effects at day 2. DEX and MPA no longer exerted any repressive effects at days 5, 6 and 7 (Fig. 3.16 C). Interestingly GILZ transactivation by DEX and MPA was maintained throughout the duration of the time course (Fig. 3.16 D), with DEX having stronger transactivation effects than MPA, suggesting that as for the previous section, factors such as loss of RNA or receptor integrity are not involved. Interestingly, basal expression levels of

IL6 and IL8 decreased over time more so than RANTES, while GILZ mRNA levels remained similar to basal conditions (EtOH) at day 2 (Appendix A, Fig. A.5.2).

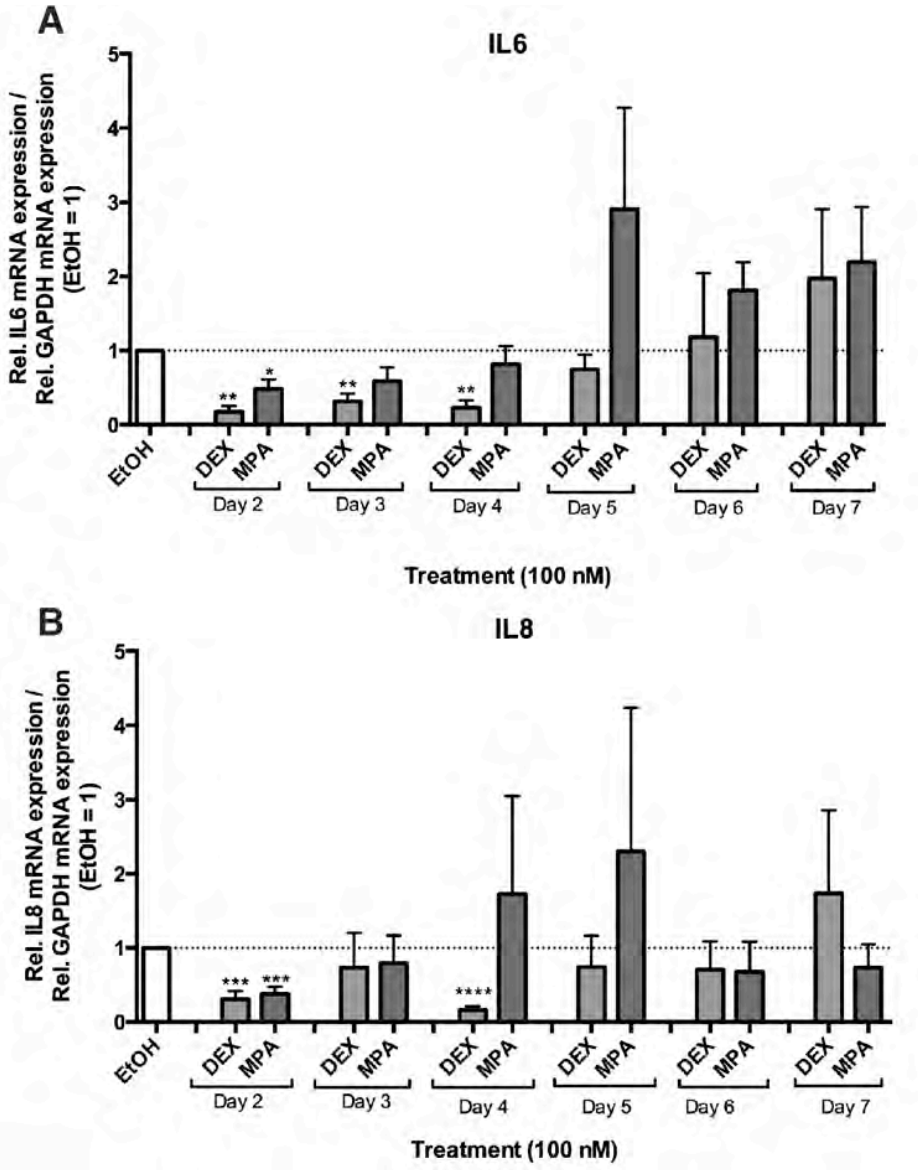


Figure 3.16: DEX and MPA-mediated repressive effects on select genes is lost over time, while transactivation effects are maintained. PHA activated PBMCs were pre-treated with 100 nM DEX, MPA or 0.1% (*v/v*) vehicle control (EtOH) for 7 days with half media exchange at day 3 and 6. Samples were harvested every day from day 2 to day 7 for RNA isolation. 250 ng RNA was reverse transcribed to cDNA and used as template in subsequent real time qPCR. Relative mRNA expression levels of IL6 (A) and IL8 (B) were normalised to GAPDH mRNA expression levels. Relative fold change in expression was determined by setting vehicle treatment (EtOH) at each day to 1. Histograms represent pooled data from four independent experiments from four female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a Dunnett's post-test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with *, **, *** and **** denoting $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ respectively.

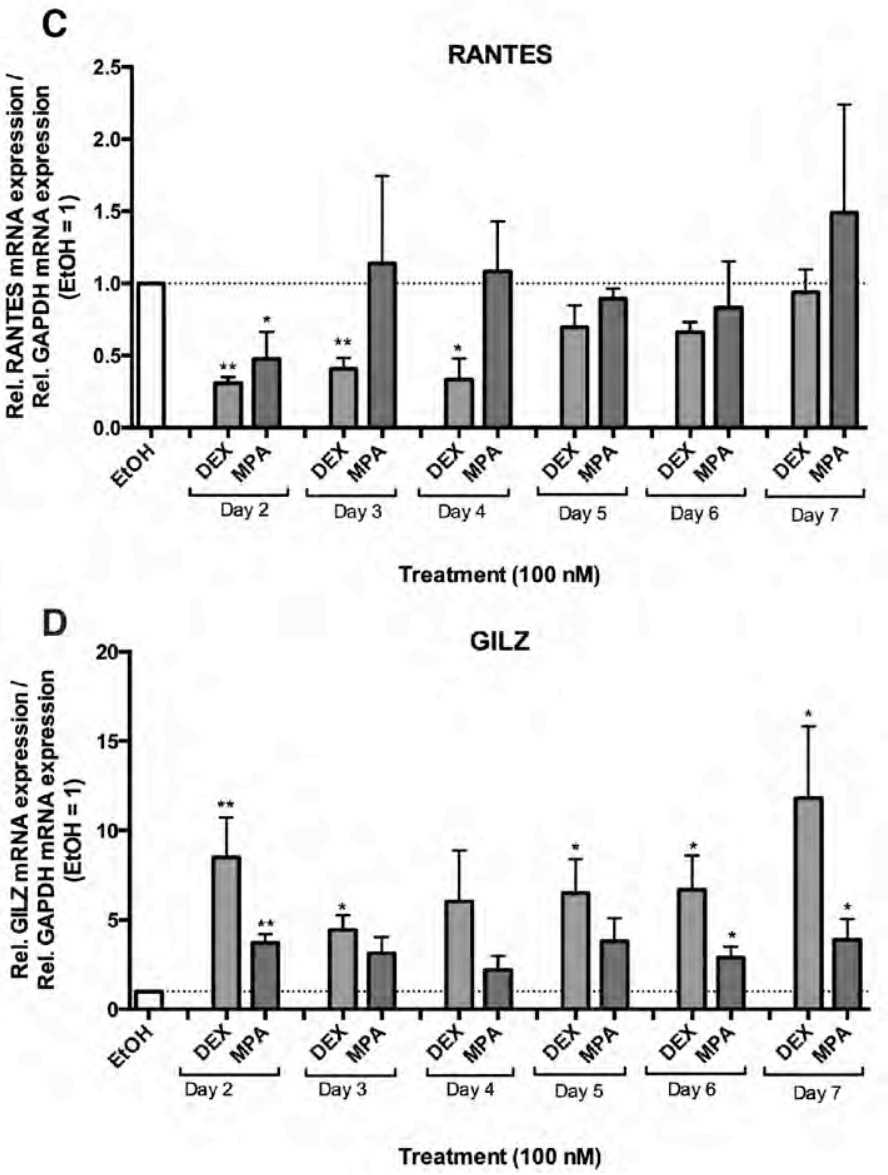


Figure 3.16 continued: DEX and MPA-mediated repressive effects on select genes is lost over time, while transactivation effects are maintained. PHA activated PBMCs were pre-treated with 100 nM DEX, MPA or 0.1% (*v/v*) vehicle control (EtOH) for 7 days with half media exchange at day 3 and 6. Samples were harvested every day from day 2 to day 7 for RNA isolation. 250 ng RNA was reverse transcribed to cDNA and used as template in subsequent real time qPCR. Relative mRNA expression levels of RANTES (C) and GILZ (D) were normalised to GAPDH mRNA expression levels. Relative fold change in expression was determined by setting vehicle treatment (EtOH) at each day to 1. Histograms represent pooled data from four independent experiments from four female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a Dunnett's post-test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with * and, ** denoting $p<0.05$, and $p<0.01$ respectively.

In order to elucidate the mechanism of this loss of IL6 mRNA repression over time, cell viability and steroid receptor expression levels were analysed. Cell viability as assessed by MTT, indicated that cell viability significantly decreased over time (Fig. 3.17 A). DEX treatment resulted in a 50% loss of cell viability, while MPA treatment resulted in a 20% loss in cell viability at the end of the time course. It could be that the loss of viability could affect gene expression, however GILZ transactivation by DEX and MPA was maintained over the time course (Fig. 3.17 D).

The effects on gene expression may not only be as a result in loss of cell viability, but a change in the relative expression levels of other transcription factors. Since it has been established that DEX and MPA mediate their effects via steroid receptors, AR, ER, MR, PR and GR levels were assessed in order to elucidate whether changes in their expression levels correlated with the changes in mRNA levels. DEX and MPA are partial agonists for the MR (Africander et al., 2011) and the AR (Africander et al., 2014). MPA is a full agonist for the PR and partial agonist for the GR, while DEX is a full agonist for the GR with no PR activity (Africander et al., 2011). Additionally, it has been shown in some studies that steroids are known to regulated the expression of other steroid receptor genes (Acconcia & Kumar, 2006; Mesiano et al., 2002; De Amicis et al., 2009). As such, receptor levels were assessed by western blot for the AR, GR and PR. Several MR antibodies were used in duration of this study, however the antibodies used were either non-specific or did not produce convincing western blots. Thus real time qPCR was also performed to investigate MR mRNA levels. In the DEX treated samples, MR mRNA levels are slightly elevated over time, with more MR mRNA levels at days 5, 6 and 7 (Fig 3.17 B). However, MR mRNA expression levels remain unchanged in the MPA treatment of samples throughout the time course, and at similar expression levels compared to the control treated samples (Fig. 3.17 B). Neither the AR nor the PR protein were detectable via western blot at any of the time points tested (data not shown), suggesting that the AR and PR are not involved in this loss of repression effect. GR was detected by western blotting at all the time points in this experimental sample set (Fig. 3.17 D). DEX-mediated turnover of the GR protein remained constant over the duration of the experiment (Fig. 3.17 C and D) while MPA treated samples did not appear to cause turnover of the GR (Fig. 3.17 C and D). Taken together the data highlight that the repressive effects on mRNA gene expression by DEX and MPA is time dependent and that cell viability decreases concomitantly with a loss of the repression observed. Additionally, the data indicate that GR, PR and AR receptor levels do not change over time in response to ligand treatment.

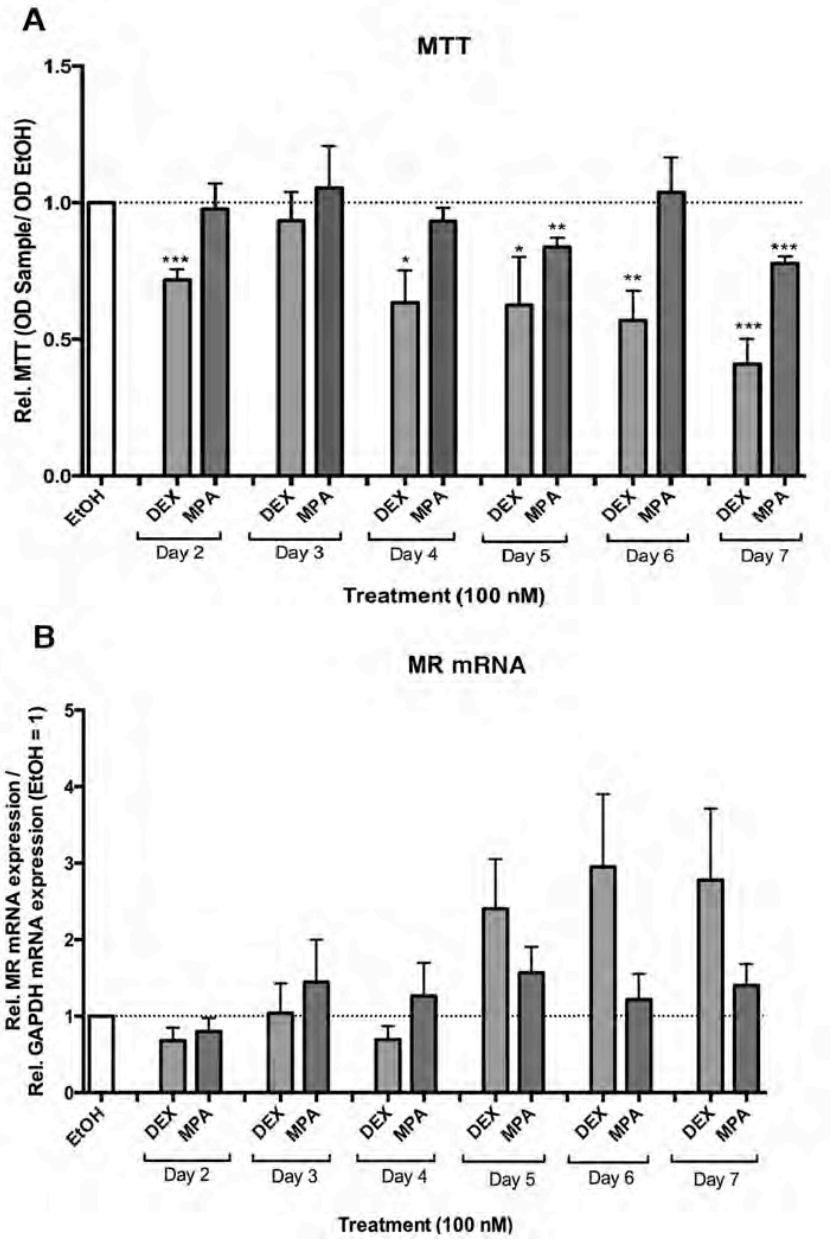


Figure 3.17: Cell viability, MR mRNA expression levels or GR protein levels do not change or correlate with the loss of DEX and MPA-mediated effects over time. PHA activated PBMCs were pre-treated with 100 nM DEX or MPA or 0.1% (*v/v*) vehicle control (EtOH) for 7 days with half media exchange at day 3 and 6. (A) MTT assays were performed every day from day 2 to day 7 and cell viability was measured spectrophotometrically. Samples were harvested every day from day 2 to day 7 for RNA and protein isolation. 250 ng RNA was reverse transcribed to cDNA and used as template in subsequent real time qPCR. Relative mRNA expression levels of MR (B) were normalised to GAPDH mRNA expression levels and relative fold change in expression was determined by setting vehicle treatment (EtOH) at each day to 1. Histograms represent pooled data from four independent experiments from four female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a Dunnett's post-test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with *, **, *** denoting $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

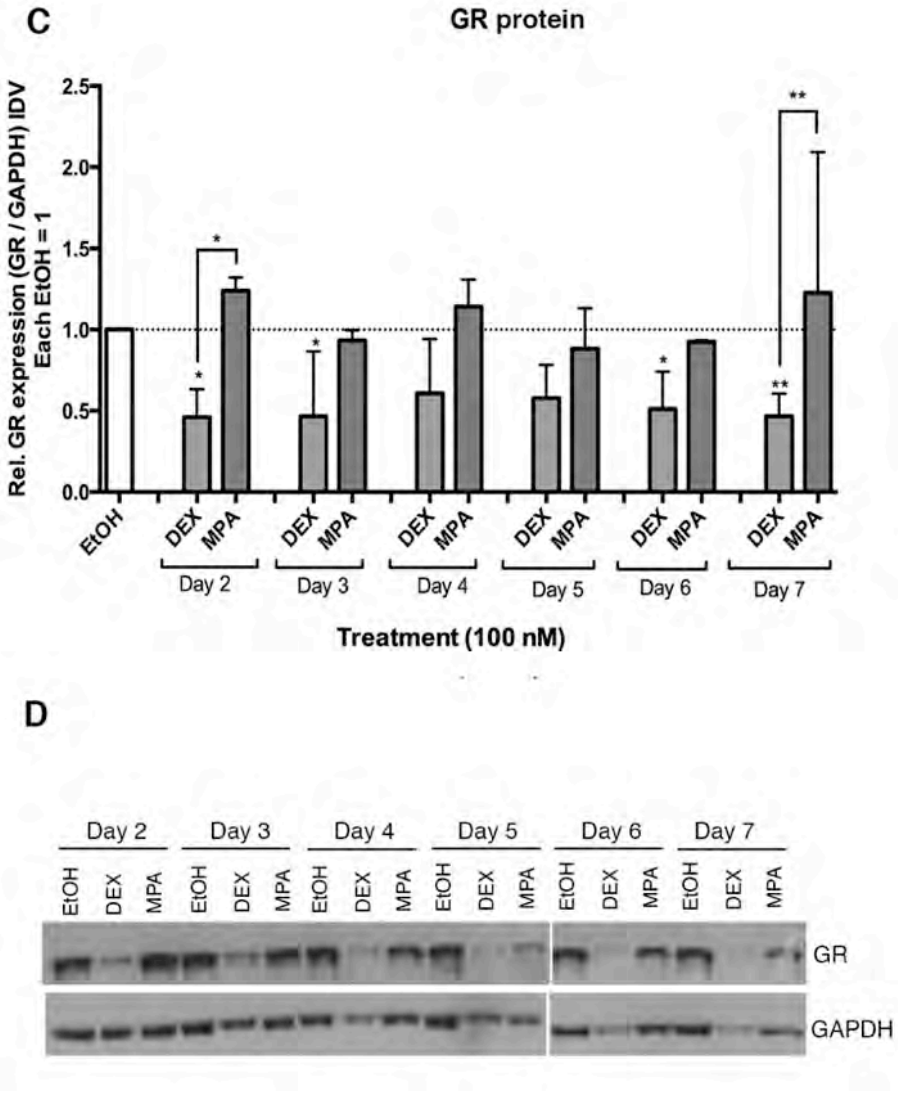


Figure 3.17 continued: Cell viability, MR mRNA expression levels or GR protein levels do not change or correlate with the loss of DEX and MPA-mediated effects over time. PHA activated PBMCs were pre-treated with 100 nM DEX or MPA or 0.1% (*v/v*) vehicle control (EtOH) for 7 days with half media exchange at day 3 and 6. Protein samples, isolated using TAPS buffer, were electrophoresed on an 8% SDS-PAGE gel before being electroblotted onto a nitrocellulose membrane and probed for GRα and GAPDH. Nitrocellulose blots were incubated for 1 minute in ECL-chemiluminescent substrate before being visualised by audio-radiography. (C) Represents the average GR protein expression normalised to GAPDH at each day, determined by densometric analysis (AlphaEC™, Alpha Innotech) with (D) a representative western blot used in the analysis. Histograms represent pooled data from four independent experiments from four female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a Dunnett's post-test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with * and ** denoting $p<0.05$ and $p<0.01$ respectively.

Discussion

PBMCs are important mediators in the systemic immunity, and contain an array of immune cells including monocytes, macrophages and T cells. These cells respond to numerous stimuli to orchestrate an effective immune response (Murphy et al., 2012). As such, PBMCs are a relevant model in which to elucidate the effects of progestogens on systemic immune function. Additionally, PBMCs consist of important target cells for HIV-1 infection, and are thus a useful model in which to study the effects of HIV-1 infection and viral dissemination (Pope & Haase, 2003; Haynes & Shattock, 2008).

The relative composition of cell types within PBMCs is variable from donor to donor. As such a caveat of PBMC research is the high variability of responses between donors, which could potentially mask significant effects. Several papers have used different strategies in which to address the high variability within PBMCs. One approach would be to measure responses from individual cell types like Huijbregts et al. (2013). The authors separated PBMCs into different cell types and analysed the effects of MPA and DEX on gene expression and HIV-1 infection (Huijbregts et al., 2013). A second approach would be to choose a donor that is representative of the effects observed, like Asin et al. (2008). The authors reported on the effects of HIV-1 replication to differing concentrations P4 and E2 in PBMCs and reported their data using one patient's responses as a representative of their experiments (Asin et al., 2008). The third approach would be to pool data together from samples in which a particular response was observed. For example, Kleynhans et al. (2011) presented only pooled donors that responded to BCG in their figures (Kleynhans et al., 2011). For this current study, no donors were excluded from the analysis and where possible, flow cytometry was performed. Additionally, where appropriate responses were grouped to assess biological differences.

The effects of progestogens on gene regulation in PBMCs

This study shows that DEX and MPA have immunosuppressive effects in modulating both mRNA and protein expression levels of key immunomodulatory genes, while P4 and NET-A have little-to-no repressive effects on these genes in PBMCs, upon incubation up to 2 days. The results from this study show for the first time that MPA, unlike NET and P4, dose (Fig. 3.2) and time (Fig. 3.1) dependently decrease IL6, IL8, RANTES (to a lesser extent) and increase anti-inflammatory GILZ mRNA expression in primary un-stimulated PBMCs. Furthermore this study indicated that IL6 protein was predominantly expressed in the CD14+ monocytes, where at 100 nM, MPA significantly repressed IL6 protein expression, while NET, P4 and LNG had no effect after 48 hours (Fig. 3.6). Birx et al. (1990) also observed that IL6 expression was predominantly from CD14+ monocytes (Birx et al., 1990), supporting the observation in this study. Similarly, MPA, like DEX, increased GILZ protein expression in CD4+ T cells, while P4, NET and LNG had no effect (Fig. 3.6). These findings are supported by several studies from the Hapgood laboratory that show that MPA compared to NET and P4 differentially affects immunomodulatory genes. Africander et al., (2011) reported that IL6 expression was down-regulated at the mRNA level in ectocervical and vaginal cell lines

treated with 1 µM MPA compared to 1 µM NET or P4 after 24 hours (Africander et al., 2011). Similarly, Govender et al. (2014) found that MPA but not NET-A or P4 decreased IL6, IL8 and RANTES, and increased GILZ mRNA and protein expression levels in an endocervical cell line after 24 hours in a dose dependent manner (1 nM – 1000 nM) (Govender et al., 2014). These results are similar to what was reported in Fig. 3.1. However, in PBMCs, the greatest repressive effect on mRNA gene expression by MPA was exerted after 48 hours (Fig 3.1), suggesting that different cell types may respond differentially to progestogens at different times. Similar to what was reported in this study, Kurebayshi et al. (2003) observed that IL6 protein secretion levels were significantly reduced in a thyroid cancer cell line after 48 hours stimulation with MPA at 10, 100 and 1000 nM (Kurebayashi et al., 2003). Additionally, Vazques-Tello et al. (2012) observed that DEX increased GILZ and decreased IL6 mRNA expression levels in PBMCs from asthmatic patients (Vazquez-Tello et al., 2012), consistent with the results observed in this study by DEX on gene expression (Fig.3.1, 3.2, 3.3, 3.6 and 3.8).

The results from this study also show that at 100 nM, MPA like DEX, significantly decreased protein expression levels of IL8, IL-1β (Fig. 3.4) and IL-1ra (Fig. 3.5) and appeared to reduce IL10 protein levels after 48 hours (Fig. 3.5 and appendix A, Fig. A1). Additionally, Figure 3.5 shows that DEX had greater effects on protein expression than MPA. DEX treatment significantly and dose dependently (at 10 and 100 nM) decreased expression levels of IL-1ra, VEGF, IL7, IL12 (p70), TNF-α and RANTES compared to the vehicle control. Like the mRNA expression data, P4 and NET had no effect on protein expression levels (Fig. 3.4, 3.5 and appendix A Fig A.1) with the exception of IL12, which was decreased with 10 nM NET compared to the vehicle control (Fig. 3.5). The results from the present study are in agreement with Huijbregts et al. (2013), where MPA, but not P4 dose dependently decreased IL6, IL-1β and IL-1ra (at 100 and 1000 nM) after 24 hours (Huijbregts et al., 2013). Interestingly, in the Huijbregts et al. (2013) paper, the authors observed no significant repression on IL8 with MPA treatment after 24 hours unlike the results from this study (Fig. 3.2 and 3.4), which found significant repressive effects by MPA on IL8 mRNA and protein expression after 48 hours. This could be due to the different time point between the studies, where repressive effects on IL8 repression were only observed after 48 hours (Fig. 3.1.). Enomoto et al. (2007) found in PBMCs that DEX (0.3 nM), unlike P4 (1 nM) and E2 (1 pM) significantly reduced Th1 cytokine production after 72 hours, reducing IL2, IFN-ɣ and IL12 protein secretion, while E2 and P4 treatment had no effect. In addition, DEX, but not E2 or P4, treatment significantly reduced select pro-inflammatory cytokines (IL-1β, IL6 and GM-CSF) and Th2 cytokine responses (IL10 and IL4, with no effect on TGF-β) (Enomoto et al., 2007). Kleynhans et al. (2011) reported that PBMCs from women using MPA had decreased IL10 and IL12 cytokine protein expression profiles compared to women not on MPA in response to BCG stimulation. In addition the authors observed in BCG-stimulated PBMCs, that MPA to a similar extent as cortisol (CORT) repressed IL6, TNFα, IL-1ra, IL-13 and GM-CSF after 6 days post infection at concentrations within the serum contraceptive range (1 nM – 100 nM) (Kleynhans et al., 2011).

The gene expression data in this study (Fig. 3.1, 3.2 and 3.3) suggest that MPA is likely to exert anti-inflammatory immunosuppressive effects via GILZ gene expression within the range of concentrations found in the serum of injectable contraceptive users (1-100 nM), as well as possibly on IL6 and IL8, particularly shortly after injection. However, the current study suggests that the immunosuppressive effects of NET-EN on PBMCs via the GR are likely to be negligible, even shortly after injection, while P4 is unlikely to exert immunosuppressive effects in PBMCs even during pregnancy (highest P4 levels) via these genes. Interestingly, the Hapgood laboratory have recently shown that the GR may also play a key role in PBMCs in discriminating between apoptotic effects by DEX and MPA, unlike NET and P4, at doses within the contraceptive range, in the absence and presence of HIV-1 infection (Tomasicchio et al., 2013). The physiological consequences of immunosuppressive and apoptotic effects of MPA on PBMCs could include a wide range of effects that are beneficial to the virus. These could include compromising the ability of the host to mount effective innate and adaptive immune responses, as well as increasing binding of HIV-1 to CCR5 co-receptors. Interestingly, mRNA expression data (Fig 3.3) suggest that MPA has slight to no effects on mRNA expression levels at 10 nM, weakly transactivating GILZ mRNA expression, and slightly reducing IL6 mRNA expression. Strikingly, this experiment shows that P4 and NET treatment may elevate the mRNA expression levels of IL6, IL8 and RANTES (although not significant), in a manner similar to Africander et al. (2011), which found some pro-inflammatory effects with P4 on IL6 and IL8 gene expression in TNF-α treated Ect1/E6E7 and Vk2/E6/E7 cell lines (Africander et al., 2011). While MPA at 10 nM does not greatly affect mRNA expression levels, its expression patterns are different to P4 and NET, indicating that even at low serum concentrations, MPA and NET might modulate gene expression differentially. In addition to concentration, the results of this study suggest that time has an important role on the immunosuppressive effects of MPA in PBMCs (Fig. 3.1), with little to no repression observed at 4 and 24 hours, and significant repressive effects at 48 hours post incubation. This could be due to changes in expression levels of other proteins involved in the responses, or changes in the relative cellular population or in their activation state over time.

The data in this study suggest that DEX, more so than MPA, has greater effects on immune gene expression than P4 or NET. The Hapgood laboratory has shown previously that MPA exhibits partial GR agonist properties, as well as exhibiting differential effects on gene repression in a promoter specific manner (Ronacher et al., 2009; Koubovec et al., 2004; Koubovec et al., 2005; Govender et al., 2014). In addition the authors provided evidence whereby ligand-selective interaction between the GR and different co-activators and co-repressors GRIP-1, SRC-1A, NCoR and SMRT was a major determinant of ligand-selective and promoter-specific differences in potency and efficacy, for both transactivation and transrepression (Ronacher et al., 2009). Thus, MPA may differentially regulate cytokine gene expression, in a promoter-specific manner, having greater effects on IL6, IL8 and GILZ mRNA and protein expression than on RANTES mRNA and protein expression, like the data in this study suggest (Fig. 3.2, 3.4, 3.5 and 3.6). In addition MPA, in the same manner, may repress IL-1ra (Fig. 3.5) and IL-1β (Fig. 3.4) protein expression more effectively than IL10 (Fig. 3.4), VEGF, IL7, IL12 and TNF-α expression (Fig 3.5). It is

apparent from this study as well as others that MPA, like DEX has strong immunomodulatory effects, and that the extent of repression depends not only on the concentration of MPA, but also on its selective potency towards target genes. This has been observed by Govender et al. (2014) where the authors found that MPA had differential potencies for different target genes in the same cell line (Govender et al., 2014). It has also been observed that MPA, unlike NET increases arterial thrombosis in ovarectomised apolpoprotein E-deficient mice. Transcriptome analysis indicated that MPA had a distinct and different transcriptional profile compared to NET treated mice (Freudenberger et al., 2014).

Taken together the results from this study are supported by the findings in the literature, and highlight that dose and time have significant implications on the effects observed by the different progestogens. Furthermore the results from this study show for the first time that MPA unlike the progestogens P4, NET and LNG differentially modulates immune gene expression in a manner similar to the glucocorticoid DEX, and these effects are more pronounced at concentrations in the range of the peak serum concentration in MPA users in PBMCs.

The repressive effects on gene expression by MPA are mediated by the GR

In order to determine the role of the glucocorticoid receptor in mediating cytokine gene repression, PBMCs were treated with 100 nM DEX or MPA in the absence and presence of RU486, a known GR/PR antagonist. In Figure 3.8, IL 6, IL 8 and RANTES repression by DEX and MPA was lifted in the presence of RU486, while DEX and MPA induction of GILZ was lifted by RU486 (Fig. 3.8 A-D). These results indicate that the GR is involved in the regulation of these cytokine genes. Similarly Huijbregts et al., (2013) found that RU486 treatment with MPA, lifted the MPA-mediated repression of IFN-γ protein expression in PBMCs and CD3+ cells. The data suggest that MPA exerts its repressive effects through the GR, and that this affects both mRNA (Figure 3.8), and protein expression (Huijbregts et al. 2013). Similar to what was observed in this current study on a GR transactivation gene, GILZ, Thomas et al. (2006) observed in a canine kidney C7 cell line that MPA, like DEX, increased SGK-1 gene expression via the GR, and that this increase in expression was lost using the GR/PR selective antagonist RU486, but not with the PR selective antagonist Org31710 (Thomas et al., 2006).

While the data from this study suggest GR involvement, RU486 is also known to antagonise the PR. However, it has been shown by Tomasicchio et al. (2013) and in this study (Fig. 3.7), that PBMCs do not express the PR. In addition, Bamberger et al. (1999) found no detectable expression of the PR (both mRNA and protein) in the primary T lymphocytes, while were able to readily detect the GR (Bamberger et al., 1999), in agreement with the results seen in Figure 3.7 and those observed in Tomasicchio et al. (2013). Thus it is most likely that the effects observed on gene expression in the presence of DEX and MPA were mediated via the GR. To further support this finding, PBMCs were nucleofected with GR-specific siRNA, to reduce the expression of the GR. The effects of reduced GR levels on mRNA transcription levels were assessed by real time qPCR (Fig. 3.9). While only moderate GR knockdown was observed in this

experiment, we show for the first time in PBMCs, that the GR knockdown supports the finding that the GR is involved in DEX and MPA mediated repression of IL6, IL8 and RANTES mRNA expression (Fig. 3.9). This data is in agreement with Govender et al. (2014) who found that upon GR knockdown in the endocervical cell line End1/E6E7, MPA and DEX repression on IL6, IL8 and RANTES was mediated by the GR at both the mRNA and protein expression levels of IL6 and IL8 (Govender et al., 2014). In addition, their results, like those obtained in this study, found that P4 and NET gene regulation were not affected by GR knockdown (Govender et al., 2014). Interestingly, a study in human myometrial cells, found that MPA at 1 μM, reduced the expression of an IL-1β-driven gene, COX-2, and that this repression was mediated via the GR even in the presence of overexpressed human PRB. Using both RU486 and a specific PR antagonist Org31710, the authors also found that P4 (at 10 μM) exerted this repression via the GR (Lei et al., 2012). This study suggests that P4 is able to exert some of its repressive functions via the GR, consistent with the findings observed by Koubovec et al. (2004), and that the concentration of the progestogen is important when considering the effects of these progestogens on immune function. Nevertheless, the data in this study suggests that P4 at 100 nM, had no effect via the GR (Fig. 3.9) and may be due to the fact that lower concentrations were used.

To further explore the role of the GR in regulating gene transcription, cycloheximide experiments were performed in this study (Fig 3.10). Cycloheximide inhibits *de novo* protein synthesis, as such one is able to determine whether the effects are due to direct effects by proteins already present, or if they require additional proteins for the response, that is, indirect effects. Govender et al. (2014) found that in an endocervical cell line, MPA-mediated effects on IL8, GILZ and RANTES mRNA levels were mediated at least in part via the GR, but required the addition of new proteins in which to fully exert the repressive effects. The authors observed that the effects on GILZ and IL6 mRNA levels were due to direct effects mediated via the GR. Additionally the chromatin immuno-precipitation (ChIP) results found that the GR was directly recruited to target sites within the promoters of IL6 and GILZ, consistent with the direct effects of the GR on transcription (Govender et al., 2014). GILZ mRNA expression in PBMCs appeared to be modulated via direct effects (Fig. 3.10), with increased levels of mRNA expression observed in both DEX and MPA treated samples, and was similarly observed in Govender et al. (2014). Interestingly, GILZ expression has been found to be involved in inhibiting T cell receptor induced IL2 receptor expression and NFκB activity (Ayroldi, 2001). In addition, it has been found that GILZ prevents NFκB translocation into the nucleus, and transcription through direct interaction with the p65 subunit in T cells (Ayroldi & Riccardi, 2009; Di Marco et al., 2007), and that overexpression of GILZ in lung epithelial cells (BEAS-2B) prevents NFκB activation by LPS, IL-1β and poly I:C (polyinosinic:polycytidylic acid) (Eddleston et al., 2007). Thus, it could be that MPA-driven repression on IL6, IL8 and RANTES could, in part, be due to the effects of GILZ protein interference on NFκB-driven genes, supporting the cycloheximide data for indirect effects modulating gene transcription in PBMCs (Fig.3.10).

The results from this study strongly support the hypothesis proposed by Hapgood et al. (2004) that MPA, at serum contraceptive concentrations, modulates key immune genes via the GR, while P4 and NET do not. This study is the first to report that the differential effects of gene expression by MPA compared to P4 and NET is via the GR in primary PBMCs.

Dose is critically important in assessing the differential effects on gene expression

The data from this study highlight the importance of dose and concentration of the progestogens. When considering contraceptive usage, MPA and NET are administered at relatively high concentrations, with MPA reaching peak serum concentrations between 2.5 - 65 nM a few days after intramuscular injection (Mishell, 1996; Mathrubutham & Fotherby, 1981; Ortiz et al., 1977; Halpern et al., 2014; Africander et al., 2011), and NET reaching a peak of 1.5 - 59 nM after injection (Africander et al., 2011; Stanczyk et al., 2013). LNG is administered at very low doses, with peak daily serum concentrations reaching 6.4 nM in oral contraceptive users, 1.6 nM in intrauterine devices (Kuhl, 2011) and 2.47 nM in implant users (Sivin et al., 2002). While 100 nM MPA and NET concentrations used in this study are close to peak serum concentrations, LNG is well above any concentration likely to be achieved *in vivo*. Endogenous P4 levels are governed by the phase of the menstrual cycle and pregnancy. P4 serum concentration levels range from 15 – 32 nM (Wira et al., 2015) during the menstrual cycle, with peak P4 levels reached in pregnancy, up to 600 nM (Africander et al., 2011). It has been reported that peak serum concentrations of MPA are prone to inter-individual variability, with some women having much higher serum concentrations than others (Mishell, 1996; Shelton & Halpern, 2014; Halpern et al., 2014; Fotherby, 1981). Thus using a range of concentrations of the different progestogens may help to elucidate the effects of the injectable contraceptives relevant to the serum concentrations.

The data from this study suggest that MPA, P4 and NET at physiological concentrations have differential effects on immune gene expression (Fig. 3.2, 3.4, 3.5, 3.6). At near-peak serum concentrations, the differential effects of MPA compared to P4 and NET on gene expression requires the GR (Fig. 3.8 and 3.9). Further this study has observed that MPA and NET at near peak serum concentrations differentially modulate immune gene expression after long term exposure (Fig. 3.12 and 3.16) and differentially affect HIV-1 replication in PBMCs (Fig. 3.14 and 3.15). P4 and LNG even at higher than peak serum concentrations (100 nM), had little effect on gene expression, while the LNG and P4 had no overall effect on HIV-1 replication compared to the control and individual responses were highly varied.

Additionally the data at lower concentrations suggest that MPA may have more selective effects on gene expression. In the dose response, MPA appeared to decrease IL6 mRNA levels at 10 nM, but had no effect on IL8 and RANTES mRNA levels after 48 hours (Fig. 3.2). Further, MPA significantly increased GILZ mRNA levels in this experiment (Fig. 3.2). P4 and NET had no effect on gene expression in the dose response experiment at 10 nM (Fig. 3.2). Interestingly, when only 10 nM concentrations of the different ligands (Fig. 3.3), it was found that while MPA had no significant effects on gene expression, while it

appeared that P4 and NET had elevated expression levels of IL6 and IL8 mRNA levels (Fig. 3.3). Interestingly, in both of these experiments MPA at 10 nM was more similar to DEX than to the other progestogens (Fig. 3.2 and 3.3). The data at low concentrations suggest that MPA has different effects on select genes compared to P4 and NET, which have no or slightly proinflammatory effects at 10 nM after 48 hours. Thus the dynamic changes in concentration and time could greatly affect the changes in gene expression by the different ligands.

Taken together the data from this study supports the hypothesis that MPA but not NET, P4 and LNG differentially modulates immune gene expression and HIV-1 replication in PBMCs at physiologically relevant concentrations.

Progestogens exert differential effects on HIV-1 infection and gene expression in primary PBMCs, with MPA, unlike NET, increasing HIV-1 replication in the majority of donors

This study is the first to report differences in HIV-1 replication by the different progestogens in parallel experiments. The results for DEX and CORT treatment were prone to inter-individual variability, with 4 to 5 out of the 10 donor samples exhibiting an increase in HIV-1 replication (Fig. 3.11). P4, and LNG were also prone to variability, with 5 out of 10 samples exhibiting an increase in viral replication to different extents in each donor (Fig. 3.11). Interestingly, NET was the only progestin that exhibited no effects on HIV-1 replication for most of the donor samples (Fig. 3.11), while for MPA, 7 out of 10 donor samples exhibited an increase in HIV-1 replication compared to the vehicle control.

The variable effects of P4 and LNG on HIV-1 replication, unlike the results for NET, suggest that these progestogens behave differently, despite their similar steroid receptor selectivity profiles (Africander et al., 2011; Stanczyk et al., 2013). Although few other studies have addressed the effects of P4 on HIV-1 replication in PBMCs, results from Asin et al., (2008) from a single donor, showed that P4 (1 nM) and E2 (100 pM) at concentrations, mimicking the mid-follicular phase, increased HIV-1 replication in female PBMCs, whereas P4 (100 nM) and E2 (1 nM), mimicking the mid-luteal phase, decreased HIV-1 replication, while P4 and E2 alone had no effect on HIV-1 replication compared to the vehicle control (Asin et al., 2008). Further the authors suggest that these differences were modulated through differential HIV-1 LTR activity, based on results using a TZM-bl assay (Asin et al., 2008). The results from the current study suggest that treatment with 100 nM P4 alone decreases HIV-1 replication levels in 50% of the donors compared to the vehicle control. While results from the current study appear contrary to that reported in Asin et al. (2008), the results reported in their study are representative of only one donor. However, the variability in HIV-1 replication in the presence of P4 and LNG could also be due to differences in cell type activation. It has been observed that P4 at high concentrations (6.3 – 63 µM), decreases pDC function by decreasing IFN-α production upon stimulation with a TLR9 agonist, to mimic a viral response (Hughes et al., 2008). Thus, it could be that at lower concentrations, P4 may have moderate to no effect on select

target cells, depending on the relative abundance of pDCs within a population of PBMCs, that may subsequently produce variable effects on HIV-1 replication.

There may several factors involved in changing the susceptibility of an individual to HIV-1 replication, such as the different relative distribution of cell type populations, different levels of endogenous GR levels, the different levels of co-factors needed to drive transcription or the differing levels of endogenous HIV-1 co-receptors present on target cells. Huijbregts et al. (2013) observed that treatment with MPA and DEX (at 100 nM and I µM) significantly increased HIV-1 replication in PBMCs depleted of CD8+ T cells, but decreased (DEX) or had no effect (MPA) on HIV-1 replication in CD4+ T cells depleted of CD14+ T cells (Huijbregts et al., 2013). Further supporting this hypothesis, Hanley and Viglianti (2011) found that DEX treatment for 24 hours, dose dependently decreased HIV-1 pseudovirus replication in monocyte derived macrophages (MDMs) and CD4+ T cells (Hanley & Viglianti, 2011). Further, the authors assessed steroid receptor involvement and found that when MDMs were co-incubated with the selective GR antagonist NEA, DEX repression of HIV-1 replication was lost (Hanley & Viglianti, 2011). Taken together, it could be that the differential effects observed in the present study on HIV-1 replication for the DEX, CORT and MPA treated samples may be in part due to GR involvement as well as the different effects these ligands have on different cell types that may be present at different levels in each donor.

The pooled results from this study suggest that there are variable effects on HIV-1 replication in PBMCs with the different ligands (Fig. 3.11). However, there appeared to be distinct differential effects within the MPA treated group on both HIV-1 replication and IL6 mRNA levels (Fig. 3.11 and 3.12). When analysing the sample set comparing IL6 mRNA levels with HIV-1 replication in the MPA treated group, there was a significant and positive correlation observed (Fig 3.13). That is, with an increase in IL6 mRNA expression, increased HIV-1 replication was observed (Fig. 3.14). A similar finding was reported by Birx et al. (1990) where high IL6 protein levels were associated with increased HIV-1 viral loads in monocytes (Birx et al., 1990). The results from the MPA treated group were separated into those with low IL6 mRNA levels and those with high IL6 mRNA levels. Three out of ten donor samples showed low relative mRNA levels (Fig. 3.15 A) and this corresponded to lower levels in HIV-1 replication (Fig 3.15 B). Additionally, in samples where MPA increased IL6 mRNA levels (Fig. 3.14 A), HIV-1 replication was significantly higher compared to the vehicle control (Fig 3.14 B). Furthermore, the majority of donor samples (7 out of 10) were found to have an increase in HIV-1 replication with MPA treatment. Most strikingly in the current study was the observation that IL6 repression observed after 48 hours was lost in 7 out of 10 donor PBMCs, and that this effect was independent of HIV-1 infection (Fig.3.14 A), after prolonged incubation for 9 days with MPA at 100 nM in activated PBMCs. Interestingly, DEX treated samples also exhibited a positive correlation between relative IL6 mRNA levels and HIV-1 replication (Appendix A, Fig. A.3.1) suggesting that these effects could be due to the glucocorticoid effects of MPA. However, no correlation was observed with CORT treatment, suggesting that the endogenous glucocorticoid acts differently to the synthetic and partial glucocorticoid agonists. IL8, RANTES and GILZ mRNA expression did not correlate with HIV-1 replication

(data not shown), for any of the ligands tested. Interestingly a recent study by Sampah et al. (2015) observed that MPA as physiological serum concentrations, dose dependently increased HIV-1 infection in un-stimulated CD3+CD8+ T cells, and that this increase in infection required the presence of CD14+ monocytes (Sampah et al., 2015). Thus it could be that MPA increases infection in CD3+CD8+ T cells, and that CD14+ monocytes could contribute to this increase in infection through an increase in IL6 expression.

The data from this study suggest that in 70% of the donor samples, MPA increases HIV-1 replication compared to the vehicle control. This may have important implications for initial viral replication and viral dissemination into the lymphatic system, and supports the hypothesis that MPA may increase disease progression in individuals. The effects of MPA on disease progression have not been fully explored. Several studies have reported that MPA has no effect on disease progression in HIV-1 positive women (Richardson et al., 2007; Heffron et al., 2013), while Stringer et al. (2009) reported that women on DMPA were more likely to qualify for anti-retroviral treatment or progress to AIDS than women not on contraception (Stringer et al., 2009). Roberts et al. (2010) reported that an increase in pro-inflammatory cytokines found in the plasma during the acute phase of HIV-1 infection could be used as a predictor of progression to AIDS, with IL7 and IL-1α levels associated with an increase in viral plasma load in women (Roberts et al., 2010). While IL6 was not significantly associated as a predictor of HIV-1 viral load and subsequent disease progression in the Roberts et al. (2010) study, it supports the finding in this current study, that suggests that the change in gene expression by MPA after prolonged exposure has significant effects on HIV-1 replication. Furthermore the results from this study support the observations by Stringer et al. (2009) that DMPA use could be associated with an increase in HIV-1 disease progression.

This is also the first study to assess the effects of prolonged exposure by different ligands on HIV-1 replication and mRNA gene expression in parallel. DEX, CORT and MPA appeared to repress IL8 mRNA expression after 9 days compared to the vehicle control. However, this repression was lost in the presence of HIV-1 infection (Fig. 3.12). Both RANTES and IL6 mRNA expression were elevated compared to the vehicle control in the absence and presence of HIV-1 after 9 days treatment with CORT, DEX or MPA (Fig 3.12). Interestingly, P4, NET and LNG had no effect on IL6, IL8, RANTES or GILZ mRNA levels compared to the vehicle control (Fig 3.11). GILZ mRNA expression levels increased in CORT, DEX and MPA treated samples and appeared to be further elevated in the presence of HIV-1 (Fig. 3.12). However this was only significant for DEX. It has been previously observed that the viral protein R (VpR) acts as a co-activator for the GR (Kino et al., 2002; Hapgood & Tomasicchio, 2010), and has been found to increase the apoptotic effects of DEX and MPA in PBMCs (Tomasicchio et al., 2013). Thus it could be that the significant increase in GILZ transactivation by DEX in the presence compared to the absence of HIV-1 could be due to VpR acting as a co-activator for the GR, increasing the transcriptional response. While CORT and MPA had smaller, non-significant increases in GILZ transactivation in the presence of HIV-1, it could be due to the lower potencies of these ligands on GILZ transcription. Consistent with this hypothesis, it has been observed by Ronacher et al. (2009) and Hadley et al. (2011), that there are ligand specific effects that drive

transactivation efficacy, GR translocation into the nucleus and subsequent GR recruitment to the promoter region of the GILZ gene, with DEX having greater effects on these factors than MPA and CORT (Ronacher et al., 2009; Hadley et al., 2011).

The results from this study found that CORT, DEX and MPA decreased PBMC viability, while P4, NET and LNG did not (Fig. 3.11 B). MPA decreased cell viability by ~ 20%. However this effect was not statistically significant, while CORT and DEX had more pronounced effects on cellular viability (~35% and ~50% respectively). Interestingly, it appeared that the effects of CORT, DEX and MPA on cellular viability were independent of the presence of HIV-1 (Fig. 3.11 B). Enomoto et al. (2007) found that DEX significantly reduced PBMC proliferation (Enomoto et al., 2007), suggesting that the results in this study may be due to a reduction of proliferation by DEX and MPA (Fig 3.11 B and Fig 3.17 A). The reduction of proliferation could be due to an increase in apoptosis, as the authors found that DEX increased the expression of the Fas ligand CD178 and the Fas death receptor CD95 in CD8+ and CD14+ cells (Enomoto et al., 2007).Tomasicchio et al. (2013) found that DEX and MPA significantly increased apoptosis in CD4+ T cells, and that this effect was exacerbated by the presence of HIV-1 or the HIV-1 viral accessory protein R (VpR) (Tomasicchio et al., 2013). While the current study did not detect any differences in cellular viability when treated with MPA, the MTT assay is not specific enough to detect the effects of apoptosis, but rather gives an indication on total cellular metabolism within the multicellular population. Thus it could be that the effects of HIV-1 on increasing DEX and MPA induced apoptosis may only be prevalent in select cell types. It could also be that changing the relative types of immune cells present, through targeted apoptosis by MPA may also contribute to differences in HIV-1 replication.

Collectively, the results from this study suggest that MPA, unlike NET, increases HIV-1 replication in the majority of donor PBMCs. Furthermore the results show that the increase in HIV-1 replication correlates with increased levels of IL6 mRNA levels in MPA treated PBMCs. While DEX was similar to MPA, the effects on viral replication and IL6 mRNA levels were more consistent in MPA treated samples. While IL6 mRNA expression correlated with HIV-1 replication in MPA treated samples, IL8, RANTES and GILZ mRNA expression did not. Interestingly, mRNA levels after 9 days exposure to CORT, DEX and MPA were different to the observations previously reported after 48 hours (Fig. 3.1, 3.2, 3.3 and 3.4), suggesting that length of time of incubation has important consequences on gene expression. P4, NET and LNG had similar effects on IL6, IL8, RANTES and GILZ mRNA expression levels. However, while NET had no effect on viral replication, LNG and P4 treatment resulted in highly varied HIV-1 replication levels. Interestingly, of the ligands tested, MPA and NET had the most consistent and opposite effects for all the donor samples.

The immunosuppressive effects of MPA are time dependent in PBMCs

The effects on cell mediated immunity and gene expression by the different progestogens and glucocorticoids could also depend on the concentration of the ligand and the length of time of exposure to the ligands. The data from this study suggests that duration of exposure to the progestogens at a constant concentration may influence the immunomodulatory effects. The early time course experiments indicated that MPA, like DEX repressed maximally at 48 hours post treatment (Fig. 3.1), and that this was dose dependent (Fig. 3.2), while gene expression data from the HIV-1 infection studies, found that after 9 days of treatment, DEX, CORT and MPA repression of IL6, IL8 and RANTES mRNA was lost (Fig. 3.12). This interesting observation led to the hypothesis that the repressive effects of DEX and MPA on select immunomodulatory effects may be time dependent. As such, activated PBMCs were stimulated with 100 nM DEX or MPA in the presence of 30U/ml IL2 to mimic the HIV-1 infection conditions and sampled every 24 hours from 48 hours (peak repression) to 168 hours (7 days). The data presented indicate that DEX exhibited strong repressive effects on IL6, IL8 and RANTES mRNA expression at days 2, 3 and 4, and less to no repression on the mRNA expression levels of these genes at days 5, 6 and 7 (Fig. 3.16). MPA had similar effects, however the repressive effects on gene expression were less effective than DEX, with loss of repression observed from day 3 onwards (Fig. 3.16). Additionally, the ability of DEX and MPA to increase GILZ mRNA expression was maintained throughout the duration of the experiment, indicating that the transactivation mechanism was consistently activated (Fig. 3.16 D). This is the first study to report these effects by MPA in PBMCs over a prolonged time course, and suggest that DEX and MPA exert similar effects on these cell types. A study in the Vk2/E6E7 vaginal cell line assayed the effects of MPA on mRNA expression levels and protein expression levels of immunomodulatory genes (Irvin & Herold, 2015). After 5 days in the presence of increasing concentrations of MPA, TNF-α, GM-CSF, IL6, IL8, CCL3 and CCL4 mRNA expression levels were significantly elevated above the vehicle control. Irvin and Herold (2015) used between 38 nM - 388 µM of MPA, and only observed significant increase in expression levels at 3.8 µM and above (Irvin & Herold, 2015). Kleynhans et al. (2011) found that in female PBMCs stimulated with exogenous BCG, IL6 protein expression was repressed with 10 µM MPA after 6 days, while MPA had no effect on IL8 protein expression, which was similar to the BCG control after 6 days (Kleynhans et al., 2011). While Kleynhans et al. (2011) reported that IL6 protein expression was reduced after 6 days, this was in the presence of BCG, suggesting that these effects may be specific for a bacterial response. The results from this current study are from PBMCs activated for one day with PHA, using 100 nM concentrations of MPA. Thus the results from the current study are the first to show, that at peak contraceptive doses of MPA and in the absence of an exogenous bacterial stress, that IL6, IL8 and RANTES mRNA expression changes over time (Fig. 3.16), and the first study to observe these effects in primary PBMCs.

To elucidate the possible mechanisms underpinning the loss of transrepression, steroid receptor expression was examined to determine whether the switch could be explained due changes in SR expression levels. It has been established that MPA is a partial agonist for the AR and weak agonist for the

MR (Africander et al., 2014; Africander et al., 2013). Additionally, Africander et al. (2011) observed that the TNF-α induced IL6 mRNA expression was elevated by MPA in ectocervical cell lines, and that this effect was partially modulated via the AR (Africander et al., 2011). Thus it was hypothesised that the AR could be involved in the pro-inflammatory responses after prolonged exposure to MPA. However AR protein expression was not observed at any of the time points assayed in any of the treatment groups suggesting that the AR may not be involved in this switch mechanism. Furthermore the results from this study suggest that the regulation of, PR or GR protein or MR mRNA levels are not involved in the switch from anti-inflammatory effects by MPA or DEX to pro-inflammatory effects (Fig. 3.17). However, the results do not exclude the possibility that different steroid receptors could be involved in this process via non-genomic actions of SRs on downstream gene expression.

While the mechanism of the switch from anti-inflammatory to pro-inflammatory responses was not determined in this study, similar observations have been reported in the literature. Glucocorticoids are widely used as immunosuppressive drugs, (Tsitoura & Rothman, 2004; Africander et al., 2011). However, after prolonged treatment, glucocorticoids, in some patients, no longer inhibit cellular proliferation and cytokine production at the site of inflammation (Tsitoura & Rothman, 2004; Goleva et al., 2002). Tsitoura and Rothman (2004) found that prolonged glucocorticoid treatment (72 hours) lead to glucocorticoid resistance in CD4+ T cells, and that this mechanism was dependent on IL2 levels and the enhancement of MEK/ERK signalling. The authors found that primary T cells incubated with DEX and IL2 showed a proliferation pattern similar to that of control T cells, compared to DEX treatment alone, which significantly reduced proliferation (Tsitoura & Rothman, 2004). This finding is contrary to the results reported in this study, where, DEX reduction of cellular viability was observed in the presence of IL2 (Fig 3.11 and 3.17). It could be that the concentration of IL2 necessary to mediate this complete reversal in the DEX response was higher than used in the present study. Pandolfi et al. (2013) found that an IL2 dose of 50 - 80 U/ml rescued different T cell populations from DEX induced apoptosis (Pandolfi et al., 2013). However, the concentration of IL2 used in this study (30 U/ml) did not reduce the DEX induced effect on cell viability. In addition, Tsitoura and Rothman (2004) found that this increase in DEX insensitivity was mediated via the activation of the transcription factor, NF-AT and activation of the MEK/ERK signalling pathway (Tsitoura & Rothman, 2004). The authors found that DEX alone did not stimulate ERK or JNK activation via phosphorylation. However in the presence of IL2, DEX increased phosphorylated ERK and JNK levels shortly after activation (Tsitoura & Rothman, 2004). Thus it could be that over time, DEX and MPA treatment in the presence of IL2, could promote the stimulation of the NF-AT pathway resulting in the production of inflammatory cytokines such as IL6, as suggested by the results in the time course study (Fig. 3.16) and infection studies (Fig. 3.12 and 3.14). In some donor samples, as observed in the infection experiments in this study, IL6 mRNA repression was maintained by DEX and MPA in the absence and presence of HIV-1 (Fig. 3.13), suggesting that the presence of IL2 alone, may not be the only factor that contributes to a loss of IL6 mRNA expression levels over time. It has also been observed that DEX treatment could promote the development of T regulatory (T reg) cells in PBMCs in the presence of IL2

(Chen et al., 2006). T reg cells are defined as suppressor cells and play a crucial role in maintenance of peripheral tolerance and prevent a number of immune-mediated diseases (Murphy et al., 2012), The authors found that co-stimulation with DEX and IL2 amplified the capacity of IL2 to expand T-reg cells and CD4+FoxP3+ cells and concluded that IL2 can rescue T reg cells from DEX-dependent cell death, and that DEX promotes the expansion of T reg cells (Chen et al., 2006). Thus it could be that the results observed in Figures 3.12, 3.14 and 3.16 are due to an increase in T reg cells.

The results suggest that DEX and MPA repression of IL6, IL8 and RANTES in activated PBMCs is present after 4 days, where after the repressive effects are lost. It was observed in this study that regulation of SRs did not contribute to this loss of repression. However the activity of SRs as assessed by phosphorylation was not measured in this study. Further, DEX and MPA significantly decreased cell viability over the time course, and suggests that apoptosis could have important implications in this loss of regulation, by targeting select cell populations for apoptosis, that may drive the switch in the response observed in this study.

Chapter 4

The effects of progestogens on immunomodulatory gene expression and HIV-1 replication in *ex vivo* cervical explants

Part of this work has been presented at the HIV R4P Conference in Cape Town South Africa, 2014 (Fig 4.2 A, B and C and part of Fig 4.11).

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Introduction

The effects of progestogens on gene regulation and HIV-1 replication have not been fully elucidated in any cervical *ex vivo* model to date. The primary *ex vivo* cervical explant model is a useful and relevant model in which to assay the effects of these progestogens on differential gene expression and HIV-1 replication, as it is a model of the local female genital mucosal immune environment.

To date, it has been established that the lower female genital tract is an important site for initial infection, with several studies finding key HIV-1 target cells present within the ectocervix, endocervix and vagina (Pudney et al., 2005; Greenhead et al., 2000; Collins et al., 2000), and that these cell types are permissible to and important in initial infection events of the HIV-1 viral cycle (Greenhead et al., 2000; Hladik et al., 2007; Maher et al., 2005). Further, it has been established that the menstrual cycle may affect the distribution of these cell types within the different compartments of the FGT (reviewed in Wira et al., 2005; Wira et al., 2010) and that explants obtained from women in the secretory phase (or luteal phase) were more permissible to HIV-1 infection and had higher viral loads (p24 levels) than explants obtained from women in the proliferative phase (follicular phase) (Saba et al., 2013), suggesting a role for high progesterone (P4) levels in increasing HIV-1 infectivity.

Research on the effects of progestogens on HIV-1 infection, immune function and gene expression has been performed in primary epithelial cells (Ferreira et al., 2014), vaginal mononuclear cells (Huijbregts et al., 2013) or in observational studies utilising biopsy samples, purified cell types or cervicovaginal secretions (CVL) (Hughes et al., 2008; Chandra et al., 2013; Huijbregts et al., 2014; Michel et al., 2015; Michel et al., 2013; Guthrie et al., 2015). Taken together, the data suggest, that MPA regulates the expression levels of key immune response genes (Huijbregts et al., 2013; Huijbregts et al., 2014; Michel et al., 2013; Guthrie et al., 2015), changes cell type distributions within the epithelium (Chandra et al., 2013), alters cell mediated immune functions in select cell types (Hughes et al., 2008; Huijbregts et al., 2014; Michel et al., 2015), increases HIV-1 transcytosis through the primary epithelium (Ferreira, Dizzell, et al., 2014), decreases vaginal epithelial thickness (Michel et al., 2013) and increases CCR5 and CXCR4 expression on key target cells (Huijbregts et al., 2014; Goode et al., 2014), all of which may lead to an increased susceptibility to HIV-1 infection.

Only a few studies have reported on the effects of progestogens other than MPA. Huijbregts et al. (2013) found that NET and LNG had no significant effects on cytokine gene expression, or cell function in VMMCs, CD4+ and CD8+ cells (Huijbregts et al., 2013). Michel et al. (2013) found that NuvaRing (containing the progestin etonorgestrel and ethinyl estradiol) users have suppressed human β-defensin 2 and 3 levels in CVL samples (Michel et al., 2013). Michel et al. (2015) found that the use of the NuvaRing was associated with a reduction IFN-α and TNF-α expression in pDCs, as well as a reduction in CD207+ Langerhan cells in the vaginal epithelium (Michel et al., 2015).

The effects of MPA, NET and LNG on gene expression and HIV-1 replication in primary models have not been established, nor have the effects of the menstrual cycle on steroid receptor expression levels in this model been elucidated.

Therefore the aims of this study were to:

1.) **Determine the steroid receptor profile in primary cervical explants at different phases of the menstrual cycle.**

Cervical samples were obtained from premenopausal women undergoing hysterectomies for benign reasons, with P4, E2, follicular stimulating hormone (FSH) and luteinising hormone (LH) serum concentration levels obtained for each patient. Using the serum concentrations of these hormones, women were grouped into either the follicular or luteal phase of the menstrual cycle. Protein was isolated from the cervical samples from women in both groups, and PR, ER, AR and GR protein expression levels were evaluated using western blots.

2.) **Determine whether the progestogens and glucocorticoids differentially modulate select immune gene expression and steroid receptor levels in different cervical compartments.**

Ectocervical explants were incubated with 100 nM CORT, DEX, MPA, P4, NET or LNG for 48 hours and RNA was isolated. RNA samples were converted to cDNA and the expression levels of IL6, IL8 and GILZ mRNA was determined using qPCR. Steroid receptor mRNA levels were determined via real time qPCR. ER, MR, AR, GR and PR levels were assessed. Protein was harvested from one patient and GR and PRAB expression levels were assessed via western blots. Endocervical samples were processed in the same manner. However due to the small sample sizes of this tissue the effects of only 100 nM DEX, MPA and NET were investigated, as well as differential regulation of IL6, IL8 and GILZ mRNA levels.

3.) **Determine if the repressive effects exerted by MPA on IL6, IL8 and GILZ mRNA expression are dose dependent in ectocervical explants.**

Ectocervical explants were treated with increasing concentrations of MPA (1 nM - 1 µM) for 48 hours. Gene expression was determined using real time qPCR. Dose response curves were analysed and potencies (EC_{50}) were determined.

4) **Determine the effects of progestogens on HIV-1 replication and gene expression in primary ectocervical explants.**

Ectocervical tissue samples were incubated for 48 hours with either 100 nM MPA or NET, before being infected with either X-tropic ($HIV_{pNL4.3}$) or R-tropic ($HIV_{BaL\_Renilla}$) HIV-1 IMCs for 2 hours. Explants were extensively washed and replaced with media with 100 nM MPA or NET. HIV-1 infection was determined by harvesting the supernatant from the explants at days 0, 3, 5, 7 and 10 post infection and assessing p24

expression levels via a p24 ELISA. Tissue was harvested at the end of the time course for RNA isolation and subsequently processed for real time qPCR, and IL6, IL8 and GILZ mRNA levels were determined.

Results

Primary ectocervical explants express all the steroid receptors and their expression levels are dependent on the menstrual cycle of the women

It has been suggested that the sex hormones E2 and P4 regulate the steroid receptor levels present in the female genital tract (Camacho-Arroyo et al., 1996; Tibbetts et al., 1998). Thus it was of interest to determine the effects of the menstrual cycle on the expression levels of the PR, AR, ER, GR and MR. Women in this study had their P4, E2, LH and FSH serum concentrations determined by ELISA at the National Health Laboratory Services (NHLS, South Africa), that allowed for the determination of the menstrual phase cycle for each donor (See Appendix C for donor information). The concentrations of each hormone from a donor were matched to the most likely phase of the menstrual cycle by comparing the concentrations of the hormones from the donor to the known ranges of each hormone within each phase of the menstrual cycle. The range of concentrations of each hormone at each phase of the menstrual cycle was supplied by the NHLS, South Africa.

Donors were sorted into follicular and luteal phases, and their cervical explant tissue was harvested for protein isolation whereafter the different steroid receptor levels were determined by western blotting. Protein isolation was optimised for this study. However protein yields were still variable (appendix E, Table E.2). For this experiment, 20 µg of protein was loaded per sample according to the Bradford assay of protein determination. However upon inspection of the GAPDH levels (Fig. 4.1. D), it was obvious that the Bradford method of protein determination was not accurate. Despite this inaccuracy, protein quantitation by densometric analysis reduced this error by normalising the intensity of the protein band of interest (IDV) to the intensity of its corresponding GAPDH protein band. After normalisation, distinct protein expression patterns emerged.

Twenty-four donors were used in this study, with 12 donors identified in the luteal phase (high P4 and low E2) and 12 donors identified in the follicular phase (low P4 and high E2). Upon densometric analysis of the western blots (as seen in Fig. 4.1. D), the total average PR levels were found to be higher in the follicular phase compared to the luteal phase. When separating total PR levels into its isoforms, it was observed that women in the follicular phase, had, on average, elevated levels of PRB (Fig. 4.1 A) and significantly higher levels of PRA compared to the luteal phase (Fig. 4.1 B). While there was more PRB in the follicular phase, there was also more PRA, suggesting that there may be more active PRB in the luteal phase, however this comparison is yet to be performed or validated. ERα levels were also regulated, with significantly higher average levels of the ERα protein present in the follicular phase than the luteal phase (Fig. 4.1 C). GR and AR protein levels were hard to detect in these samples, and may be due to the low abundance of the GR and AR compared to the ERα and the PR isoforms. Additionally, it could also be due to the inaccuracy of the Bradford assay in protein quantitation that resulted in less protein per sample added to each well of the SDS-PAGE gel that

could affect the detection of the GR and AR via western blotting. As such, it may be that a more accurate method of protein determination is needed to ensure equal loading. Additionally, it could also be that higher concentrations of total protein are needed for the visualisation and subsequent quantitation of the GR and AR (Fig. 4.1 D).

The representative western (Fig. 4.1 D) shows the variation in the steroid receptor levels within a donor (each lane) as well as between donors (across lanes). However, this variation may not be as a result of donor variation but could be from the isolation method used. It could be that the isolation method from the Qiagen® AllPrep isolation kit, which uses a heavy metal (Zn^{2+}) precipitation step could preferentially precipitate charged proteins. It could also be that some proteins have undergone proteolysis within the sample. Additionally, it could also be that the GR and AR are more labile than the ER and PR, and are thus more difficult to detect. Due to cross-reactivity of the MR antibodies used in this study, the effects of the menstrual cycle on MR regulation are yet to be determined.

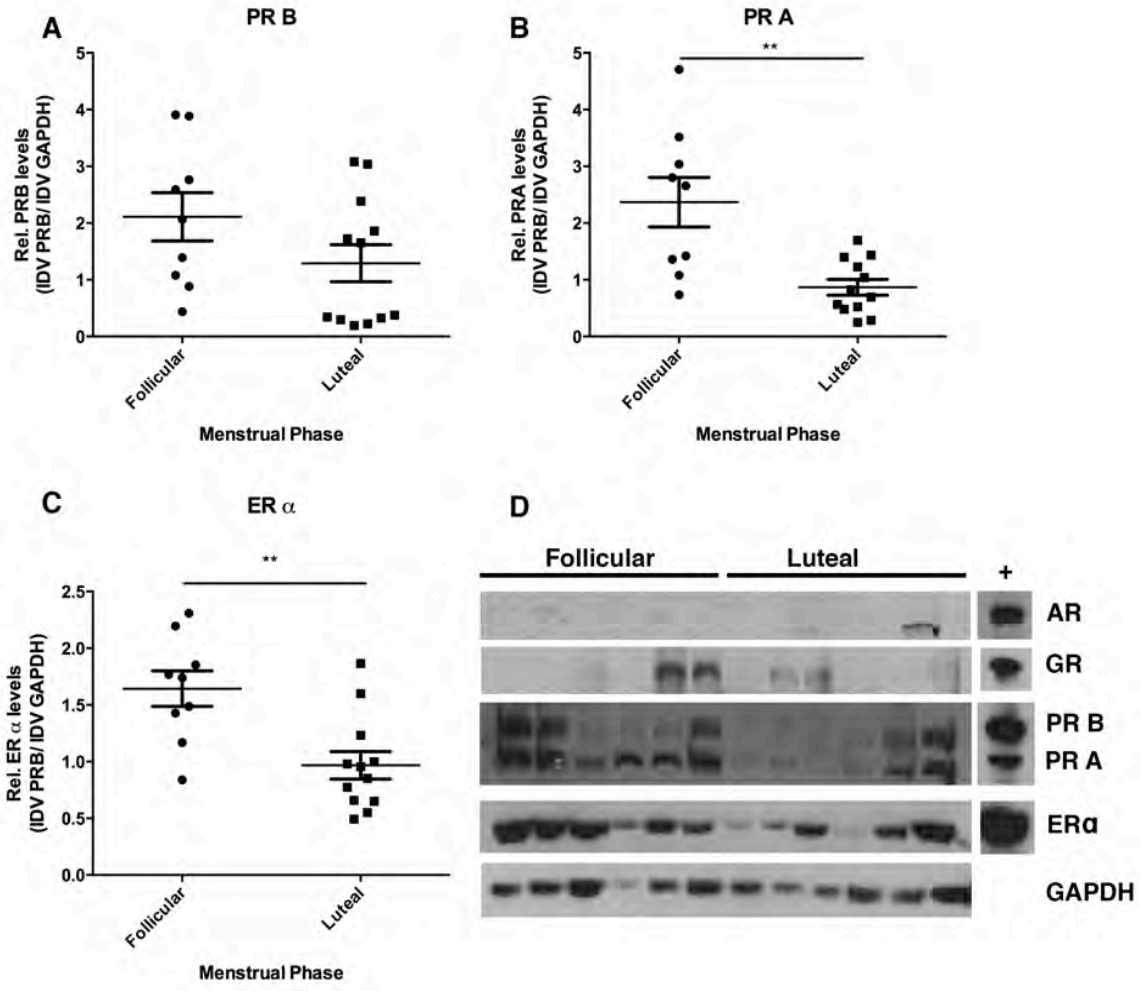


Figure 4.1: Endogenous SR profiles in ectocervix are differentially modulated during the menstrual cycle. Primary ectocervical explants were homogenised in 800 µl Buffer RLT (Qiagen, Netherlands) and processed for protein isolation. Protein concentration was determined using the Bradford assay, where 20 µg total protein was loaded onto an 8% SDS-PAGE gel and electrophoresed for 1 hr and 30 min at 120 V. The gel was subsequently electroblotted onto a nitrocellulose membrane, and probed for the steroid receptors with the appropriate primary antibodies. The nitrocellulose membranes were incubated for 1 minute in ECL-chemiluminescent substrate and visualised using audio-radiography. Relative PR B (A), PR A (B), ER (C) levels were determined using densomitry analysis software (AlphaEC™) with each sample normalised to its' corresponding GAPDH levels. (D) A representative blot showing 12 donors (6 in the follicular and 6 in the luteal phase) with differing steroid receptor expression levels and GAPDH. COS-1 positive controls for each steroid receptor was electrophoresed with each sample set to confirm steroid receptor detection in the ectocervical samples and is donated as + in the representative western blot.

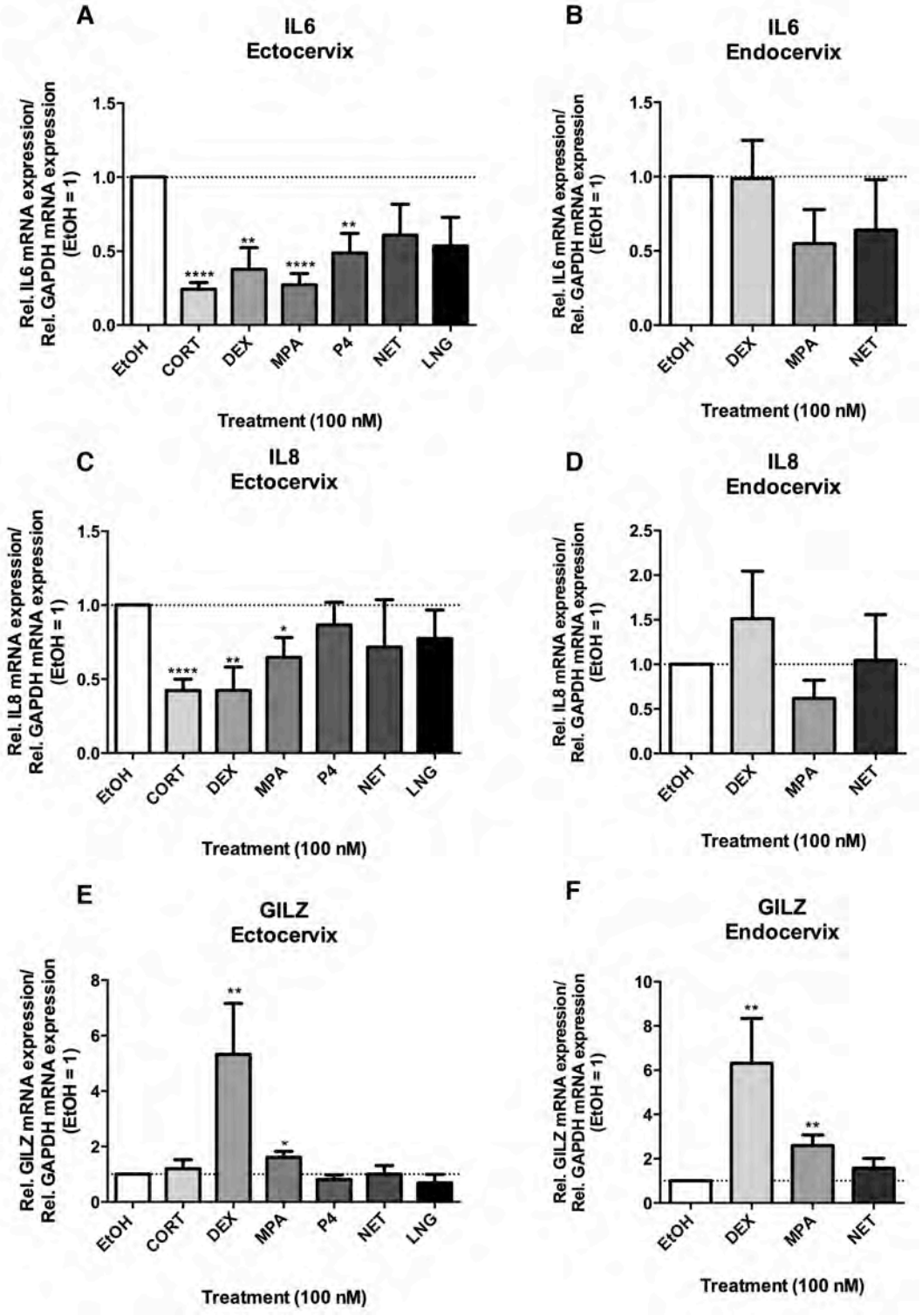
Progestogens differentially regulate mRNA and protein expression levels of key immunomodulatory genes in ectocervical and endocervical explants

Having established in PBMCs that MPA, unlike P4 and NET, repressed mRNA levels of immunomodulatory genes at 48 hours (Fig. 3.1), the mRNA expression of IL6, IL8 and GILZ after 48 hours incubation was assessed in two different compartments of the lower female genital tract, the ectocervix and endocervix. Before these experiments could be performed, RNA isolation and tissue conditions needed to be optimised. Many RNA isolation techniques were tested. However homogenisation with the Qiagen TissueRuptor® in combination with the RNA isolation solution, TriReagent®, consistently produced good quality and high quantity total RNA in comparison to the

other methods (appendix E, Fig. E.2.1 and Table E.2). Furthermore, it was also established that fresh tissue should be used for all incubation experiments. It was found during optimisation studies, that freeze/thawing of the tissue greatly decreased the tissue viability compared the fresh tissue counterpart (data not shown).

In the ectocervical tissue explants, CORT, DEX, MPA and P4 significantly decreased IL6 mRNA levels (Fig. 4.2 A), while only CORT, DEX and MPA significantly repressed IL8 mRNA levels (Fig. 4.2 C) compared to the vehicle control. NET and LNG had no significant effect on IL6 mRNA levels and P4, NET and LNG had no significant effect on IL8 mRNA levels (Fig. 4.2 A and C). GILZ mRNA expression in the ectocervical explants was significantly increased by DEX and MPA treatment, and slightly elevated with CORT, while P4, NET and LNG had no effect on gene expression (Fig. 4.2 E). RANTES mRNA expression was not readily detected in the explants, with C(t) values only detectable after 40 cycles in some donor samples (data not shown), thus RANTES levels were not assessed.

The endocervix samples were smaller than the ectocervical samples, as such limited incubations could be performed per experiment. In the endocervical tissue, DEX and NET had no effect on IL6 or IL8 mRNA levels compared to the vehicle control after 48 hours (Fig. 4.2 B and D), while MPA appeared to reduce IL6 and IL8 mRNA levels. Both DEX and MPA significantly increased GILZ mRNA levels in the endocervix, while NET had no effect compared to the vehicle control (Fig. 4.2 F). The data suggest that there could be compartment specific effects of these ligands on gene expression, with more pronounced effects by MPA in the ectocervix than the endocervix. Cytokine expression levels were determined via Luminex, however due to the unreliability of the standard curve, accurate concentrations could not be determined and thus the results are not presented in this study (data not shown).

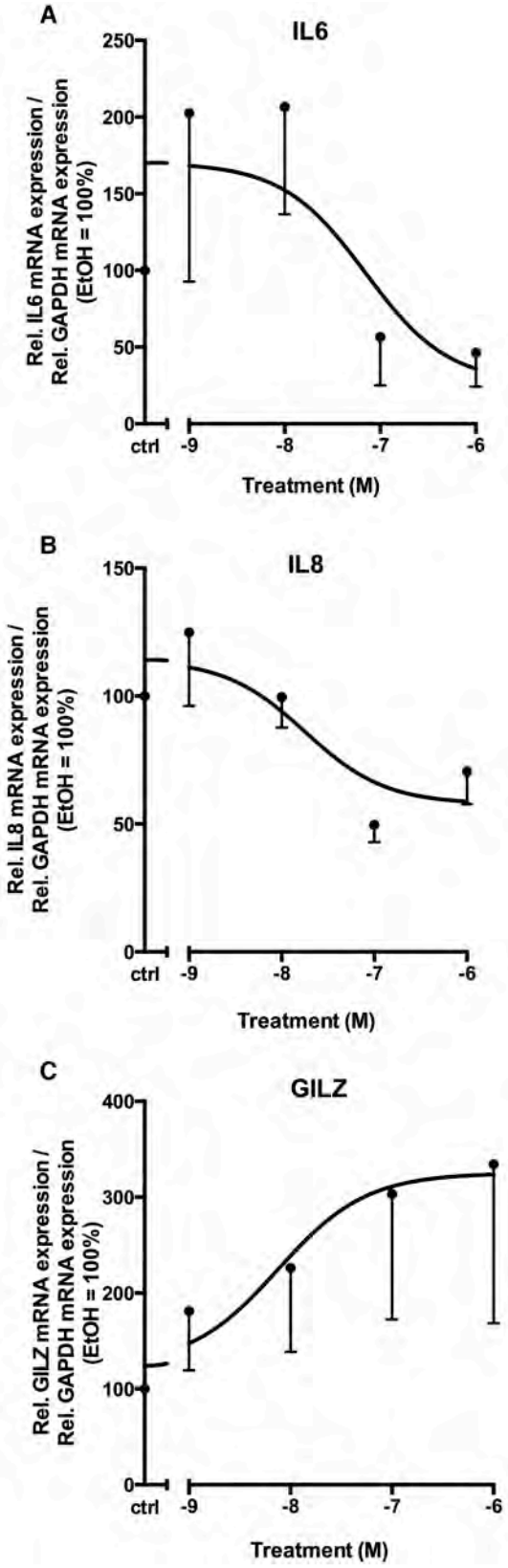


<u>Figure 4.2:</u> IL6, IL8 and GILZ expression in ectocervical and endocervical explant tissues are differentially modulated by glucocorticoids and progestogens. Ectocervical (A, C and E) or endocervical explant tissue (B, D and F) samples were stimulated with 100 nM cortisol (CORT), DEX, MPA, P4, NET or LNG or with vehicle i.e. 0.1% (*v/v*) ethanol (EtOH), for 48 hours after which RNA was isolated, cDNA was synthesised and relative IL6, IL8, RANTES and GILZ mRNA levels determined by real time qPCR, normalised to GAPDH. Relative fold change in expression was determined by setting vehicle control to 1. In the ectocervix relative mRNA expression levels of (A) IL6, (C) IL8 and (E) GILZ were determined with all the ligands. Due to the small amount of tissue in the endocervix relative expression levels of (B) IL6, (D) IL8 and (F) GILZ were determined for 100 nM DEX, MPA and NET treated samples. (A-F) shows histograms of pooled results from four donors (Prog 0010, 0011, 0024 and Prot 0001) with vehicle set to one, plotted as mean +/- SEM. A one-way ANOVA with either a Dunnet's post hoc test (A and C) or student's t-test (B and F) was performed. Statistical significance is denoted by *, ** or **** to indicate $P< 0.05$, $P< 0.01$ or $P< 0.0001$, respectively.

MPA dose dependently affects immunomodulatory mRNA levels in ectocervical explants

Ectocervical explants were treated with increasing concentrations of MPA in order to determine dose-dependent effects of MPA on relative IL6, IL8 and GILZ mRNA expression. Dose response curves give useful information on the efficacy (maximal response) and potency (EC_{50} or half maximal response) of the ligand on a particular gene (Africander et al., 2011; Hapgood et al., 2013). This information gives insight into the concentrations necessary to exert a transcriptional response. There was both inter- and intra-individual variation on the dose response curves, with some samples exhibiting pro-inflammatory effects by MPA on the target genes at the lower concentrations (Fig. 4.3).

The dose response analysis on IL6 mRNA expression levels indicated that MPA repressed IL6 mRNA expression between 100 nM and 1 µM, with a calculated EC_{50} of ~70 nM (Fig. 4.3 A). MPA repression on IL8 mRNA expression appeared to be more sensitive, with mRNA expression levels reduced between 10 - 100 nM, with a calculated EC_{50} of ~18 nM (Fig. 4.3 B). GILZ mRNA expression levels increased with increasing concentrations of MPA, although the error was high in this data set (Fig. 4.3 C). The non-linear regression curve fit indicated that the EC_{50} for transactivation by MPA on GILZ mRNA expression was ~7.5 nM. The data suggest that MPA may exhibit dose and gene selective sensitivities on gene expression (Fig. 4.3). The dose response experiments in primary ectocervical explants showed high inter-individual variation in the responses measured from the samples of the different donors, resulting in the large error found in this experiment. Thus, dose responses should be repeated for more accurate potency determinations on each gene assayed.



<u>Figure 4.3:</u> MPA appears to regulate IL6, IL8 and GILZ mRNA expression in a dose dependent manner in ectocervical explant tissues. (A-C) Ectocervical explants were treated in triplicate with 0.1% (*v/v*) ethanol (EtOH or ctrl), or increasing concentrations of MPA (1 nM, 10 nM, 100 nM and 1 µM) for 48 hours after which RNA was isolated, cDNA was synthesised and relative IL6 (A), IL8 (B) and GILZ (C) mRNA levels determined by qPCR, normalised to GAPDH. Dose response curves show pooled results from three donors (Prog 0026, 0027 and Prog 0028) with vehicle set to 100%, plotted as mean +/- SEM. A (log) agonist vs. response - variable slope (four parameters) non-linear regression analysis with a HillSlope set at 1, was performed on the data set, with vehicle treatment set as 100%. (A) IL6 dose response for MPA, (B) IL8 dose response (C) GILZ dose response.

Progestogens and glucocorticoids do not alter the gene expression profiles of the different steroid receptors in primary ectocervical explants after 48 hours incubation

Primary ectocervical explants were assessed for mRNA expression of the AR, ERα, GRα, MR and PRAB as well as the protein expression of GRα, PR A and PR B. Ectocervical explants had detectable mRNA expression levels of all the SRs (Fig. 4.4). Additionally, GRα and PR protein levels were assessed. However isolation of total protein from the TriReagent® fraction was only successful in one donor (Fig. 4.5).

The SRs did not appear to be differentially regulated by the different ligands at an mRNA expression level after 48 hours (Fig. 4.4). This also appears to be the case at the protein expression level for the GR and PR (Fig. 4.5). However the results of the protein levels must be cautiously interpreted since they are only from one donor. AR mRNA levels appeared to be highly expressed. However detection of the AR protein via western blotting was not successful in this experiment. AR mRNA levels appeared to be slightly elevated by all treatments except LNG, which was similar to the vehicle control (Fig. 4.4 A). ERα levels (Fig. 4.4 B) were all similar to the vehicle control in the different treatment groups. Interestingly, MR mRNA appeared to be expressed at a low level in all the treatment groups (Fig. 4.4 F). Thus, the quantitation of the MR by qPCR should be cautiously interpreted due to the low expression levels observed (Fig. 4.4 C and 4.4 F). GRα mRNA expression levels appeared to be increased in the P4 treated group after 48 hours post treatment, while results for the other treatment groups were similar to the vehicle control (Fig. 4.4 D). PRAB expression also appeared to be slightly elevated in all groups compared to the vehicle (EtOH) control, except in the P4 group which had similar expression levels to the control (Fig. 4.4 E).

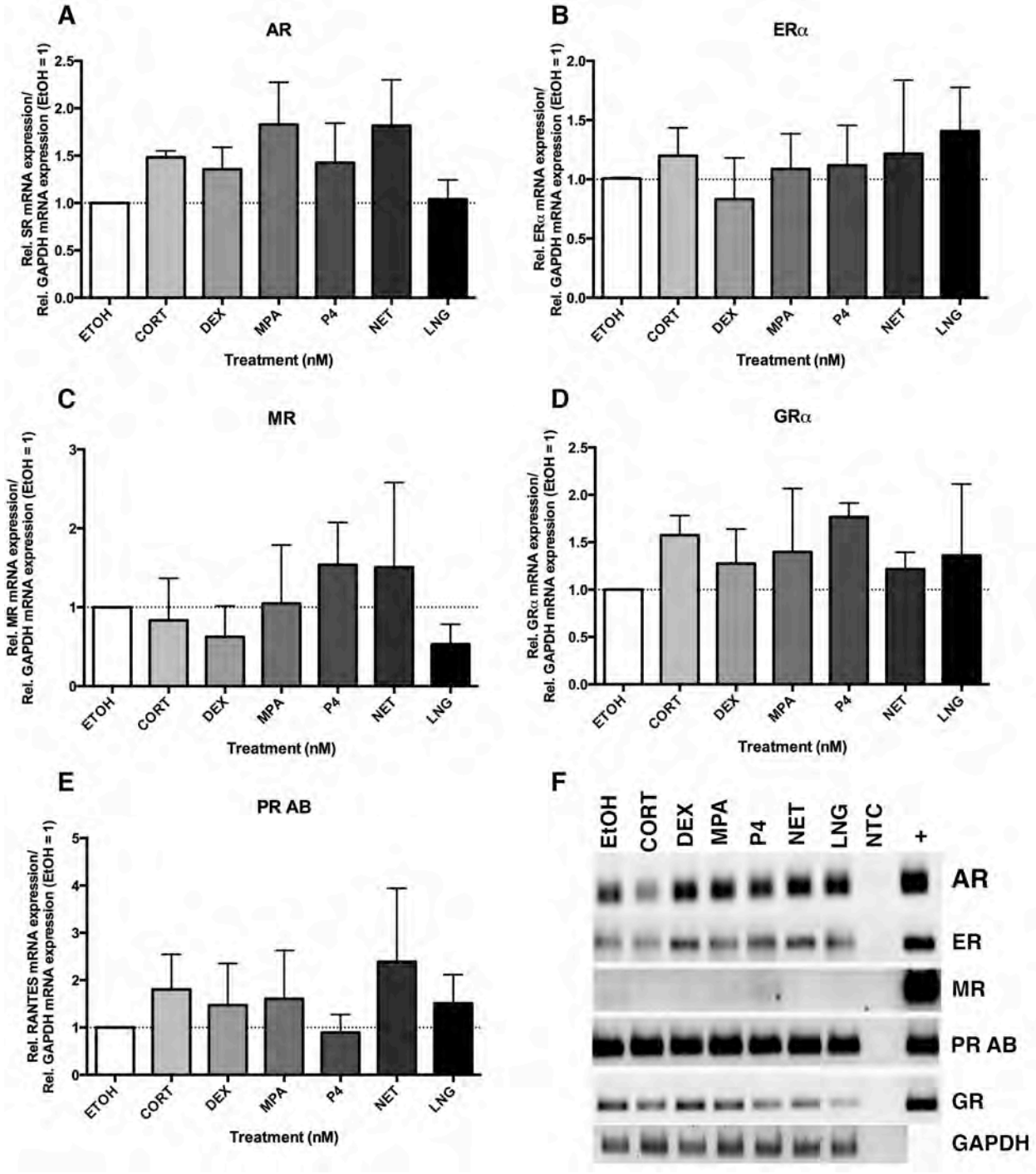


Figure 4.4: Progestins do not appear to modulate SRs mRNA levels in primary ectocervical explants. Primary ectocervical explants were incubated with vehicle i.e. 0.1 % (*v/v*) ethanol (EtOH), or treated with 100 nM cortisol (CORT), DEX, MPA, P4, NET or LNG for 48 hours after which explants were homogenised in 800 µl TriReagent® (Sigma). RNA was isolated and 250 ng was used for cDNA synthesis. Relative (A) AR, (B) ERα, (C) MR, (D) GRα or (E) PR AB mRNA levels were determined by qPCR and normalised to relative GAPDH, with vehicle treatment set to 1. Five times sample application buffer (Fermantas) was added to each qPCR reaction and 10 µl of the samples were loaded onto a 1% Agarose-1 X TAE gel and electrophoresed for 1 hour at 60 V. Agarose gels were visualised under 256 nM UV light in a Syngene, G:Box (Vacutec, England) and images acquired using GeneSnap from synGene, version 7.08 (SynGene, England). A representative gel is shown for each SR and GAPDH control (F). Histograms represent pooled data from three donors (Prot 0001, Prog 0010 and Prog 0015) with treatments performed in triplicate, plotted at mean ± SEM.

In order to compare the mRNA expression levels of the SRs with protein levels, western blotting was performed. As previously mentioned, these results are representative of one donor. Total protein isolated from the phenol layer of the TriReagent® fraction was only successful in one donor, while the protein isolated from the other donors appeared to be degraded upon western blotting analysis (data not shown). From this donor, it appeared that PR A was more abundant that PR B in this sample set across treatment (Fig. 4.5 D). PR A (Fig. 4.5 B) and B (Fig. 4.5 A) protein expression levels appeared to be elevated in MPA and P4 treated samples, while NET treatment appeared to have lower levels than the vehicle control. CORT appeared to elevate PR A expression levels only (Fig. 4.5 B), while DEX and LNG appeared to have no effect on PR protein expression (Fig. 4.5 A and B). Similarly it appeared that GRα levels were slightly elevated in P4 treated samples (Fig. 4.5 C), while the other ligands had no effect on relative protein expression levels. Interestingly, DEX turnover of the GR, was only observed in 1 of the 3 replicates (Fig. 4.5 D). More repeats need to be performed to elucidate the regulation of the SRs in response to the different ligands. Taken together, it appears that SR levels are not modulated in response to glucocorticoid or progestogen treatment after 48 hours.

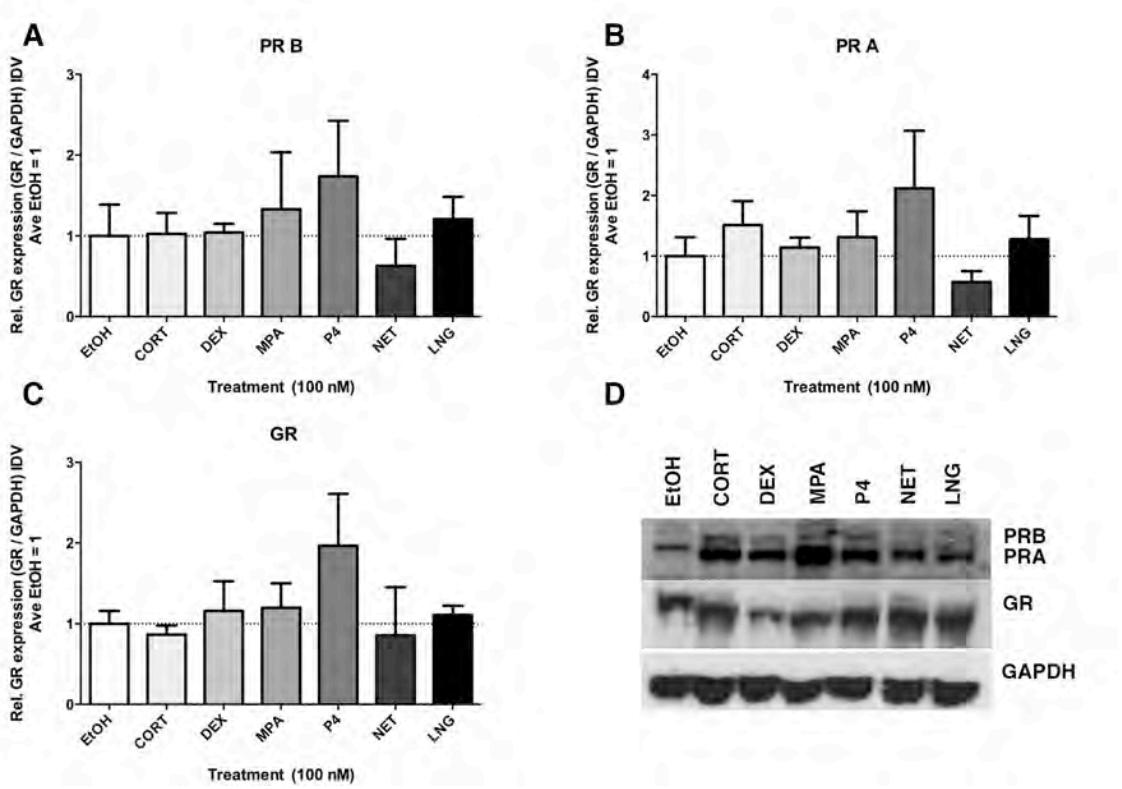


Figure 4.5: Progestins do not appear to modulate GR and PR protein levels from one donor. Primary ectocervical explants were incubated with vehicle i.e. 0.1% (*v/v*) ethanol (EtOH), or treated with 100 nM cortisol (CORT), DEX, MPA, P4, NET or LNG for 48 hours after which explants were homogenised in 800 µl TriReagent® (Sigma-Aldrich, South Africa). Protein samples, isolated from the phenol fraction, were electrophoresed on an 8% SDS-PAGE gel before being electroblotted onto a nitrocellulose membrane and probed for GRα, PR A B and GAPDH. Nitrocellulose blots were incubated for 1 minute in ECL-chemiluminescent substrate before being visualised by audio-radiography. (A-C) Represent the average PR B (A), PR A (B) or GR (C) expression normalised to GAPDH, determined by densometric analysis (AlphaEC™, Alpha Innotech, version 3.1.2) with a representative blot of one of the three replicate samples for each treatment group (D). Due to the isolation method, GR and PR protein bands appear to migrate slightly differently, possibly due to different amounts of salt and phenol contamination in each sample, and not indicative of the different isoforms of these proteins. Histograms represent pooled data from one donor (Prog 0024) with treatments performed in triplicate, plotted at mean ± SD.

Progestogens do not significantly modulate IL6 and IL8 mRNA levels after prolonged exposure in the absence and presence of HIV-1

It was observed in PBMCs that MPA no longer repressed IL6 mRNA levels after 9 days in the presence and absence of HIV-1, and that the expression of IL6 mRNA levels by MPA in these samples, correlated with an increase in HIV-1 infection levels in PBMCs. It was thus of interest to determine whether similar effects were observed in the primary genital mucosa using primary ectocervical explants. However, due to the high biological variability between donors and within donor treatments, no correlations could be made in this study. Furthermore, it was challenging to isolate good quality RNA from primary cervical explants after long term treatment (see appendix E, Fig. E.2.1 B). As such, the results should be cautiously interpreted due to both the methodological limitations and donor variability observed in this study.

After 12 days incubation with 100 nM MPA or NET, gene expression from 4 donors was analysed. The data suggest that in the presence of HIV-$1_{BaL\_Renilla}$ at 10 days post infection, 100 nM MPA appeared to elevate relative IL6 and IL8 mRNA levels compared to the vehicle control; however this was not significant (Fig. 4.6 A and B). Additionally, 100 nM NET treatment had no effect on IL6 or IL8 mRNA levels. GILZ mRNA expression appeared to increase with MPA treatment, while NET had no effect (Fig. 4.6 C). These experiments need to be confirmed with additional repeats, in the absence of HIV-1, so that the effects of the progestogens on gene expression after long term incubations can be determined in primary ectocervical explants.

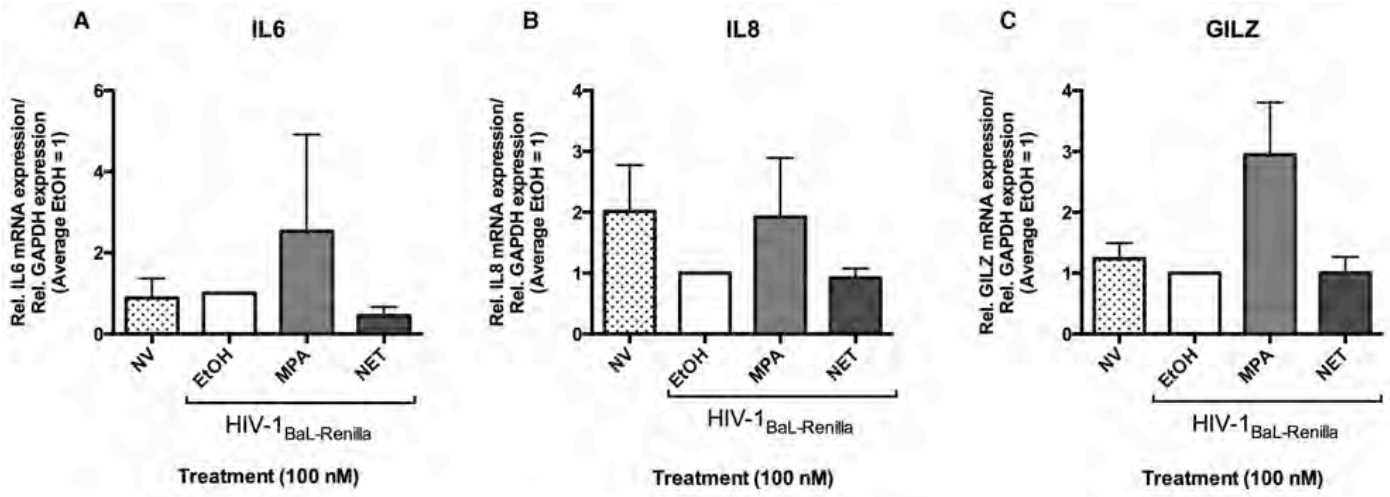
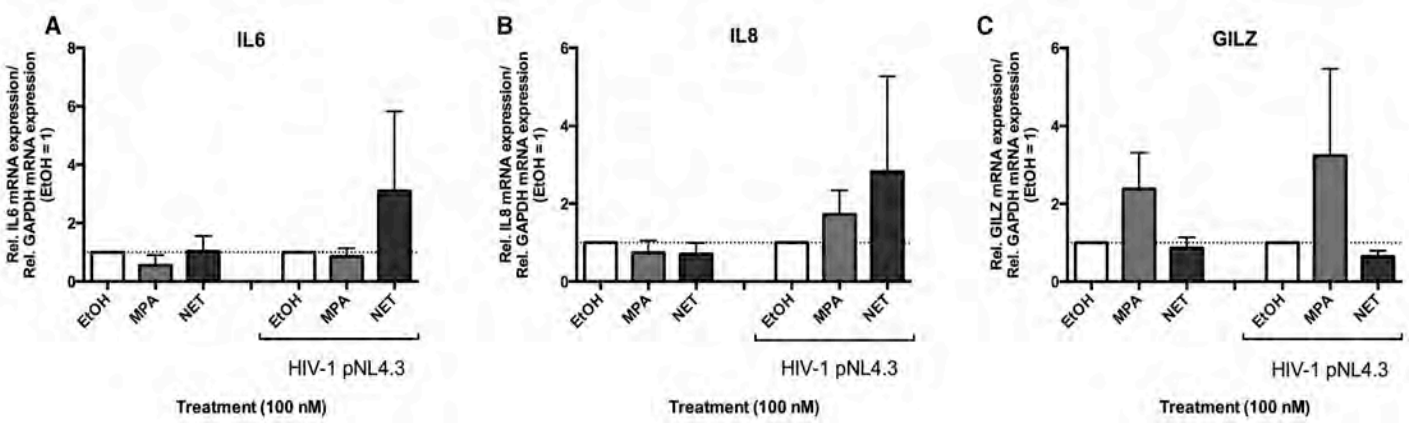


Figure 4.6: MPA and NET do not significantly modulate IL6 and IL8 mRNA expression, while MPA appears to transactivate GILZ mRNA expression in ectocervical explants after 12 days in the presence of HIV-$1_{BaL\_Renilla}$. Ectocervical tissue was treated for 48 hours with vehicle i.e. 0.1% (*v/v*) ethanol (EtOH), 100 nM MPA, or NET after which 500 IU/ml HIV-$1_{BaL\_Renilla}$ was added for 2 hours. Explants were washed 4 X with 1 X PBS, and media was replaced. The explants were cultured for a further 10 days, in the presence of ligands. Tissue was harvested in TriReagent® at 10 days post infection, where RNA was isolated and cDNA synthesised for use in subsequent real time qPCR. Relative IL6 (A), IL8 (B) and GILZ (C) mRNA expression levels were normalised to GAPDH. Relative fold change expression levels were determined by setting the vehicle control to 1. A - C are histograms representing pooled results from four donors, with each treatment performed in triplicate (Prog 0025, Prog 0028, Prot 0003 and Prog 0042), plotted as mean ± SEM.

Primary ectocervical explants were infected with the X4 tropic IMC, HIV-$1_{pNL4.3}$, and gene expression was assessed after 12 days. In this experiment, mRNA levels were assessed in the presence and absence of HIV-1.

The data from 4 donors after 12 days incubation with ligand indicated that in the absence of HIV-$1_{pNL4.3}$ infection, MPA appeared to reduce the mRNA levels of IL6, but had no effect on IL8 mRNA levels compared to the vehicle control (Fig. 4.7 A and B). In the presence of HIV-1, MPA appeared to have no effect on IL6 and IL8 mRNA levels compared to the vehicle control (Fig. 4.7 A and B). NET treatment in the absence of HIV-1 infection, appeared to have no effect on IL6 and IL8 mRNA expression levels. However, it appeared in the presence of HIV-1 that NET treatment elevated IL6 and IL8 mRNA levels (Fig. 4.7 A and B). MPA treatment after 12 days in primary ectocervical explants appeared to elevate GILZ mRNA expression levels compared to the vehicle control in both the absence and presence of HIV-1, however this was not significant (Fig. 4.7 C). NET had no effect on GILZ mRNA expression in any of the treatment conditions (Fig. 4.7 C).

Taken together, the data suggest that after 12 days in the presence of HIV-1, 100 nM MPA does not repress IL6 and IL8 mRNA levels (Fig. 4.6 A, B and 4.7 A, B), unlike the repressive effects observed after 48 hours. Similarly, 100 nM MPA appears to have no effect on IL6 and IL8 mRNA levels in the presence of HIV-1, 10 days post infection (Fig. 4.6 A, B and 4.7 A, B). While it appears that 100 nM MPA is able to transactivate GILZ mRNA expression this increase in expression is not significant (Fig. 4.6 C and 4.7 C). Additionally, it appears that virus tropism has no effect on MPA and NET driven gene expression (Fig. 4.6 and 4.7).



<u>Figure 4.7:</u> MPA and NET do not affect IL6 and IL8 mRNA expression, while MPA appears to transactivate GILZ mRNA expression in ectocervical explants after 12 days in the absence and presence of HIV-$1_{pNL4.3}$. Ectocervical tissue was treated for 48 hours with vehicle i.e. 0.1% (*v/v*) ethanol (EtOH), 100 nM MPA, or NET after which 500 IU/ml HIV-$1_{pNL4.3}$ was added for 2 hours. Explants were washed 4 X with 1 X PBS, and media was replaced. The explants were cultured for a further 10 days, in the presence of ligands. Tissue was harvested in TriReagent® at 10 days post infection, where RNA was isolated and cDNA synthesised for use in subsequent real time qPCR. Relative IL6 (A), IL8 (B) and GILZ (C) mRNA expression levels were normalised to GAPDH. Relative fold change in expression was determined by setting vehicle control (EtOH) in each group to 1. A - C are histograms representing pooled results from four donors (Prog 0035, Prog 0037, Prog 0039 and Prog 0040) with each treatment performed in triplicate, plotted as mean ± SEM.

MPA treatment exhibits variable effects on HIV-1 replication, while NET treatment appears to cause consistently reduced HIV-1 replication in tissue from most donors

Some observational studies as well as the current meta-analyses suggest that MPA increases HIV-1 acquisition in women (Polis et al., 2014; Ralph et al., 2015; Morrison et al., 2015). The lower female genital tract is a key site of initial HIV-1 transmission (Haynes & Shattock, 2008; Jespers et al., 2010), having a multitude of target cells present (Greenhead et al., 2000; Haynes & Shattock, 2008; Hladik & Hope, 2009). As such it was next sought to assess the direct effects of MPA and NET on HIV-1 replication in primary ectocervical explants, consisting of the upper epithelial layer as well as the underlying stroma. The advantage of using primary explants to assess the effects of the different progestins on HIV-1 viral replication is that this eliminates most of the confounding behavioural issues inherent in observational studies. Additionally these experiments allow one to determine the direct effect of a progestogen on cervical tissue as apposed to the indirect effects observed in *in vivo* studies.

Optimisation of the infection method for these experimental conditions was extensive. In the initial pilot experiments, tissue explants were infected individually. However due to the low titre of viral stocks produced in this study, the amount needed to obtain measurable productive infection was more than the volume of the 96 well. Therefore before proceeding with the experiments in this section, a better infection strategy was needed. A method from Arakelyan et al. (2013) was adapted for this study (Arakelyan et al., 2013). After 48 hours incubation with the different ligands, explants in triplicate treatments were removed and placed into a well of a 24 well plate, whereafter samples were infected for 2 hours with an appropriate IMC. After infection, samples were washed and placed back into the same 96 well plate. It was found that infecting each triplicate group in one well greatly reduced the error between the replicates when measuring p24 levels (appendix E, Fig. E.2.2 B). Further, the infection trend between the two methods (infecting individually versus infecting in triplicate) was similar (appendix E, Fig E.2.2 A and E.2.2 B) suggesting that the infection method did not change the outcome in infection. This method was subsequently used as the infection method for the results presented in this chapter.

In addition to the infection method, it was important to establish that the tissue explants were productively infected. One pilot experiment showed that the R5 HIV-1 entry inhibitor, Maraviroc, appeared to reduce HIV-$1_{BaL\_Renilla}$ replication levels over time compared to the vehicle control (appendix E, Fig.E.2.4). However no such studies were performed with HIV-$1_{pNL4.3}$. In order to assess productive infection levels accumulative plot curves for the vehicle control conditions in each experiment was assessed for a linear trend. That is, if the infection levels in the control group increased over time, a linear trend should establish that the increase was significant and had a positive slope. If the increase was not significant, and or the slope of the trend was negative, then the experiment was excluded from all subsequent analyses (see appendix E, Fig E.2.3 for examples).

Furthermore, in two of the experiments where explants were infected with HIV-$1_{pNL4.3}$, samples were taken immediately after infection (plotted as day 0 post infection). Here, it was shown that HIV-1 replication levels at days 3, 5, 7 and 10 post infection are greater than at day 0 post infection (Fig. 4.9 G and H). Taken together, these controls show that the tissue explants were productively infected in this current study.

Ectocervical explants were incubated with either 100 nM MPA or NET for 48 hours, before being infected for 2 hours with HIV-1 (X-tropic HIV-$1_{pNL4.3}$ or R-tropic HIV-$1_{BaL\_Renilla}$ IMCs). Explants were cultured for a further 12 days in the presence of the ligand, with supernatants collected at various time points. It was evident in this experiment that there was significant inter-individual variation in the response to progestin treatment on HIV-1 replication and gene expression. When analysing the individual plots of the relative infection (p24) levels (Fig. 4.8 and Fig. 4.9) for each HIV-1 virus type (HIV-$1_{BaL\_Renilla}$ or HIV-$1_{pNL4.3}$) it appeared that there were two types of responses to MPA treatment; those that appeared to have increased HIV-1 viral replication, and those that had no effect to decreased HIV-1 viral replication.

For the HIV-$1_{BaL\_Renilla}$ results, samples from donors Prot 0003 (Fig. 4. 8 A), Prog 0044 (Fig. 4. 8 B) and 0043 (Fig. 4. 8 C) appeared to have elevated HIV-1 replication levels in the MPA treated group compared to the vehicle control over time. Samples from donors Prog 0042 (Fig. 4. 8 D), 0025 (Fig. 4. 8 E) and 0049 (Fig. 4. 8 F) appeared to have similar or lower levels of HIV-1 replication in the MPA treated group compared to the vehicle control over time. At an individual level, NET treatment appeared to increase viral replication in one donor sample, less than MPA (Prot 0003, Fig. 4.8 A), and appeared to increase viral replication at day 10 post infection more than MPA in another donor sample (Prog 0043, Fig. 4.8 C). In samples from donors Prog 0044, 0042, 0025 and 0049 (Fig. 4.8 B, D, E and F respectively), NET treatment resulted in lower or similar effects to the vehicle control at all time points.

It appeared that phase of menstrual cycle did not contribute to the outcome of HIV-1 replication in MPA or NET treated samples. Five out of six donors were in the follicular phase, with 2 of these samples showing an increase in infection with MPA (Prot 0003 and Prog 0045), one showing a decrease in HIV-1 replication with MPA (Prog 0025), and two samples showing no difference in HIV-1 replication in the MPA treated samples compared to the vehicle control (Prog 0042 and Prog 0049). Donor Prog 0043 showed an increase in HIV-1 replication in the MPA treated samples and the donor was in the early ovulatory phase.

In this experimental subset, the average donor age was 45.8 years old (95% CI; 43.1 - 48.6), the average LH levels were 10.6 IU/L (95% CI; 1.0 - 20.1), FSH levels were 9.5 IU/L (95% CI; 5.1 - 14.0), E2 527.6 pmol/L (95% CI; -488.6 - 1543.8) and P4 levels were on average 2.3 nmol/L (95% CI; -1.2 - 5.8).

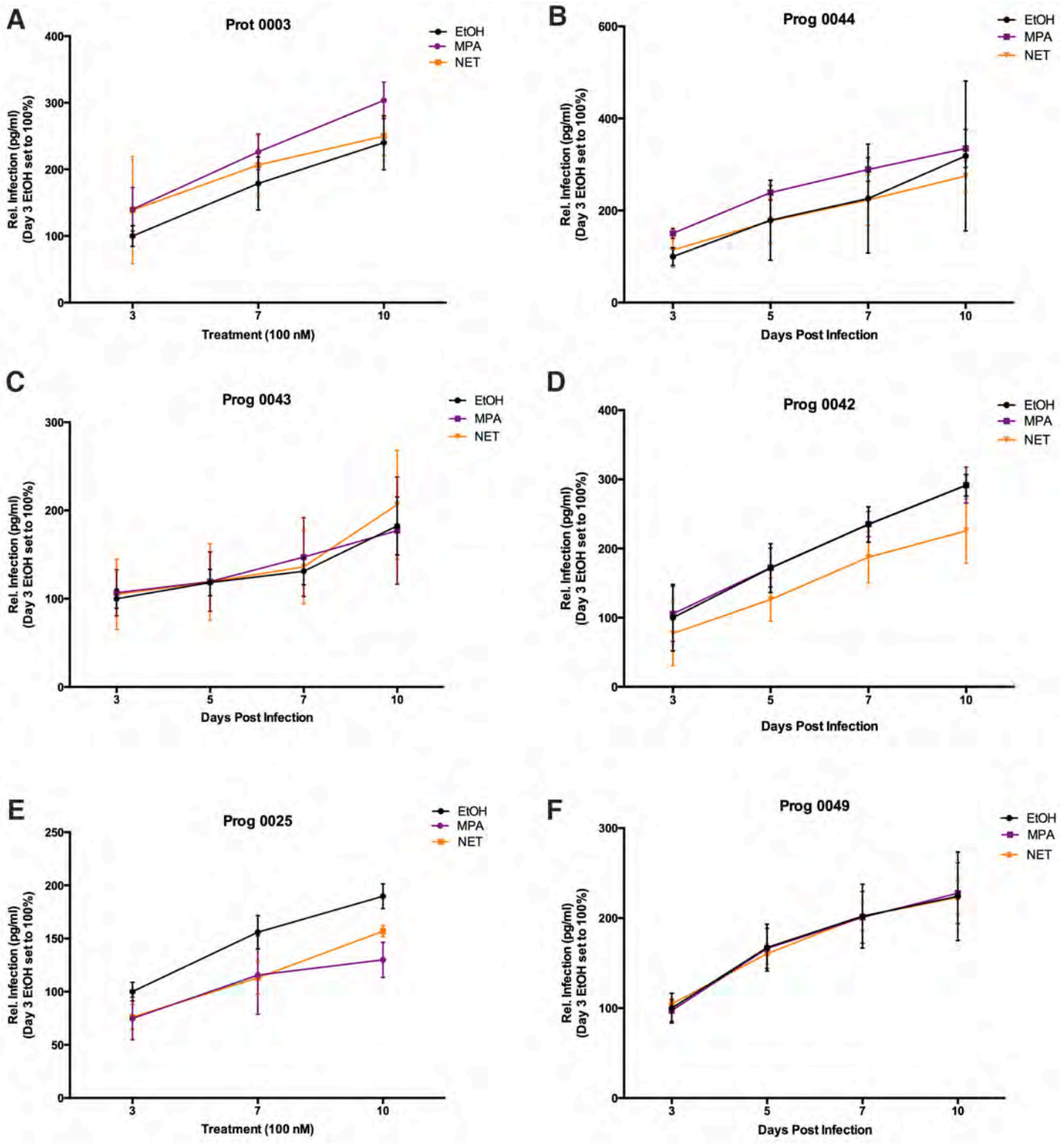


Fig. 4.8: Individual data plots showing ectocervical explants infected with HIV-$1_{BaL\_Renilla}$ over time, suggest that MPA and NET result in varied responses within and between donor samples. Ectocervical tissue from each donor was incubated in triplicate with vehicle i.e. 0.1% (*v/v*) ethanol (EtOH), or stimulated with 100 nM MPA, or NET for 48 hours after which 500 IU/ml HIV-$1_{BaL\_Renilla}$ was added for 2 hours. Explants were cultured for a further 10 days, in the presence of ligands, and infection was measured by p24 ELISA (Innogenetics, Belgium). (A-F) shows the relative infection levels of each donor at time points 3, 5 (only 4 donors were sampled at this time), 7 and 10 days post infection with HIV-$1_{BaL\_Renilla}$ with Day 3 EtOH set to 100% infection. A - F are pooled XY graphs of one donor representing relative infection plotted as mean +/- SD. See appendix C for individual donor information regarding hormone levels.

For the HIV-$1_{pNL4.3}$ infection data set, samples from donors Prog 0035 (Fig. 4.9 A), 0037 (Fig. 4.9 B), 0042 (Fig. 4.9 E), 0049 (Fig. 4.9 F) and 0050 (Fig. 4.9 G), appeared to have increased HIV-1 replication in the MPA treated group compared to the vehicle control. Samples from Prog 0039 (Fig. 4.9 C), 0040 (Fig. 4.9 D) and 0051 (Fig. 4.9 H) exhibited similar or lower levels of HIV-1 replication in the MPA treated group compared to the vehicle control. NET treatment appeared to increase HIV-1 replication compared to the vehicle control, but less than MPA, in one donor sample (Prog 0050, Fig. 4.9 G), while NET treatment of Prog 0035, 0037, 0039, 0042, 0049, and 0051 samples, resulted in no or lower levels of HIV-1 viral replication compared to the vehicle control.

As observed in the HIV-$1_{BaL\_Renilla}$ infected donor group, phase of menstrual cycle did not appear to affect the outcomes in HIV-$1_{pNL4.3}$ replication in the MPA treated samples. Of the 5 donor explants that showed an increase in HIV-1 replication, 3 were in the follicular phase (Prog 0042, Prog 0049 and Prog 0050), one was in the luteal phase (Prog 0035) and one donor explant was perimenopausal (Prog 0037). In donor explants that results showed a decrease or no effect in HIV-1 replication with MPA, two donors were in the luteal phase (Prog 003 and Prog 0051) and one was in the follicular phase (Prog 0040).

Interestingly, the effects by MPA and NET on HIV-$1_{pNL4.3}$ viral replication appeared to be more pronounced compared to the effects by the progestins on HIV-$1_{BaL\_Renilla}$ viral replication. That is, the differences between MPA and NET were greater in the samples infected with HIV-$1_{pNL4.3}$ compared to the differences between the samples in the HIV-$1_{BaL\_Renilla}$ infected group. At day 10 post infection, the average fold change difference of infection in the MPA group compared to the vehicle control was 1.1, while NET treatment was 0.78. In comparison, the mean relative fold change difference of infection at day 10 post infection with HIV-$1_{BaL\_Renilla}$ in the MPA treated group was 0.99 compared to the vehicle control, while the mean relative infection level in the NET treated group was 0.93.

It was apparent in the HIV-$1_{BaL\_Renilla}$ and HIV-$1_{pNL4.3}$ data sets the effects on viral replication by MPA and NET were prone to large intra-individual variation as evidenced by the large standard deviation at each time point within each patient (Fig. 4.8 and Fig 4.9).

In this experimental subset, the average donor age was 44.4 years old (95% CI; 41.1 - 47.7), the average LH levels were 8.3 IU/L (95% CI; -1.3 - 17.8), FSH levels were 12.3 IU/L (95% CI; -5.2 - 29.7), E2 240.0 pmol/L (95% CI; 108.5 - 371.6) and P4 levels were on average 4.4 nmol/L (95% CI; -1.5 - 10.3).

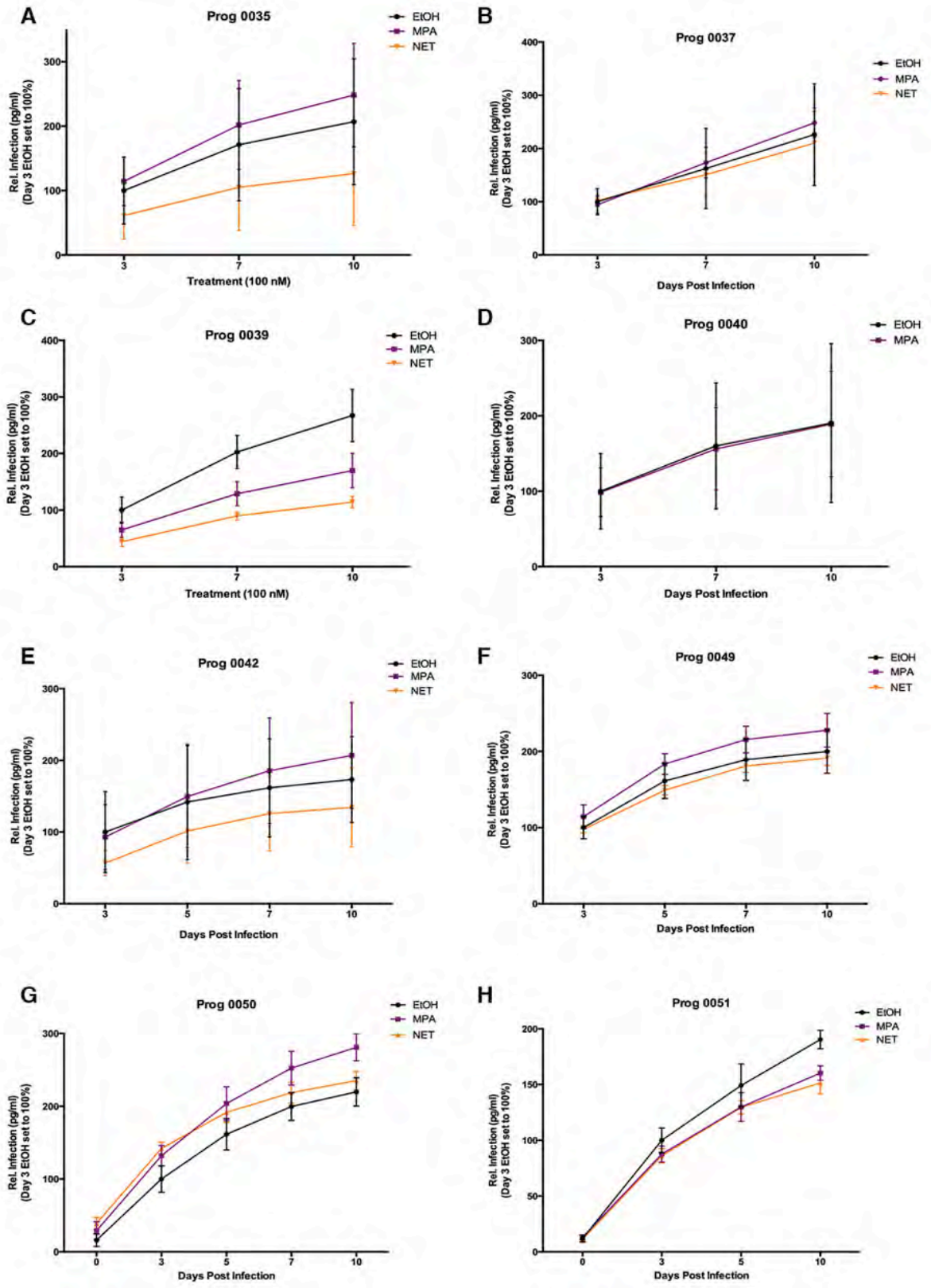


Fig. 4.9: Individual data plots showing ectocervical explants infected with HIV-$1_{PNL4.3}$ over time, suggest that MPA results in more varied responses compared to NET treatment between donors. Ectocervical tissue from each donor was treated in triplicate for 48 hours with vehicle i.e. 0.1% (*v/v*) ethanol (EtOH), 100 nM MPA, or NET after which 500 IU/ml HIV-$1_{PNL4.3}$ was added for 2 hours. Explants were cultured for a further 10 days, in the presence of ligands, and infection was measured by p24 ELISA (Innogenetics, Belgium). (A-H) shows the relative infection levels of each donor at time points 0 (only 2 donors were sampled at this time) 3, 5 (only 4 donors were sampled at this time), 7 and 10 days post infection with HIV-$1_{PNL4.3}$ with Day 3 EtOH set to 100% infection. A-H are pooled XY graphs of one donor representing relative infection plotted as mean +/- SD. See appendix C for donor information regarding individual donor hormone levels.

MPA increased HIV-1 replication in the majority of explant samples, while NET causes consistently reduced viral replication levels in explant samples

The individual infection data from the different donor samples strongly suggested that MPA may have different effects on HIV-1 replication. As such, the donor explant data were grouped as described above into groups where MPA appeared to increase viral replication or into groups where MPA appeared to have no or reduced effects on relative HIV-1 viral replication levels.

In the HIV-$1_{BaL\_Renilla}$ group where the results suggested an increase in viral replication (Fig 4.10 A, purple line), MPA treatment resulted in significantly higher levels of HIV-1 replication compared to the vehicle control and NET treatment at all the time points assayed (Appendix B, Table B3; n= 3, Day 3 pi, $p<0.001$; Day 7 pi, $p<0.001$ and day 10 pi, $p<0.05$). NET treatment (Fig. 4.10 A, orange line) showed significantly lower levels of HIV-1 replication compared to MPA treatment at all the time points assayed and at days 7 and 10 post infection to the vehicle control (Appendix B, Table B3). Additionally, time, treatment and the interaction thereof, were significant factors in the analysis (Appendix B, Table B3). In the HIV-$1_{BaL\_Renilla}$ group where MPA treatment resulted in similar or decreased levels of relative p24 levels, MPA treatment showed significantly lower levels of HIV-1 viral replication compared to the vehicle control at day 10 post infection (Fig. 4.10 B and Appendix B Table B4, n = 3, $p<0.05$). Interestingly, in this grouping, vehicle, MPA and NET treatment were similar at most time points, with only time contributing significantly to the analysis (Appendix B, Table B4).

HIV-$1_{pNL4.3}$ data indicated that in the donor samples that appeared to show an increase in HIV-1 viral replication (Fig. 4.10 C, purple line, n = 5), MPA treatment showed significantly higher levels of HIV-1 viral replication compared to the vehicle control (Appendix B, Table B5; Day 3: $p < 0.05$, Day 7 and 10 pi: $p < 0.0001$) and the NET treated group at all of the time points assayed (Appendix B, Table B5; Day 3: $p < 0.05$, Day 7 and 10: $p < 0.0001$). In addition, time, treatment and time X treatment interaction were all significant factors in the analysis (See appendix B, Table B5). NET treatment (Fig. 4.10 C, orange line) resulted in significantly lower HIV-1 replication than the vehicle control (Day 7: $p< 0.001$ and Day 10 pi: $p<0.0001$) and MPA treatment (Appendix B, Table B5; Day 3 and 7 pi: $p < 0.001$, and 10 pi: $p < 0.0001$). When grouping the samples that appeared to show similar or decreased levels in HIV-1 viral replication (Fig. 4.10 D, n = 3), MPA treatment (Fig. 4.10 D, purple line) resulted in significantly lower levels of HIV-1 replication compared to the vehicle control group at day 10 post infection ($p<0.05$, appendix B, Table B6). In addition, time, treatment and time X treatment interaction were all significant factors (See appendix B, Table B6). Unfortunately, the NET group only had 2 replicates, and as a consequence the statistical analysis could not be performed on this data set.

When comparing the relative infection levels in the group that showed an increase in HIV-1 viral replication (Fig. 4.10 A and C) to the group that showed a decrease in viral replication with MPA (Fig.4.10 B and D), MPA treatment resulted in higher relative levels of p24, compared to the group where MPA treatment decreased viral replication (Fig. 4.10 A and C compared to B and D

respectively). In the HIV-$1_{BaL\_Renilla}$ group, it appeared that the relative levels were about 2 fold higher, with the MPA relative p24 levels at day 10 post infection at a mean of 300% relative infection in the groups showing an increase (Fig. 4.10 A) versus the 160% relative infection in the group that showed a decrease in viral replication at day 10 post infection (Fig 4.10 B). Similar trends were observed between the vehicle control groups (Fig 4.10 A and B). However, NET relative infection levels between the groups appeared to be similar (Fig. 4.10 A and B).

As observed in the HIV-$1_{BaL\_Renilla}$ group, similar differences in viral replication levels were observed between the HIV-$1_{pNL4.3}$ groups (Fig. 4.10 C and D). In the group that showed an increase in HIV-1 replication with MPA (Fig. 4. 10 C), the mean relative infection level at day 10 post infection was 230%. In the group that showed a decrease in HIV-1 replication in the MPA treated group (Fig. 4.10 D), a mean infection level of 150% was observed at day 10 post infection. The data suggests that there is a 1.5 fold difference in HIV-1 replication between these groups.

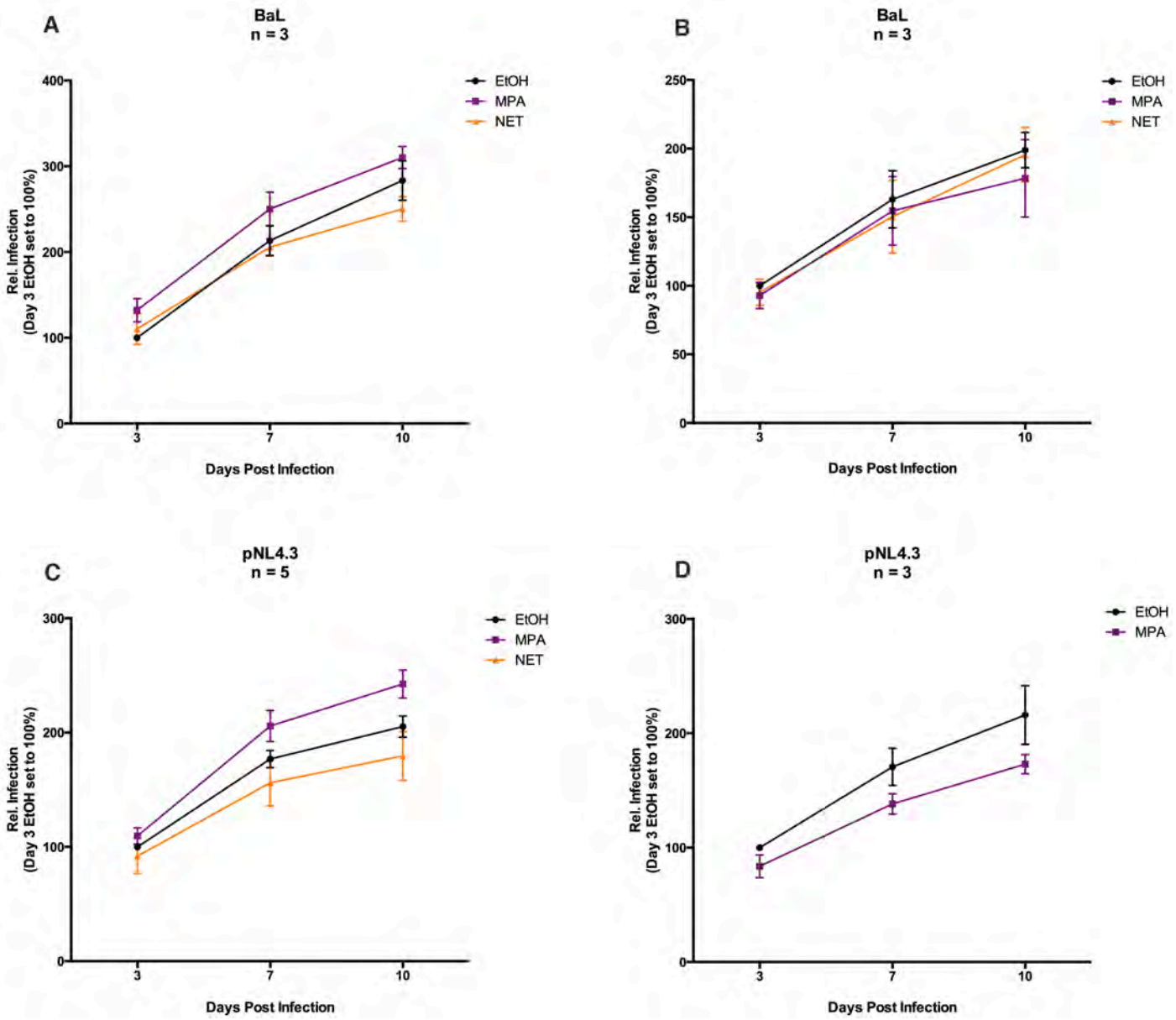


Figure 4.10: Treatment with MPA either significantly increases or significantly decreases HIV-1 viral replication in primary ectocervical tissue infected with either HIV-1$_{PNL4.3}$ or HIV-1$_{BaL_Renilla}$. Ectocervical tissue was incubated with vehicle i.e. 0.1% (*v/v*) ethanol (EtOH), or stimulated with 100 nM MPA, or NET for 48 hours after which 500 IU/ml HIV-1$_{PNL4.3}$ or HIV-1$_{BaL_Renilla}$was added for 2 hours. Explants were cultured for a further 10 days, in the presence of ligands, where infection was measured by p24 ELISA (Innogenetics, Belgium). (A-B) shows the relative infection levels of three donors at time points 3, 7 and 10 days post infection with HIV-1$_{BaL_Renilla}$. (C) Shows the relative infection levels of five donors at time points 3, 7 and 10 days post infection with HIV-1$_{PNL4.3}$ with Day 3 EtOH set to 100% infection. (D) Shows the relative infection levels of three donors at time points 3, 7 and 10 days post infection with HIV-1$_{PNL4.3}$. A repeated measures two way ANOVA was performed on these data sets (see Appendix B, Tables B3 - B6 for statistical outcomes). A - D are pooled XY graphs of three to five donors representing relative infection plotted as mean ± SEM.

When pooling all the data, MPA showed no significant effect on HIV-1 replication, while NET treatment resulted in significantly reduced HIV-1 replication levels in primary ectocervical explants

The grouping analysis suggested that while MPA treatment had differential effects on HIV-1 replication, NET treatment appeared to result in consistently reduced HIV-1 replication. The data from the HIV-$1_{PNL4.3}$ or HIV-$1_{BaL\_Renilla}$ were pooled to determine the overall effect of these progestogens on HIV-1 replication for all the explant experiments showing productive infection.

When ectocervical explants were infected with HIV-$1_{BaL\_Renilla}$, MPA treated explants showed similar effects on HIV-1 replication compared to the vehicle control (EtOH) (Fig. 4.11 A). However, results of NET treatment appeared to be different to both MPA and the vehicle control (Fig. 4.11 A).

In order to analyse in more detail and determine the statistical significance of the effects of MPA and NET on HIV-1 replication over time in the ectocervical explants, a repeated measure two-way ANOVA was used. Only matched time points could be utilised in this analysis. As such only relative p24 levels from days 3, 7 and 10 post infection were analysed (See Appendix B). The statistical analysis for the HIV-$1_{BaL\_Renilla}$ data set showed that the NET treated group had significantly lower relative infection levels ($p < 0.05$) compared to the MPA treated group at day 7 post infection (Fig. 4.11 B, and Appendix B Table B1). In addition, the repeated measure two-way ANOVA analysis revealed that time was a significant factor, while treatment was not (see Appendix B, Table B1). At day 10 post infection, the vehicle control (EtOH) had a mean relative infection level of 241.2% (95% confidence interval (CI) 184 - 298.4). The MPA treated group had a mean of 239.1 (95% CI 175.5 - 302.7) and the NET treated group had a mean relative infection level of 221.9% (95% CI 177.8 - 266.1). The confidence intervals, at day 10 post infection, suggest that the inter-individual variation within each data set is substantial.

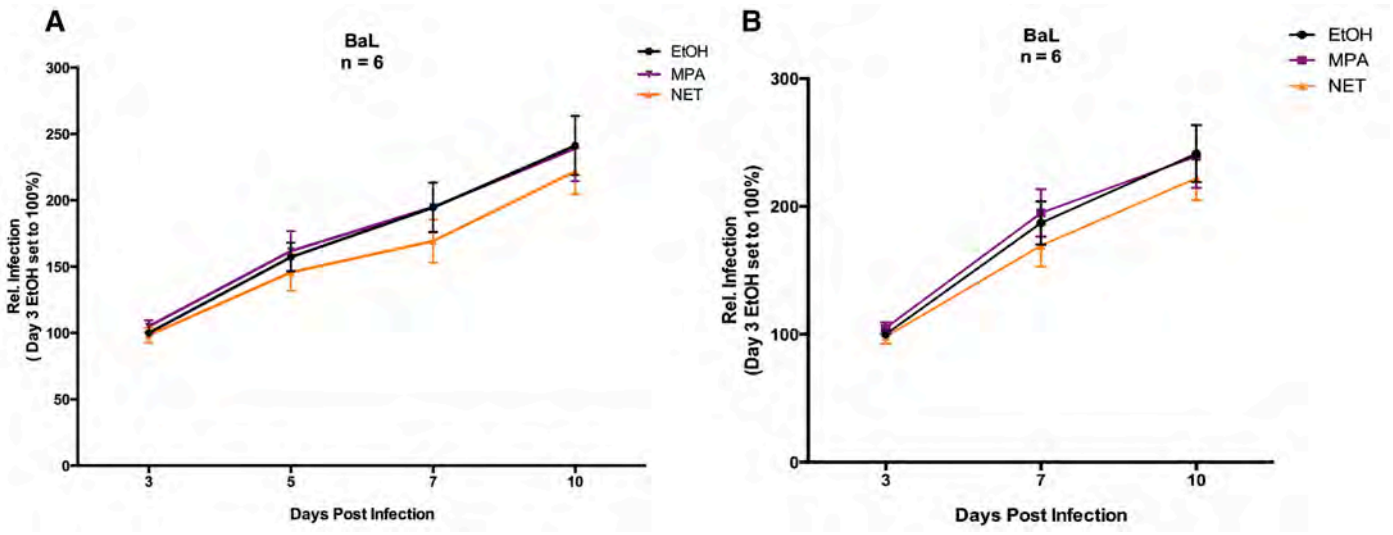


Figure 4.11: NET treatment significantly reduces HIV-$1_{BaL\_Renilla}$ replication, while MPA does not in pooled data from all primary ectocervical explants. Ectocervical tissue was treated in triplicate for 48 hours with vehicle i.e. 0.1% (*v/v*) ethanol (EtOH), 100 nM MPA, or NET after which 500 IU/ml HIV-$1_{BaL\_Renilla}$ was added for 2 hours. Explants were cultured for a further 10 days, in the presence of ligands, and infection was measured by p24 ELISA (Innogenetics, Belgium). (A) Shows the relative infection levels of six donors at days 3, 5, 7 and 10 post infection with HIV-$1_{BaL\_Renilla}$ with Day 3 vehicle control (EtOH) set to 100% infection. (B) Shows the data set used for statistical analysis, with matched data sets for six donors at time points 3, 7 and 10 days post infection with HIV-$1_{BaL\_Renilla}$ with Day 3 vehicle control set to (EtOH) 100% infection. A repeated measure two way ANOVA was performed (see appendix B, Table B1, for statistical outcomes). A and B are pooled XY graph of six donors representing relative infection plotted as mean ± SEM.

Ectocervical explants were infected with an X-tropic HIV-1 infectious molecular clone, HIV-$1_{pNL4.3,}$ and HIV-1 p24 levels were measured. The data suggest that MPA treated samples infected with HIV-$1_{pNL4.3}$ (Fig. 4.12 A) had similar viral replication levels compared to the vehicle control at all the time points assayed (Fig. 4.12 A). Although the results from the MPA and the vehicle control groups were similar, the MPA treated group had slightly higher mean values at the later time points (days 5, 7, and 10 post infection). In contrast, relative infection levels in the NET treated group were lower than the MPA and vehicle control groups (Fig. 4.12 A). Interestingly, when p24 levels were assessed directly after infection (taken as day 0 post infection) for two donors, results suggest that the predominant amount of p24 expression occurs between 0 - 5 days post infection. This is evident when comparing the slope of the curve at day 0 – 5 and the slope from day 5 – 10 days post infection (Fig. 4.12. A).

For the HIV-$1_{PNL4.3}$ data set, it was evident that at days 3, 7 and 10 post infection, the NET treated group relative p24 levels were lower than the MPA and vehicle treated groups (Fig. 4.12 A and B). This was confirmed in the repeated measure two-way ANOVA (Fig. 4.12 B), where NET treatment was found to result in significantly lower HIV-1 replication levels compared to MPA treatment and the vehicle control at all the time points assayed (Day 3: $p < 0.05$, day 7 and 10: $p < 0.0001$; Appendix B, Table B2). In addition the two way ANOVA analysis suggested that both time and time X treatment interaction were significant factors in this analysis (see appendix B, Table B2), with treatment having a

modest but not significant contribution (p = 0.0564) to the analysis. The mean relative infection levels at day 10 post infection for each group was, 214.4% (95% CI 189-4 - 239.4) for the vehicle control group (EtOH), 214.9% (95% CI 176.8 - 253) for MPA and 166.02% (95% CI 123.4 - 209.1) for NET. Once again, the confidence intervals suggest that the variation within each treatment group is high.

Taken together the data suggest that while the effects of MPA on the majority of samples may be masked when grouping the results from all the donors (Fig. 4.11 B and 4.12 B), the effect of NET on HIV-1 replication is consistently and significantly lower compared to the MPA treated and the vehicle control groups in primary ectocervical explants infected with either R- or X- tropic HIV-1 (Fig. 4.11 B and 4.12 B).

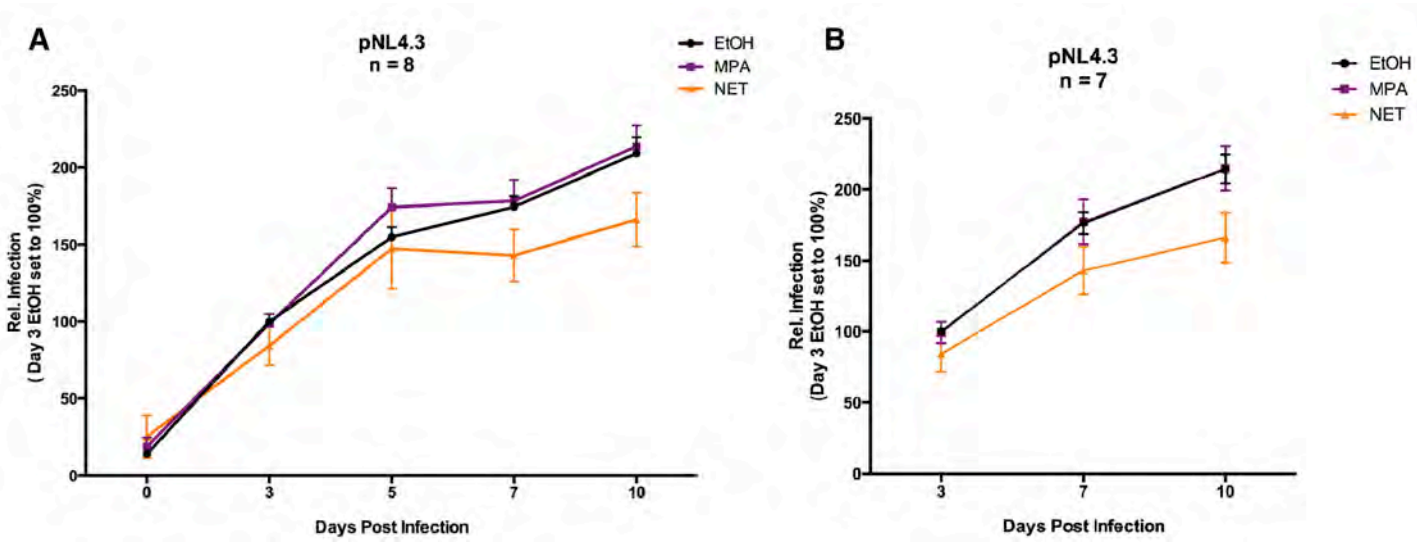


Figure 4.12: NET treatment has significantly reduced levels of HIV-$1_{PNL4.3}$ replication compared to the vehicle control while MPA appears to have no effect on HIV-$1_{PNL4.3}$ replication in pooled results from all infected primary ectocervical explants. Ectocervical tissue was incubated in triplicate for 48 hours with vehicle i.e. 0.1% (*v/v*) ethanol (EtOH), or treated with either 100 nM MPA, or NET after which 500 IU/ml HIV-$1_{PNL4.3}$ was added for 2 hours. Explants were cultured for a further 10 days, in the presence of ligands, and replication was measured by p24 ELISA (Innogenetics, Belgium) at various time points. (A) Shows the relative infection levels of eight donors at time points 0 (only 2 donors were sampled at this time) 3, 5 (only 4 donors were sampled at this time), 7 and 10 days post infection with HIV-$1_{PNL4.3}$ with Day 3 vehicle control (EtOH) set to 100% infection. (B) Shows the matched relative infection levels of seven donors at time points 3, 7 and 10 days post infection with HIV-$1_{PNL4.3}$ with Day 3 vehicle control (EtOH) set to 100% infection, used for the statistical analysis. A repeated measure two way ANOVA was performed on both data sets (see appendix B for statistical outcomes). A and B are pooled XY graph of eight (A) or seven (B) donors representing relative infection plotted as mean ± SEM.

Discussion

The female genital tract (FGT) is a major site for heterosexual infection and transmission in women. As such, the effects of different progestogens on immune gene expression and their effects on HIV-1 replication are important to establish in light of the recent observational and meta-analysis studies which suggest that MPA increases HIV-1 susceptibility in women (Noguchi et al., 2014; Noguchi et al., 2015; Ralph et al., 2015; Morrison et al., 2015). Furthermore, recent studies have observed that women on DMPA have a distinct immunosuppressive cytokine profile in their CVL (Ngcapu et al., 2015), as well as distinct cytokine profiles that are associated with an increased risk in HIV-1 infection (Morrison et al., 2014). However, these studies have not assessed the direct effects of different progestogens on immune gene expression or HIV-1 replication. This study utilised primary cervical explants from pre-menopausal women to assess the direct effects by the different progestogens on cervical tissue.

Primary cervical explants are useful models of the FGT to address the direct effects of progestogens in the genital mucosa (Hladik et al., 2007; Hladik & Hope, 2009; Merbah et al., 2011; Anderson et al., 2010; Hapgood, 2013). They are multicellular models consisting of epithelial cells, stromal cells, T cells, dendritic cells, Langerhan cells and macrophages (Hladik & Hope, 2009; Miller & Shattock, 2003; Pudney et al., 2005). However the relative distribution of these immune cells is dynamic within the ectocervix and endocervix (Anderson et al., 2010; Pudney et al., 2005; Merbah et al., 2011), posing challenges in biological reproducibility within a donor and between donors. Despite these challenges, this study found significant effects on gene expression in explant tissue from 5 donors upon short term incubation with the different progestogens. While the effects on infection were varied, when pooling for different types of responders, significant differences were observed within and between treatment types.

The menstrual cycle differentially affects steroid receptor levels in primary cervical explants

It was of interest to determine whether there are differences in the steroid receptor levels during the different phases of the menstrual cycle. As previously mentioned, progestogens are able to bind to and have differential activity towards steroid receptors other than the PR (Africander et al., 2011; Stanczyk et al., 2013; Hapgood et al., 2004). Further to this, it has been reported that changes in the steroid receptor concentration could change the apparent affinity (IC_{50}) and the potency (EC_{50}) of the ligand for the receptor (Hapgood et al., 2013). Thus it was hypothesised in this study, that the differential effects of P4 and E2 on the immune response could be mediated through changes in SR expression. In this study, pre-menopausal women that were undergoing hysterectomies for benign reasons were recruited. During consent, P4, E2, LH and FSH levels were obtained to accurately cycle women (see Appendix C). Select patients were chosen in the different phases of the menstrual cycle (follicular or luteal) and total protein was isolated from ectocervical tissue for downstream analysis by western blot. The data from this study suggest that the ERα and the PR isoforms, PRA and PRB, are

differentially expressed, with significantly lower expression levels of these receptors found in the luteal phase during the menstrual cycle compared to the follicular phase (Fig. 4.1). While the results in this study are preliminary, they support the results observed in Snijders et al. (1992), where the authors observed maximal PR and ER expression by immunohistochemistry in the glandular epithelial and stromal cells of the endometrium in the proliferative or follicular phase of the menstrual cycle (Snijders et al., 1992). Tibbets et al. (1998) observed that E2 stimulation in the mice uterus had compartmental effects on ERα expression increasing levels in the luminal epithelial cell layers, while decreasing in the stromal and glandular epithelial cells. E2 stimulation also increased PR levels in the stroma and myometrium. The authors found that P4 stimulation, negatively regulated PR levels in all the sites of the mice reproductive tract tested (Tibbetts et al., 1998). Similarly it has been observed that E2 increases PR mRNA expression levels, while P4 decreases PR levels in the cerebral cortex of ovarectomised rabbits (Camacho-Arroyo et al., 1996), and that there are estrogen response elements in the promoter regions of the PR (Kastner et al., 1990). It has also been observed in a breast cancer cell line, that PRB is able to repress ERα mRNA transcriptional levels via binding to a 1/2 PRE site in the promoter region of the ERα gene (De Amicis et al., 2009). Lastly, it has been observed in a vaginal epithelial tissue model, that E2 increased PRB expression levels (Ayehunie et al., 2015). Interestingly, these authors used ectocervical explants as controls, and observed that ERα expression within the ectocervix was predominantly expressed in the epithelial cells, while PRB expression was predominantly in the basal stromal layer (Ayehunie et al., 2015). These studies corroborate the data observed in this present study, whereby there are higher ERα, PRA and PRB levels in the follicular phase, where E2 is high and P4 low, compared to the luteal phase, where E2 is low and P4 is high. It may be that when E2 levels are elevated in the follicular phase, ERα, PRA and PRB levels increase through an increase in ER-driven transcription of the ER and PR genes in the epithelial and stromal cells of the ectocervix. While PR and ERα are easily and detectably expressed in the primary ectocervical explants in this study, the expression levels of GRα and AR are low (Fig. 4.1). As such, GRα and AR expression levels could not be accurately determined (Fig. 4.1). This is the first study to report GR and AR protein expression in primary human ectocervical tissue. Further, this study suggests that MPA, NET and LNG may be able to induce transcriptional responses through their partial agonism towards the GR (MPA only) or the AR in the ectocervix. While the detection of AR and GR was not possible in all donor explants, either due to biological variation or methodological limitations, it may be that the relative levels of the GR and AR are donor dependent.

Progestogens differentially modulate immune gene expression and cause distinct expression profiles in the ectocervix and the endocervix

Having investigated the expression levels of steroid receptors, the difference in gene expression of selected immune function genes by the different progestogens compared to glucocorticoids was investigated after 48 hours incubation. Additionally, the effects of the progestins on gene expression in different cervical compartments, as well as changes in steroid receptor profiles, were assessed.

When investigating the effects of progestogens on gene expression in primary cervical explants, it was evident that compartment specific regulation occurs between the ectocervix and the endocervix (Fig. 4.2). In the ectocervical explants 100 nM CORT, DEX, MPA and P4 treatment significantly decreased IL6 mRNA expression. NET and LNG also appeared to slightly reduce IL6 mRNA expression levels, although this was not significant. CORT, DEX and MPA significantly repressed relative IL8 mRNA expression levels compared to the vehicle control, while P4, NET and LNG had no effect after 48 hours. These results suggest that there could be different sensitivities towards different genes by the different ligands in the ectocervix. Additionally only MPA and DEX significantly increased GILZ mRNA levels (Fig. 4.2), suggesting that in the ectocervix, MPA, like DEX, exerts glucocorticoid effects most likely via the GR. Thus it could be that in the ectocervix, 100 nM MPA has a greater effect on IL6 and IL8 mRNA expression than NET, P4 and LNG through differential steroid receptor usage, as well as the recruitment of different co-repressors to the promoter elements of the genes, to elicit different responses. DEX and MPA were able to significantly transactivate GILZ mRNA expression after 48 hours in the ectocervix. P4, NET and LNG had no effect on GILZ mRNA expression (Fig. 4.2). Interestingly, CORT (the natural glucocorticoid ligand) also had no effect on GILZ mRNA expression in the ectocervix, showing that DEX and MPA are more efficacious than CORT on GILZ mRNA transactivation in the ectocervix. Hadley et al. (2011) reported similar effects with CORT, DEX and MPA on GILZ transactivation in an airway epithelial cell line (A529), and found that these ligands had differential effects on GR translocation and recruitment to the promoter regions of the GILZ gene (Hadley et al., 2011). Thus it could be that DEX, more so than MPA, more so than CORT, increases nuclear translocation and subsequent GR recruitment to the promoter element of the GILZ gene, increasing transcription in ectocervical explants.

When assessing the mRNA expression levels in the endocervix after 48 hours incubation with the different ligands, it was interesting to observe that MPA appeared to exhibit less repressive effects on IL6 and IL8 mRNA expression levels compared to the ectocervix, and that DEX treatment did not repress IL6 or IL8 mRNA expression (Fig. 4.2). DEX and MPA significantly increased GILZ mRNA levels suggesting that the transactivation mechanism of action by DEX and MPA is more effective in the endocervix compared to the transrepression mechanism of action, or that the factors necessary for transactivation via the GR are present in the endocervix, while factors necessary for transrepression may be limiting in the endocervix. Govender et al. (2014) observed in an endocervical cell line that DEX

and MPA significantly repressed IL6, IL8 and RANTES mRNA expression levels, and that this was via a GR dependent mechanism, while NET-A had no effect on mRNA levels for these genes (Govender et al., 2014). While the data from this study did not report significant repressive effects by MPA on IL6 and IL8 mRNA levels, the results from Govender et al. (2014) suggest that repressive effects may occur in the epithelial cells of the endocervix. Additionally, Verhoog et al. (2011) found that in End1/E6E7 cells activated with TNF-α, IL6 mRNA expression levels were significantly reduced by DEX treatment, and that this repression was via the GR (Verhoog et al., 2011). However, of interest is that DEX at the same concentration as MPA had no effect on transrepression in the endocervix, supporting the idea that factors necessary for GR dependent gene repression may be limiting in the endocervix, compared to the ectocervix. The lack of repression by DEX, and the reduced repression by MPA (no significant effect) on IL6 and IL8 mRNA levels in the endocervical explants compared to the ectocervical explants also suggests that there could be cell type differences present in the endocervix compared to the ectocervix, as well the differential expression levels of steroid receptors in these cell types, which may govern the level of responses observed.

In a study comparing the effects of P4, MPA and NET on mRNA levels in different cell types, Africander et al. (2011) found in ectocervical and vaginal cell lines that 1 μM P4 increased TNF-α induced IL6 mRNA expression levels, while 1 μM MPA and NET had no effect in both cell lines after 24 hours (Africander, et al., 2011). The authors also found that TNF-α induced IL8 mRNA expression was significantly upregulated by P4 and MPA, but not NET-A in ectocervical cells, while the ligands had no effect on IL8 mRNA expression in vaginal cells (Africander et al., 2011). While the results from the latter study appear contrary to the current findings, the ectocervical explant experiments are in the context of a multicellular environment with different cell types such as epithelial cells, dendritic cells, stromal cells, T cells, and macrophages within each explant tissue (Pudney et al., 2005; Greenhead et al., 2000), which most likely contribute to the total effect of mRNA gene expression observed (Fig. 4.2). The concentrations of ligands used in this current study were also lower than those reported by Africander et al. (2011) and mRNA levels were assayed at a longer time point in this study. In addition, the results in Fig.4.2 are from experiment in the absence of immune activation, while Africander et al. (2011) performed experiments in the presence of TNF-α (Louw-du Toit et al., 2014a; Africander et al., 2011). Collectively, these results highlight that the progestogens may exert differential effects in individual cell types, suggesting that this may be due, in part, to the differential expression levels of steroid receptors.

Few studies have used primary cell systems to assess the changes in gene expression by different progestins. In one such study, Huijbregts et al. (2013) found in vaginal mucosal mononuclear cells (VMMCs) that IL6 protein expression levels were reduced, while IL8 protein levels were unaffected by MPA treatment. Interestingly, P4 significantly increased IL8 protein expression at 100 nM after 24 hours treatment (Huijbregts et al., 2013). These results agree with some of the mRNA expression data observed in this study, whereby MPA but not P4, decreased IL6 mRNA expression in both ectocervical (significantly)

and endocervical explants (Fig. 4.2) and suggest that MPA directly represses several proinflammatory responses after 24 – 48 hours in different cell types in the female genital tract. However the results from the current study suggest that the significant repression of IL8 mRNA expression by MPA after 48 hours in the ectocervical explants may not be via the mononuclear cells. Additionally the results suggest that length of incubation with MPA may be important in determining the outcome of repression on IL8 mRNA levels. Results from the PBMC short time course (Fig. 3.1) in this study also showed repression with MPA on IL8 mRNA levels after 48 hours, but not at 24 hours. Moreover, this data suggest that variation in the repressive effects of MPA on key immune gene may due to the relative levels of the different cell types present within the tissue sample. In a recent transcriptome analysis on endometrium and cervical transformation zone tissue obtained from women on DMPA and LNG for at least 6 months (IUD), Goldfien et al. (2015) observed that DMPA and LNG differentially regulated gene expression. Interestingly, the authors found that both LNG and DMPA had greater transcriptional effects in the endometrium, with little expression changes observed in the cervical transformation zone (Goldfien et al., 2015). The authors propose that the progestins alter the immune milieu in the upper female genital tract, that changes the recruitment of target cells, and key signalling pathway responses that may increase the risk in sexually transmitted diseases, including HIV-1 (Goldfien et al., 2015). LNG in this current study, had weak to no effects on IL6, IL8 and GILZ mRNA expression (Fig 4.2), while MPA had significant effects on the mRNA expression levels of these genes in ectocervical explants. Goldfien et al. 2015) reported in their global analysis, that there were greater effects on gene expression in the ectocervix than the endocervix which is in agreement with the results of this current study whereby greater effects on gene expression were observed in the ectocervix compared to the endocervix.

When comparing the gene expression results of ectocervical and endocervical explant results to the results obtained in PBMCs after 48 hours using the same concentration of ligands, it is interesting to observe that the results in the ectocervical explants are more similar to the results observed in PBMCs than to the endocervical explant results. This could be due to differential cell type distributions, and the different relative expression levels of SRs, particularly the presence of the GR, in mediating the DEX and MPA repression on IL6 and IL8 mRNA levels after 48 hours. Furthermore, the current study shows that ectocervical explants express the PR, unlike PBMCs, suggesting that NET, P4 and LNG may have more pronounced effects in the local mucosa compared to the systemic system through exerting more prominent progestational effects via the PR. What is not known is the SR expression levels in endocervical explants, which would provide further insight into the site specific regulation of IL6, IL8 and GILZ mRNA levels in different immune environments. However the data suggest that the repressive effects by DEX, CORT and MPA in the ectocervical explants are most likely via the GR, similar to what was observed in PBMCs.

Taken together, the data from this study suggest that progestogens and glucocorticoids have differential effects on gene expression in different mucosal compartments. The data suggest that

differential cell type distribution, and consequently steroid receptor and co-factor expression may determine the effects on gene expression by the different progestogens and glucocorticoids. Furthermore the results of this study suggest that the effects of MPA on gene expression after 48 hours in the ectocervical explants are most likely mediated via the GR.

Progestogens do not appear to regulate SR expression after 48 hours in the ectocervix

The results from the current study suggest that there is compartment specific gene regulation within the different sites in the FGT. Further this suggests that the different progestogens, at the same concentration within the same tissue environment, have differential effects on gene expression. These differences could be due to the differential expression levels of the steroid receptors that may be regulated by the different ligands. The effects of these progestogens and glucocorticoids on steroid receptor expression levels in ectocervical explants were investigated in order to elucidate some of the mechanisms underpinning the gene regulation observed. Due to sample constraints, mRNA levels in the endocervical explants could not be determined. Consequently, the differences in the relative steroid receptor levels in the endocervix compared to the ectocervical explants could not be established.

The results suggest that the progestogens do not significantly alter mRNA levels of the AR, ERα, GRα, MR or PR after 48 hours (Fig. 4.4). Additionally, the data from one donor sample indicated that DEX increases turnover of the GR, while no other ligand had any effect on GR expression levels. It also appeared that PRA protein levels were greater than PRB protein levels in this donor sample. However, there was little difference in the protein expression patterns of these isoforms upon ligand treatment (Fig. 4.5). Only NET treatment appeared to affect the protein expression levels of PRB and PRA (Fig. 4.5). As previously mentioned, P4 is known to negatively regulated PRB expression in myometrial cells (Camacho-Arroyo et al., 1996; Tibbetts et al., 1998), as such it was thought that the treatment with the progestogens may also decrease the PR expression levels in primary cervical explants. More repeats are needed to elucidate the regulation of the SRs by different progestogens. Surprisingly P4, MPA and LNG did not exert strong effects on PR mRNA or protein expression, after 48 hours. However, it could be that effects may be observed at longer time points with these progestogens on SR regulation. While the expression of the PR is important, it is the expression of the different isoforms that may contribute to the differential effects of the different progestogens on gene expression. Mesiano et al. (2002) found that when comparing the ratios of PRA to PRB and gene expression in myometrium, obtained from labouring and non-labouring women, P4 responsiveness in the myometrium was, in part, controlled by the relative ratio of PRA to PRB (Mesiano et al., 2002). Tan et al. (2012) found that the relative ratio of PRA to PRB correlated with the transcriptional effects in a myometrial cell line. The authors found that increasing levels of PRA compared to PRB resulted in a change from anti-inflammatory actions via PRB to pro-inflammatory responses via PRA through inhibiting PRB, in response to P4 (Tan et al., 2012). While it was not possible to discriminate between PRA and PRB mRNA levels in the current study (Fig. 4.4), the protein results from one donor sample suggested that the PRA to PRB ratios may be different upon MPA, P4, NET and LNG treatment in primary ectocervical

explants (Fig. 4.5). Thus, it could be that the different ratios of PRA to PRB could also contribute towards the transcriptional responses observed in this study by the different progestogens.

Regulation of steroid receptor mRNA and protein levels is not the only mechanism by which these ligands may affect SR mediated responses. It may be that these ligands activate the SRs differentially via phosphorylation. For example, Avenant et al. (2010) found that 100 nM MPA but not NET resulted in GR phosphorylation after 24 hours, and positively correlated with transactivation and transrepression potential on promoter-reporter constructs (Avenant et al., 2010). Thus it could be that ligand selectivity for phosphorylation of SRs could play a deterministic role on the outcome of the response. Indeed, Chen et al. (2008) found that the extent of phosphorylation of the GR at the different serine residues was concentration dependent and resulted in differential effects on mRNA expression levels on GR-driven genes (Chen et al., 2008). Thus it could be that the ligands differentially modulate SR phosphorylation in a concentration dependent manner that mediates their differential effects on gene expression in ectocervical explants.

Taken together the results from this study suggest that these ligands have no effect on SR mRNA or protein expression levels, although more repeats are needed to elucidate this issue. While not investigated in this study, it could be that these ligands may affect SR activation through post-transcriptional or post-translational effects that may modulate their transcriptional responses, either by changing relative ratios of PRA to PRB or GR phosphorylation (Avenant et al., 2010; Tan et al., 2012; Chen et al., 2008; Mesiano et al., 2002).

The effects of MPA on gene expression in the ectocervix are dose dependent

In order to fully understand the effects of concentration of the progestogens on mRNA gene expression, dose response analyses were performed. This is vitally important, as the concentration of MPA within different tissue environments is not known. Circulating serum concentrations have been elucidated in several studies, and have indicated that between individuals, MPA serum concentrations vary substantially (Shelton & Halpern, 2014; Halpern et al., 2014). Studies have shown that MPA concentrations peak a few days after subcutaneous injection ranging from 2.5 - 65 nM (Ortiz et al., 1977; Mishell, 1996; Hiroi et al., 1975; Mathrubutham & Fotherby, 1981), before plateauing to ~ 2.5 nM for the remainder of the prescribed course (Africander et al., 2011; Mishell, 1996). As such using a range of concentrations is important to reflect the possible concentrations of MPA in the ectocervix. Due to the limited availability of tissue in this study, a dose response was performed with only MPA in primary ectocervical explants.

This is the first study to report a dose response analysis in primary ectocervical explants with MPA treatment. The data suggested that repression by MPA was more potent on IL8 mRNA gene expression than IL6 mRNA gene expression. That is, a low concentration of MPA may significantly decrease IL8 mRNA levels while have no effect on IL6 mRNA levels at the same concentration. A potency (EC_{50}) of ~ 18 nM was calculated for IL8 compared to an EC_{50} of ~70 nM for IL6 gene expression (Fig. 4.3).

Interestingly, it is apparent that at the lower concentrations of MPA gene expression was more variable with some samples showing an apparent increase in IL6 mRNA expression at 1 and 10 nM concentrations, with variation of IL8 mRNA expression at 1 nM MPA only. In addition, MPA was able to transactivate GILZ mRNA expression at low concentrations, with an EC_{50} of ~ 7.5 nM (Fig. 4.3). This data also highlights the gene specific effects of MPA, with some pro-inflammatory genes being more sensitive to MPA treatment than others. Due to the limited availability of tissue for this study, only four concentrations were used in order to determine the $EC_{50,}$ as such the potencies determined for this study should be cautiously interpreted. Irvin and Herold (2015) observed in vaginal Vk2/E6E7 cell line, that MPA (70 – 388 µM) dose dependently increased the mRNA and protein expression levels select immune modulators (Irvin & Herold, 2015). While these data are contrary to the data obtained in the current study that shows increased concentrations of MPA lead to an increase in repressive effects, the Irvin and Herold (2015) study used concentrations of MPA well above the range reported in this study. Further, the authors assessed transcription at 5 days post treatment (Irvin & Herold, 2015) compared to this study that assessed transcriptional effects at 2 days post treatment (Fig. 4.3). However, their data together with those reported in this study suggest that time and concentration of ligands used may greatly affect the outcome of gene expression. The results in this current study are near peak serum MPA concentrations in women, and thus may be more indicative of what is most likely to occur *in vivo*. Huijbregts et al. (2013) found that at increasing doses of MPA (10, 100 nM and 1 µM) increased the repression on IL6 protein levels in primary vaginal mononuclear cells (Huijbregts et al., 2013). While the authors did not perform a dose response analysis, it appeared in their study that the EC_{50} for MPA on IL6 protein expression is most likely between 10 – 100 nM, similar to what was observed in this study in primary ectocervical explants. Their data, together with the data in this study, suggest that in the lower FGT, the effects of MPA on IL6 mRNA levels occur at peak serum concentrations. Dose response analyses performed by Govender et al. (2014) in an endocervical cell line observed potencies (EC_{50}) of ~21 nM for IL6 mRNA and ~ 4 nM for IL8 mRNA expression by MPA after 24 hours incubation (Govender et al., 2014). While Govender et al. (2014) reported more potent EC_{50}s for MPA on IL6 and IL8 repression, MPA was more potent on IL8 than IL6 mRNA levels, as found in this current study. The differences in the potency values reported in this study compared to those reported by Govender et al. (2014) could be due to the different models used. In this current study, a multicellular ectocervical explant system was used compared to an endocervical cell line used by Govender et al. (2014). Additionally, it has been reported by Govender et al., (2014) that the endocervical End1/E6E7cell line, only expresses the GR, while primary ectocervical explants express the AR, ER, GR and PR at an mRNA and protein level (Fig. 4.1, 4.4 and 4.5). Interestingly, transactivation of GILZ by MPA in the endocervical cell line had a potency of ~24 nM (Govender et al., 2014), compared to ~7.5 nM observed in this study, suggesting a stronger transactivation potential in primary ectocervical explants.

Taken together, the data suggest that MPA at concentrations similar to and below peak serum concentration (100 nM) has distinct gene specific effects after 48 hours. The data suggests that

concentration of MPA is important in determining the range and extent of the effects on gene expression. At peak serum concentrations, MPA may exert significantly repressive effects on IL6 and IL8 gene expression and increase GILZ mRNA expression, while at lower concentrations, MPA may exert significant repressive effects on IL8 but not IL6 mRNA expression, while maintaining the increase in GILZ mRNA expression. The EC_{50} observed in this study for IL6, IL8 and GILZ are similar to what was reported by Govender et al. (2014). Additionally the profile of MPA on IL6 and IL8 are similar profile to that observed by Huijbregts et al. (2013).

The immunosuppressive effects on pro-inflammatory genes of MPA are reduced or lost after long term exposure in the absence and presence of HIV-1

It was observed in the PBMC results that MPA no longer repressed IL6 mRNA levels in the majority of donors over a longer time course in the absence and presence of HIV-1. It was therefore of interest to determine the effects on gene expression by MPA and NET in primary cervical explants over a longer time course. However, RNA isolated from donors after long term incubation with different ligands was of poor quality (appendix E, Fig E.2.1 B). As such the RNA data obtained after 12 days in this study had large variability. In comparison, short term incubation with the ligands in ectocervical explants produced good quality RNA and the data obtained had significant differences between the treatment groups (Fig. 4.2 and appendix E, Fig E.2.1 A). Therefore, the data obtained after 12 days are preliminary and should be interpreted cautiously.

Data from the HIV-$1_{BaL\_Renilla}$ group showed that after 10 days post infection, MPA did not repress IL6 or IL8 mRNA expression levels in the presence of HIV-$1_{BaL\_Renilla}$, where in some cases it appeared to be pro-inflammatory. MPA treatment appeared to increase GILZ mRNA expression at day 10 post infection relative to the vehicle control (Fig. 4.6). NET had no effect on gene expression in this group (Fig. 4.6). In the HIV-$1_{pNL4.3}$ group, gene expression in the absence and presence of HIV-1 was determined. In this data subset, MPA treatment appeared to lose its repressive effects on IL6 and IL8 mRNA levels in the absence and presence of HIV-1 (Fig. 4.7). Interestingly, MPA induction of GILZ mRNA levels was varied in the presence of HIV-1 and was not significant (Fig. 4.7), unlike that observed at a shorter time point (Fig. 4.2). Additionally NET had no effect on gene expression in the absence of HIV-1, however, it appeared in some donors that in the presence of HIV-1, IL6 mRNA expression increased (although this was not a significant trend) (Fig. 4.7).

Taken together, the data suggests that MPA does not decrease IL6 or IL8 mRNA levels in the presence of HIV-1 after 12 days. In some cases NET had apparent pro-inflammatory effects on IL6 mRNA in both the absence and presence of HIV-$1_{pNL4.3}$ after 10 days. Interestingly, this was not observed in HIV-$1_{BaL\_Renilla}$. There could be differential effects on gene expression by MPA and NET that may be in part regulated by the type of virus. Further, while not significant, it appeared that MPA increased GILZ mRNA transcription in the absence and presence of HIV-1. Due to the high variability in gene expression in this study, statistical analysis did not reveal any significant effects.

MPA significantly increases HIV-1 replication is a subset of donor explants, while NET significantly and consistently decreases HIV-1 replication in primary ectocervical explants

Having established that there are differential effects on gene expression by the different progestogens, the next step was to focus on the direct effects of MPA and NET, the two most common injectable contraceptives in South Africa (Tomasicchio et al., 2013; United Nations, Department of Economic and Social Affairs, Population Division, 2011) on HIV-1 replication in primary cervical explants. This study is the first to report on the direct differential effects of MPA and NET on HIV-1 replication in primary cervical explants. While observational studies have provided useful evidence on the effects of these progestogens *in vivo,* these studies have unavoidable confounders; such as the reporting of contraceptive type, adherence and switching of contraceptive type, sexual behaviour and condom usage (Ralph et al., 2015; Morrison et al., 2015; Polis et al., 2014) which affects the outcome of the results. Furthermore, the individual observational studies vary in sampling times, population groups and types of progestogens tested (Polis et al., 2014). Thus *ex vivo* methodologies are useful in determining the direct effects of these progestogens on HIV-1 replication as they eliminate most of the behavioural confounders present in the observational studies.

The HIV-1 replication studies were performed on ectocervical explants. While it has been reported that the endocervix and the transformation zone may be the most receptive to HIV-1 infection (Pudney et al., 2005; Haynes & Shattock, 2008), Carias et al. (2013) reported using *ex vivo* tissue models and rhesus macaque infection models, that HIV-1 is able to efficiently traverse the squamous epithelial barrier through passive percolation, into areas where target cells like T cells or dendritic cells may be found and productively infected (Carias et al., 2013). Using an X-tropic (HIV-$1_{pNL4.3}$) or an R tropic (HIV-$1_{BaL\text{-}Renilla}$) HIV-1, explants were infected 2 days post treatment with either MPA or NET, where supernatant was collected on days 3, 7 and 10 post infection.

When assessing the effects of MPA and NET on HIV-1 replication in explants from different donors (Fig. 4.8 and 4.9), it was apparent that in some samples MPA increased HIV-1 viral replication (R5 and X4), while in other cases it appeared that MPA had no effect or decreased HIV-1 replication compared to the vehicle control. In the group infected with HIV-$1_{BaL\_Renilla}$ 3 out of 6 donor samples showed an increase in HIV-1 replication in the MPA treated group. Interestingly, the effect on viral replication by MPA in this group was modest. However at day 7 post infection, results for MPA were significantly different compared to the vehicle control. NET treatment had no effect to lower levels of viral replication (Fig. 4.10 A), and had significantly lower levels of HIV-$1_{BaL\_Renilla}$ replication compared to MPA and the vehicle control at days 3, 7 and 10 post infection (Fig. 4.10 A). In the HIV-$1_{pNL4.3}$ group, 5 out of 8 donor samples had significantly increased viral replication in the MPA treated group at all the time points compared to both the vehicle control and NET (Fig 4.10 C). The grouping suggests that the differential effects of MPA on HIV-1 viral replication is donor specific, occurring in 8 out of the 14 infection experiments in this study. Interestingly, in two instances, the same donor

explant was infected with both HIV-$1_{pNL4.3}$ and HIV-$1_{BaL\_Renilla}$. While MPA increased HIV-$1_{pNL4.3}$ replication it had no effect on HIV-$1_{BaL\_Renilla}$ replication in the same donor sample. While this was only observed in a small subset of donors, this result suggests that there may be HIV-1 subtype specific effects. In contrast, NET treatment had consistent effects on HIV-1 viral replication, having no effect or lower levels of viral replication throughout the time course in most of the donor samples (Fig. 4.8, 4.9 and 4. 10 A, B and C). The differences in results for MPA could be due to distinct biological differences within a population, such as the relative levels of HIV-1 target cell types present, the state of activation of the cells as well as the relative co-receptor (CCR5 and CXCR4) expression levels on HIV-1 target cells. Additionally, it has been observed in one study that not all explant samples are productively infected (Saba et al., 2013), and that this could be due, in part, to the phase of the menstrual cycle. In this study, four donor tissues were not productively infected. Saba et al. (2013) found that tissue in the luteal phase of the menstrual cycle (low E2, high P4), was more permissible to HIV-1 infection than tissue obtained from the follicular phase (High E2, low P4) (Saba et al., 2013). Most donor samples that were productively infected in this study were in the follicular phase (appendix C). However all 4 donor samples not productively infected, were from donors with low P4 levels. Two of the four donors were in the follicular phase, one donor was in the ovulatory phase and the other donor was perimenopausal (Appendix C, donors Prog 0041, 0043, 0045 and 0052). Thus, while phase of menstrual cycle did not play a significant role in the differences in viral replication between the different treatment groups observed in this study, it may contribute towards the donor sample becoming productively infected. In addition to the phase of menstrual cycle, meta-analyses performed on some of the observational studies assessing the effects of progestogens on HIV-1 risk in women, found that inflammation and women being in high risk areas may be factors that could also contribute to the increased risk of HIV-1 susceptibility in DMPA users (Heffron et al., 2011; Ralph et al., 2015; Morrison et al., 2015). Women recruited in this current study were not on contraception, were HPV and HIV-1 negative, and most women were HSV-2 negative (only 2 women were HSV-2 positive by serum ELISA in this cohort, see Appendix C). In these experiments, non-activated tissue was used to determine the differential effects of MPA and NET on HIV-1 replication. Thus the differences observed between donor samples are unlikely to be due to activation by the infections tested for in this study, but rather are most likely reflective of other differences within a population. These could include infections with other STIs not measured in this study and/or *Bacterial vaginosis* and/or other genetic or environmental differences.

This is the first study to use an X4 and an R5 tropic IMC to assess the effects of MPA on HIV-1 replication in primary cervical explants. Interestingly, the differences between MPA and NET on HIV-1 replication levels were more pronounced in the donors infected with HIV-$1_{pNL4.3}$ (Fig. 4.10 C and D). This could be due to tissue specific differences within the tissue that may favour X4 replication over R5 replication with MPA treatment. These tissue specific differences could include the immune activation state of the donor, the cell types present, co-receptor expression levels as well as different

levels of steroid receptors present within the tissue of each donor. Interestingly, some studies have observed that in primary tissue explant models, R5 viruses may have higher replication levels compared to X4 viruses. However there were no differences in replication competency for the pooled data from all the explant samples between the X4 and R5 viruses in this study (Fig. 4.8, 4.9 and 4.10). Le Tortorec et al. (2008) found that an X4 tropic strain of HIV-1 was less efficient in productively infecting and replicating in prostate explants compared to an R5 tropic strain of HIV-1 (Le Tortorec et al., 2008). Additionally, Schmitt et al. (2006) observed in mice thymic explants that X4 and R5 viruses were able to productively infect the tissue. However there were cell type differences in infection, with X4 strains restricted to certain cell types more so than an R5 strain (Schmitt et al., 2006). These two studies suggest that there may be differences in HIV-1 subtype replication in tissue explant samples, with R5 viruses showing higher viral infection levels than X4 viruses (Le Tortorec et al., 2008; Schmitt et al., 2006). The data from this current study appear to be contradictory to the findings from Le Tortorec et al. (2008) and Schmitt et al. (2006). However, several studies have reported that both CCR5 and CXCR4 are expressed on select immune cells in the ectocervix (Patterson et al., 2002; Trifonova et al., 2014; Chandra et al., 2013; Pudney et al., 2005), suggesting that both X4 and R5 HIV-1 viruses can infect cells within the ectocervix. Taken together, the data from this current study suggests that while there are no differences in replication competency, there may be virus subtype specific effects. However this preliminary observation needs to be validated, as the differences were observed in a small subset of donor explants within this study. As such the differences in HIV-1 subtype replication differences may be due to biological and or technical error. Importantly, this was not observed in cervical explants with NET treatment. In fact, NET treated explants infected with either HIV-$1_{pNL4.3}$ or HIV-$1_{BaL\_Renilla}$, had significantly lower levels in viral replication compared to those treated with MPA and the vehicle control (Fig. 4.10).

When pooling the results of the tissue from the different types of responders per virus type, the results suggested that MPA treatment did not have an effect on HIV-1 replication in primary ectocervical explants compared to the vehicle control (Fig. 4.11 and Fig. 4.12). However, NET treatment resulted in significantly lower levels of viral replication at 3, 7 and 10 days post infection in both HIV-1 groups relative to the vehicle control and MPA (Fig. 4.11 and Fig. 4.12, appendix B). This is the first study to report such differences between MPA and NET on HIV-1 replication using R5 and X4 HIV-1 IMCs in primary ectocervical explants.

Interestingly, primary ectocervical explants were used to eliminate the behavioural confounders present in observational studies. However, large variations in HIV-1 replication were still observed. While these results in this study are direct measurements on HIV-1 replication and not on observational HIV-1 risk or acquisition in women, it is interesting to observe that the explant methodology is still prone to large variations. The data from this current study suggest that it may not only be behavioural confounders that contribute to the variations in results observed in the

observational studies, but also inherent biological variations within a population. In a recent observational study by Noguchi et al. (2015) the authors directly compared the effects of MPA versus NET usage on HIV-1 acquisition. The authors found that MPA users had a higher risk of HIV-1 acquisition than NET users (Noguchi et al., 2015). The results from the current study showed that NET had lower viral replication levels than MPA in primary ectocervical explants. Taken together, observational studies as well as this present study suggests that NET has a lower HIV-1 risk profile than MPA due to direct effects of progestogens in the FGT.

Taken together the infection data indicates that there is donor variability in MPA-driven effects on HIV-1 replication in *ex vivo* explants, and that there is a significant increase in HIV-1 replication in 8 out of 14 of the explants treated with MPA compared to the vehicle control. Interestingly, NET treatment appears to cause significantly lower HIV-1 replication levels compared to the vehicle control in this system, and this effect appears consistent across donors.

Chapter 5

Conclusions and Future Perspectives

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Introduction

Recent research into the effects of hormonal contraceptives on HIV-1 acquisition in women suggests that the injectable contraceptive, MPA, increases HIV-1 risk, while NET and oral contraceptives do not (Morrison et al., 2015; Ralph et al., 2015; Noguchi et al., 2015). Thus identifying the differential actions of these progestogens may provide insight into why different progestogens have differential effects on HIV-1 susceptibility. This study is the first step to understanding some of the complex mechanisms contributing to the differential direct effects of progestogens on gene expression and HIV-1 replication in *ex vivo* models. A table below summarises the main findings of this study (Table 5.1).

MPA unlike P4, NET and LNG differentially modulates gene expression in PBMCs and primary ectocervical explants

The results from this study highlight the striking contrast between MPA and the other progestogens, and its differential effects on gene expression over time. This study shows that MPA, unlike the endogenous ligand P4, and other progestogens NET and LNG, differentially modulates expression of select immune genes at concentrations similar to peak serum ranges of MPA users after 48 hours in ectocervical explants and PBMCs.

This study found that in PBMCS, MPA, at near peak serum contraceptive concentrations, significantly modulated IL6, IL8, RANTES and GILZ mRNA expression levels, while P4 and NET did not (see Table 5.1). Select Luminex and MSD® ELISAs were performed on PBMC supernatants in this study. The results showed that MPA, like DEX, significantly decreased IL8, IL-1β and IL-1ra protein expression levels after 48 hours, while P4 and NET had no effect. Further, flow cytometry indicated that MPA, like DEX significantly decreased IL6 protein expression levels in CD14+ monocytes, and significantly increased GILZ protein expression levels in CD4+ T cells. The data from this study is consistent with the data in the literature that suggests that MPA has a general anti-inflammatory profile after short term incubations in PBMCs (Huijbregts et al., 2013; Huijbregts et al., 2014; Michel et al., 2013; Michel et al., 2015; Cherpes et al., 2008; Vicetti Miguel et al., 2012; Quispe Calla et al., 2015). Huijbregts et al. (2013) analysed the effects of MPA compared to P4 and NET in PBMCs and found that MPA, unlike P4, decreased IL6 and IL8 protein expression after 24 hours (Huijbregts et al., 2013), and that MPA, unlike P4 and NET, decreased IL6 and IL8 protein expression levels in CD2/CD3/CD28 activated mononuclear cells (Huijbregts et al., 2014). To date, the effects of MPA have been found by identifying changes in secreted protein expression levels in PBMCs (Huijbregts et al., 2013; Huijbregts et al., 2014; Michel et al., 2013; Michel et al., 2015; Cherpes et al., 2008; Vicetti Miguel et al., 2012; Quispe Calla et al., 2015). The results from this study are the first to report on the direct effects of MPA, P4, NET and LNG in parallel on mRNA levels in PBMCs.

Table 5.1: Summarised findings from this study

	IL6		IL8		RANTES		GILZ		Other genes		HIV-1 replication	
Experiments	**PBMCs**	**Explants**	**PBMCs**	**Explants**	**PBMCs**	**Explants**	**PBMCs**	**Explants**	**PBMCs**	**Explants**	**PBMCs**	**Explants**
MPA 48 hrs mRNA levels	Decreased expression at 100 nM. Most likely via the GR	Decreased levels at 100 nM in ectocervical explants. EC_{50} of ~70 nM. No effect in the endocervix	Decreased expression at 100 nM. Most likely via the GR	Decreased llevels at 100 nM. EC_{50} of ~ 18 nM in ectocervical explants. No effect in the endocervix	Decreased expresssion at 100 nM in some instances. Most likely via the GR	Not detected	Increased expression at 10 nM and 100 nM. Via the GR	Increased leevels at 100 nM, EC_{50} of ~ 7.5 nM in ectocervical explants.		No effect on mRNA expression levels of SRs		
MPA 48 hrs protein levels	Decreased levels in CD14+ monocytes		Decreased levels at 100 nM		No significant effects		Increased levels at 100 nM in CD4+ T cells		Decreased IL-1β and IL-1ra levels at 100 nM			
MPA > 2 days mRNA levels	Repression lost after 3 days		Repression after 3 days		Repression lost after day 2		transactivation maintained		MR mRNA and GR protein levels constant			
P4, NET & LNG 48 hrs mRNA levels	No significant effects	P4 decreased expression at 100 nM. NET and LNG had no effect	No significant effects	No effect	No significant effects	Not detected	No significant effects	No effect		No effect on mRNA expression levels of SRs		
P4, NET & LNG 48 hrs protein levels	No significant effects		No significant effects		No significant effects		No significant effects					
MPA ≥ 9 days mRNA levels ± HIV-1	Increased expression levels	No repression	Reduced levels minus HIV-1, but elevated in presence of HIV-1	No repression	No repression	Not detected	transactivation maintained	Elevated, but not signifcant			Increased replication in majority of donors (7/10)	Increased replication in majority of donors
NET ≥ 9 days mRNA levels ± HIV-1		No significant effects		No significant effects		Not detected		No effect			No increase in replication	No increase in replication
P4 & LNG ≥ 9 days mRNA levels ± HIV-1	No significant effects		No significant effects		No significant effects		No significant effects				Variable effects on replication	

Several studies by others have also reported that MPA at peak serum concentrations significantly regulates several important cytokines and chemokines in PBMCs, that have key roles in modulating the immune response, including IFN-α, INF-γ and TNF-α (Vicetti Miguel et al., 2012; Cherpes et al., 2008; Quispe Calla et al., 2015; Huijbregts et al., 2013; Huijbregts et al., 2014), suggesting that MPA may affect the mounting of an immune response *in vivo*. However, the results from this study found no significant effects on INF-γ and TNF-α protein expression in PBMCs after 48 hours with MPA treatment. There are several factors that may contribute to the differences in gene expression by the different progestogens, such as; time, activation and cell type specific effects. Huijbregts et al. (2013) observed that INF-γ was significantly repressed after 24 hours with 100 nM MPA in PBMCs and that TNF-α was significantly repressed in select cell types or in activated PBMCs (Huijbregts et al., 2013). The effects of gene expression by MPA in Cherpes et al. (2008) and Quispe-Calla et al. (2015) were shown to occur in CD8+ memory T cells and in dendritic cells. Taken together, it could be that the results from this current study using non-activated PBMCs could be masking the repressive effects found in select cell types within the population for some genes. Interestingly, Huijbregts et al. (2013) found more significantly repressive effects on cytokine protein expression after 24 hours in activated PBMCs with 100 nM MPA compared to the results found in this study after 48 hours. It could be that length of incubation time is critical to the repressive effects of MPA on protein expression levels. It could also be that the secreted proteins in the supernatant used in the Luminex assay in the current study were degraded at the time of the assay. Nevertheless, it is evident from both the literature and the current study that the effects of MPA on cytokine and chemokine gene expression are generally anti-inflammatory after 1 to 2 days of exposure to MPA.

This study also found similar anti-inflammatory effects with MPA in primary cervical explants after 48 hours, as found for PBMCs (see Table 5.1). This is the first study to report on the direct effects of different progestogens on select immune genes in cervical tissue explants. In primary ectocervical explants, MPA, unlike NET and LNG, significantly decreased IL6 and IL8 mRNA levels and significantly increased GILZ mRNA levels. In endocervical explants, MPA had reduced anti-inflammatory effects, but significantly increased GILZ mRNA levels after 48 hours. Multiple epithelial cell line studies have found that MPA, unlike NET, decreases pro-inflammatory gene expression after 24 hours (Africander et al., 2011; Koubovec et al., 2004; Louw-du Toit, et al., 2014b; Govender et al., 2014), however there were some cell type specific effects found between cell lines (Africander et al., 2011). A study in the endocervical cell line, End1/E6E7 found that MPA, like DEX, decreased IL6, IL8 and RANTES mRNA levels (Govender et al., 2014). The results from this study found no significant effects on IL6 and IL8 mRNA levels with MPA treatment after 48 hours in the endocervical explants. These differences could be due to the differences in the models used. Govender et al. (2014) used an endocervical cell line, while the results from this study were from multicellular endocervical tissue explants. If the results from the endocervical cell line are indicative of what may occur in primary endocervical epithelial cells, then the results in the endocervical explants suggest that the responses from the epithelial cells may be masked. Consistent with this observation is that RANTES mRNA

levels in both endocervical and ectocervical explants were not readily detected in this study. However, RANTES mRNA was detectable and regulated by MPA treatment in both ectocervical and endocervical cell lines (Africander et al., 2011; Govender et al., 2014).

The data from this current study suggest that there are distinct expression effects with progestogens in different immunological environments. In both primary ectocervical explants and PBMCs, MPA, unlike P4 and NET, differentially modulates key immune response genes. However, some differences were observed in the gene expression profiles between PBMCs and ectocervical explants after 48 hours of exposure. While P4 treatment in PBMCs had no effect on gene expression at any of the concentrations used in this study, it was found to significantly reduce IL6 mRNA levels in primary ectocervical explants after 48 hours. Interestingly, the results from primary ectocervical explants were more similar to the results from PBMCs than to the results from endocervical explants.

When comparing the cervical explant and PBMC data from this study to select clinical studies that measured changes in secreted protein levels in the CVL of women, some differences were observed. While IL6 and IL8 mRNA levels were significantly modulated after 48 hours after *ex vivo* MPA exposure in both PBMCs and ectocervical explants, recent clinical studies found no effect on these genes in the CVL of women on injectable contraceptives (Ngcapu et al., 2015; Morrison et al., 2014). In addition, Morrison et al. (2014) found that RANTES levels were increased in DMPA users, while Ngcapu et al. (2015) found no difference in RANTES expression levels. In contrast, this study found that RANTES mRNA expression was undetectable in endocervical and endocervical explants, while 100 nM MPA significantly reduced RANTES mRNA levels in PBMCs after 48 hours *ex vivo* exposure. Importantly in the study by Ngcapu et al. (2015) the effects on gene expression were representative of both MPA and NET users, thus the effects of MPA on some genes may be masked due to the inclusion of NET users in the analysis. The differences in gene expression in the PBMCs and ectocervical explants compared to the CVL from women could also be due to the differences in the concentration and the length of exposure to MPA. Furthermore, the results from this study investigated the direct effects of MPA on target cells, while the results from the clinical studies are representative of indirect effects taken from a secretory sample of the entire lower female genital tract (FGT). As such it may be that the results found in the CVL of women may not accurately reflect the direct effects of MPA on some genes in distinct regions in the FGT. However, the results from these clinical studies are similar to the results observed in the endocervical experiments after 48 hours. This suggests that perhaps the endocervix may be more indicative of the general expression profile, which the CVL from women represents. Importantly, protein expression levels were not determined in this study, nor were the effects of MPA and NET on genes other than IL6, IL8 and GILZ investigated in cervical explants. More research to assess the direct effects of different concentrations of MPA and NET on protein expression levels of key immunomodulatory genes on distinct cell populations in the FGT would be

informative. Nevertheless this study provides evidence for the first time for direct effects with different progestogens on mRNA levels.

Future areas of research should investigate why differential effects on gene expression are found in different immune environments. In particular, how DEX and MPA are able increase gene expression of key genes across systems (GILZ) but are only able to decrease mRNA levels of select genes (IL6 and IL8 mRNA levels) in some models. As mentioned previously, the differences in gene expression with the same ligand the same time point observed in different cellular models suggest that there may be differential co-factor availability, differences in GR activation as well as differences in the relative abundance of the GR and other steroid receptors in these different immune environments. Therefore future research should investigate the above issues, including identification of co-factors that are important in mediating these different responses. Additionally, it may be of use to determine the effects of MPA on factors important in mediating the viral response. Using a PCR array specific for the HIV host response (SABiosciences, Qiagen, Netherlands), one could analyse the global change on immune response genes by MPA in both PBMCs and primary cervical explants. In parallel, one could perform Luminex assays and determine the protein secretion levels of the different cytokines and chemokines. This may help to provide a better understanding in the gene expression effects of MPA on the immune response.

The results from this study suggest that time of exposure to MPA may have profound effects on the immune response. Experiments in PBMCs showed that while MPA and DEX significantly repressed IL6, IL8 and RANTES mRNA expression after 2 to 3 days, this repression was lost after day 4. Further, results in ectocervical explants suggest that IL6 and IL8 mRNA levels are no longer repressed by MPA after 12 days incubation. Collectively, the data from this study strongly suggest that the effects of MPA may be time dependent in PBMCs and primary cervical explants, with anti-inflammatory and immunosuppressive effects observed initially up to 2 days, followed by no effects or even pro-inflammatory effects after longer times of incubation. This is of particular importance as MPA, as an injectable contraceptive, is administered every three months. It has been shown that MPA concentrations change over time, with peak serum concentrations reached soon after injection (Mishell, 1996; Hiroi et al., 1975). For two months, MPA is at serum concentrations of around 2.6 nM (Mishell, 1996; Africander et al., 2011). The results from this study highlight that the length of incubation could have significant downstream effects on changes in gene expression.

Therefore, it will be of further interest to investigate the differential and time dependent effects of MPA on gene regulation in both PBMCs and primary cervical explants. Additionally, the effects of low concentrations of MPA over a long time course should be investigated. Figure 5.1 is a model suggesting some of the mechanisms that may be involved in mediating the transcriptional effects of long and short term incubations with MPA in PBMCs. Some studies have reported pro-inflammatory effects of MPA at different times and concentrations (Louw-du Toit, et al., 2014a; Africander et al., 2011;

Morrison et al., 2014), while many others have reported potent anti-inflammatory effects of MPA on gene expression (Koubovec et al., 2004; Koubovec et al., 2005; Hapgood et al., 2004; Ronacher et al., 2009; Avenant et al., 2010; Govender et al., 2014; Huijbregts et al., 2013; Huijbregts et al., 2014; Michel et al., 2015). This is the first study to suggest that there may be a switch from anti-inflammatory responses to pro-inflammatory responses with MPA treatment over time; however this finding must be confirmed. Several studies have observed the development of glucocorticoid resistance in different cellular systems over time (Tsitoura & Rothman, 2004; Cvoro et al., 2011; Van Bogaert et al., 2010; Cruz-Topete & Cidlowski, 2015). The authors observed that glucocorticoid resistance correlated with an increase in T reg cells over time (Chen et al., 2006; Goleva et al., 2002; Tsitoura & Rothman, 2004) and an increase in NF-AT expression (Tsitoura & Rothman, 2004). It could be both an increase in T reg cells over time (Chen et al., 2006; Goleva et al., 2002; Tsitoura & Rothman, 2004), as well as a possible escape mechanism whereby NF-AT transcription factors are up-regulated in response to MPA over time, to exert pro-inflammatory responses, which are not repressed via the GR tethering mechanism (Tsitoura & Rothman, 2004; Camargo et al., 2009). Future research should include identifying whether there is a shift in cellular populations over time in PBMCs in response to MPA treatment using flow cytometry. These experiments would shed light into why both pro-inflammatory and anti-inflammatory effects are observed by MPA. Additionally, some reports suggest that there is also selective modulation by the GR to initiate some pro-inflammatory actions through crosstalk with other steroid receptors such as the ER (Beck et al., 2009; Bolt et al., 2013; Cvoro et al., 2011), or through the GR itself (reviewed in Cruz-Topete & Cidlowski, 2015). The effects of time and treatment are important in understanding the differential effects of MPA-induced gene expression in PBMCs. MPA, having both anti- and pro- inflammatory effects over time on gene expression may have important implications for HIV-1 susceptibility and disease progression. An increase in select cell types as well as a dynamic change in pro- and anti-inflammatory effects may create time dependent favourable environments for HIV-1 replication and dissemination.

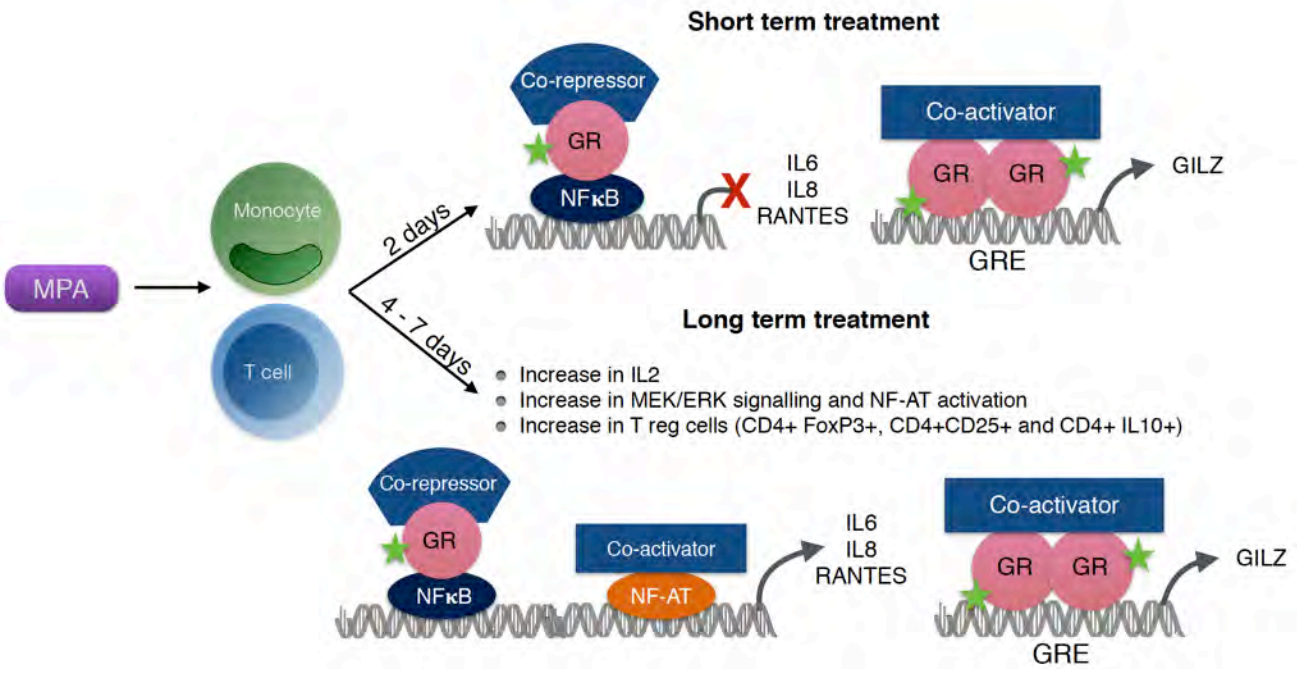

Figure 5.1: A proposed model for the differential effects of gene expression by MPA over time. The data from this study in PBMCs, suggests that at 48 hours stimulation (short term treatment), MPA via the GR, decreases IL6, IL8 and RANTES gene expression. This decrease in expression is most likely to occur through a tethering mechanism of the GR to NFκB transcription factors (Van Bogaert et al., 2010; De Bosscher & Haegeman, 2009; Mahita & Cidlowski, 2013; Govender et al., 2014). GILZ mRNA expression is increased upon stimulation with MPA, via direct binding of GR dimers to glucocorticoid response elements (GRE) in the promoter of the gene (Ayroldi & Riccardi, 2009; Berrebi, 2002; Di Marco et al., 2007; Smit et al., 2005). After prolonged MPA exposure, glucocorticoid resistance develops through an increase in T regulatory (T reg) cells and a shift from Th-1 to Th-2 cells (Chen et al., 2006; Goleva et al., 2002; Tsitoura & Rothman, 2004; Kaushic et al., 2003). In addition, there may be a shift in transcription factor use, through an increase in MEK/ERK signalling via an IL2 dependent pathway, that leads to an increase of NF-AT transcription factors (Tsitoura & Rothman, 2004; Kam et al., 1993; Cruz-Topete & Cidlowski, 2015). NF-AT is able to bind to its cognate binding sites in target pro-inflammatory genes and increase expression, such as IL6, IL8 and RANTES (Tsitoura & Rothman, 2004).

In injectable contraceptive users, the concentration of the progestogen changes over time. Shortly after injection, MPA and NET concentrations peak, where after, they decline and plateau (Mishell, 1996; Fotherby, 1981; Stanczyk et al., 2013; Africander et al., 2013). It was thus of interest to determine the effect of dose on gene expression. The results from this study found that in PBMCs, MPA had significant effects on immunomodulatory gene expression from 10 to 1000 nM, while P4, NET and in some cases LNG had no significant effect on gene expression at any of the concentrations after 48 hours (see Table 5.1). Furthermore, the glucocorticoid DEX had the most pronounced effects on gene expression in PBMCs with significant effects found from 1 nM to 1000 nM. In ectocervical explants, dose response curves were performed with MPA only. Similar to PBMCs, MPA appeared to decrease IL8 mRNA, and increase GILZ mRNA expression at 10, 100 and 1000 nM. Interestingly it appeared that MPA had reduced effects on IL6 mRNA expression in the ectocervical explants, appearing to reduce expression at 100 and 1000 nM only.

In the ectocervical dose experiments, potencies, which indicate the half maximal response of ligand (Hapgood et al., 2013), were calculated. The potency of MPA, for IL8 was ~ 18 nM, but ~ 70 nM for

IL6 in ectocervical explants. For the PBMC study, it appeared that the potency for both IL6 and IL8 in PBMCs is between 10 – 100 nM. Interestingly in PBMCs, GILZ potency appeared to be between 1 – 10 nM, similar to the potency for GILZ in the ectocervical explants, which was ~ 7.5 nM. Thus it appears that MPA may be more potent for IL6 repression in PBMCs than in the ectocervical explants, suggesting that cellular environment may contribute towards the potency of the ligand. Further, the data suggest that there could be significant differences in the gene expression profiles soon after injection where MPA serum levels peak around 2.5 – 65 nM (Ortiz et al., 1977; Mishell, 1996; Hiroi et al., 1975; Mathrubutham & Fotherby, 1981), compared to that for the remainder of the three month period, where MPA serum levels plateau to 2.6 nM (Mishell, 1996; Africander et al., 2011). These dramatic changes in serum concentrations may indicate that there could be a window period, whereby immunomodulatory effects of MPA are more pronounced. Taken together, at near peak serum concentrations of MPA, MPA has stronger immunomodulatory effects than at lower concentrations, while the potency is gene specific, with different genes showing a different dependency on MPA dose.

When looking at the effects of dose by P4 and NET in PBMCs, the data from this study suggest that at high concentrations (100 – 1000 nM), these progestogens have no effect on gene expression. However, at lower concentrations (1 – 10 nM), these progestogens appeared to have slight (but non-significant) pro-inflammatory responses. Interestingly, at 100 nM, P4 significantly reduced IL6 mRNA levels in the ectocervix. This suggests that while P4 has no significant effect in PBMCs at any of the concentrations used in this study, P4 may have immunomodulatory effects in the FGT. This result further highlights that at the same concentration, progestogens have differential effects in different systems. Therefore more research is needed on the effects of the different progestogens at different concentrations on gene expression in different primary systems. Additionally, the effects of LNG at lower concentrations are yet to be established, and as such future research should investigate the effects of low concentrations of LNG on gene expression in primary systems.

Of particular importance, is that while serum concentrations, for injectable contraceptive users, are known, there is no information on the concentrations of MPA NET or LNG in the female genital tract (FGT). Further there is no information on whether the concentration of these progestogens varies between different tissue compartments in the FGT. Understanding whether there are tissue specific differences in concentrations of progestogens within the FGT, as well how this changes during different physiological states, may shed light into which areas are more permissible to changes with MPA. Furthermore, knowing the concentrations within the tissue will aid in interpreting *ex vivo* data and design of experiments. These studies need to be performed urgently so that more informed studies using primary tissue explants can be performed to better address the question of whether MPA increases the risk in HIV-1 acquisition. Additionally, more dose response experiments are needed to obtain more accurate curves to confirm the potencies obtained in this study. Further, dose response curves should be performed with other progestogens in primary cervical explants.

The effects of MPA on gene expression after 48 hours are most likely via the GR

This study found that the immunomodulatory effects by MPA, like DEX, on IL6, IL8, RANTES and GILZ are mediated via the GR in PBMCs using antagonist and knockdown strategies. This data is in agreement with the literature that suggests MPA is a partial agonist for the GR (Koubovec et al., 2005; Ronacher et al., 2009; Govender et al., 2014; Avenant et al., 2010). Further the results from this study suggest that the effects of MPA on IL6, IL8 and GILZ mRNA expression in the ectocervix and GILZ in the endocervix are most likely via the GR. While all the SRs are expressed in the ectocervix, MPA, like the glucocorticoids DEX and CORT, significantly decreased IL6 and IL8 mRNA levels, and increased expression of a GR-driven gene, GILZ. Interestingly, while DEX and MPA increased GILZ mRNA expression in endocervical explants, DEX and MPA did not significantly decrease IL6 or IL8 mRNA levels.

While the relative abundance of SRs in the endocervical explants was not examined, the differential effects of MPA on different genes in the same system, suggest that there may be different activation effects of the GR and or differential levels of co-factor availability or recruitment in these different systems. Ronacher et al. (2009) observed in the COS-1 cell line, that there were ligand selective differences for transactivation and transrepression via the GR that were in part dependant on ligand selective interaction with different GR co-factors (Ronacher et al., 2009). Further Avenant et al. (2010) found that MPA, but not NET increased GR phosphorylation at key serine residues (S211 and S226) that correlated with an increase in both transactivation and transrepression on promoter-reporter constructs (Avenant et al., 2010). Additionally, Avenant et al. (2010) observed that phosphorylation of the GR, at any of the serine residues tested, was required for interaction with a key GR co-factor, GRIP-1 (Avenant et al., 2010). Thus it could be that the differences observed in the gene expression effects, could not only be due to the relative abundance of the different SRs, but in part due to the activation state of the GR and the availability of co-factors for optimal transcriptional activity.

It is therefore important that the role of the GR in mediating the differential effects by MPA on gene expression is determined in primary ectocervical and endocervical explants. It may be difficult to perform siRNA knockdown strategies in whole tissue; as such it may be useful to use selective antagonists to determine GR involvement on differential gene expression within the different cell types. Additionally how MPA, P4, NET and LNG may differentially activate the GR through differential phosphorylation at key serine residue sites within the GR has not been established in PBMCs or in primary cervical explants. Furthermore, the relative abundance of important co-factors has not been established in these systems. Investigating the effects of phosphorylation and co-factor recruitment by MPA compared to P4, NET and LNG in these systems will provide important mechanistic insight into why there may be differential effects on gene expression in these cellular systems.

Further it may be important to investigate the role of the GR in different cell types in PBMCs. Studies by Huijbregts et al. (2013) and Quispe-Calla et al. (2015) found that pDCs and CD8+ T cell cellular functions

are altered in response to MPA at serum concentrations. It would therefore be of interest to determine whether the GR is involved in mediating this process in pDCs and CD8+ cells, as it will give further weight to the mechanism of action of MPA.

This study found that GR levels did not change over time in PBMC samples treated with MPA. However, the differential activation states of the GR or the involvement of other transcription factors and co-factors were not investigated. It would thus be of interest to investigate the role of the GR as well as the role of other transcription factors like, NF-AT on MPA dependent gene expression in PBMCs over time. Using both real time qPCR, knockdown, antagonist and chromatin co-immunoprecipitation (ChIP) assays, one will be able to elucidate the change in transcription factor use over time, as well as the role of NF-AT in mediating the pro-inflammatory responses observed at a later time point.

The role of steroid receptors in mediating the effects of progestogens in the systemic and local immune environments

This study found that PBMCs had detectable protein expression levels of the GR, with detectable mRNA expression levels of the GR, MR and ERα. Primary ectocervical explants contained detectable protein expression levels of the AR, ERα, GRα, PRA and PRB, and mRNA expression levels of all the steroid receptors listed as well as MR. The differential levels of the steroid receptors within these different cellular systems may help to explain the differences on the effects of gene expression in this study. It was also observed that the expression levels of the PR isoforms and ERα were significantly different in the luteal phase compared to the follicular phase in primary ectocervical explants. However, what is not known are the relative expression levels of SRs in the endocervix, and if these SRs are differentially expressed at the different phases of the menstrual cycle. Furthermore it must also be established whether AR, MR and GR protein levels are regulated by the menstrual cycle in ectocervical explants.

Of importance is that the role of steroid receptors in mediating these differential effects has not been elucidated. Several cell line studies have shown that the GR is important in mediating transcriptional effects in response to MPA treatment (Govender et al., 2014; Louw-du Toit et al., 2014a; Ronacher et al., 2009; Koubovec et al., 2004), while others have found that the AR may be involved in some of the MPA-driven effects (Africander et al., 2011; Africander et al., 2014). The results from this study found that the GR was important in mediating the effects of MPA on IL6, IL8, RANTES and GILZ mRNA levels in PBMCs. However the role of the GR in mediating gene effects by MPA in primary cervical tissue has not been established. While it may be difficult to perform siRNA knockdown strategies in whole tissue, it may be useful to use different selective SR agonists and antagonists to determine which SRs may be important in gene modulation in response to progestin treatment. One could also digest the tissue and separate using filtering to isolate different cellular populations. Upon separation, one could treat with the different progestins and different antagonists (e.g. RU486: GR/PR,

hydroxyflutamine: AR) and using a combination of flow cytometry and real time qPCR, determine steroid receptor involvement on differential gene expression within the different cell types. Nevertheless, the findings that MPA acts more like the glucocorticoid DEX, than P4 or NET, in the measured responses, coupled with the known relative activity of the progestogens via the different SRs, strongly suggest that MPA is acting predominantly via the GR in the ectocervical explants, and not the PR or AR, which do not discriminate between MPA and NET. This is despite the presence of both the GR and PR in these tissues.

The differential levels of steroid receptors expressed within the menstrual cycle may help to explain why there are differential outcomes in immune responses at the different phases. Interestingly, it has been proposed that there is a window of vulnerability to HIV-1 infection, 7 - 9 days post ovulation during the luteal phase (Wira & Fahey, 2008; Vishwanathan et al., 2011). It has been hypothesised by the Hapgood group that differential ratios and relative abundance of the steroid receptors in the ectocervix during the menstrual cycle, could contribute to the changes in susceptibility to HIV-1 infection, through mediating effects via the different steroid receptors. It has been established that MPA is a partial agonist for the GR (Ronacher et al., 2009; Koubovec et al., 2005; Avenant et al., 2010). Since MPA induces changes that mimic the luteal phase of the menstrual cycle, it could be that the steroid receptor profile is similar and could contribute to the effects of MPA on HIV-1 susceptibility. Indeed, it has been shown that MPA more so than P4, increases HIV-1 and HSV-2 viral replication (Trunova et al., 2006; Kaushic et al., 2003) and suppresses cell mediated immunity (Kaushic et al., 2003; Gillgrass et al., 2003; Hughes et al., 2008; Michel et al., 2013; Huijbregts et al., 2013; Huijbregts et al., 2014). It has also been established that E2 and P4 are able to alter the expression of immunoglobulins within the FGT, with P4 decreasing the expression of immunoglobulins, and depressing the maturation of B cells (Lü et al., 2003). It has also been observed in naturally cycling women, that during the luteal phase (high P4), IL6 protein levels increase in the cervical mucus (Franklin & Kutteh, 1999). It could be if GRα: PRA/PRB ratios are greater (that is more GR available) in the luteal phase, the effects on cell mediated immunity could be greater, through mediation via the GR by MPA, which mediates the inflammatory response (Beck et al., 2009; De Bosscher & Haegeman, 2009). The results from this study found no significant regulation of SR mRNA by the different progestogens after 48 hours in primary cervical explants. Additionally, the results from the long time course experiment suggested that mRNA levels of the MR and protein expression levels of the GR were not significantly modulated by MPA. However the relative ratios and the different activation states of these SRs were not investigated in this study. Therefore, further investigation into the role of the steroid receptors and their relative ratios and expression profiles at the different phases of the menstrual cycle as well as in response to progestogen treatment, on immune gene expression must be investigated in both PBMCs and primary cervical explants.

The effects of MPA on select pro-inflammatory gene expression and HIV-1 replication in PBMCs

The direct effects of the different progestogens on HIV-1 replication were assessed in PBMCs. The results from this study found that both the progestogens and glucocorticoids used in this study had varied effects on HIV-1 replication after 7 days post infection. Half of the donor samples assayed showed increased levels of HIV-1 replication with DEX, CORT, P4 and LNG treatment. Seven out of ten donor samples treated with MPA had showed an increase in HIV-1 replication. Interestingly, NET was the only progestogen that had no effect on HIV-1 replication. When grouping the data from seven donor samples, only MPA significantly increased HIV-1 replication in these seven donor samples, while CORT, DEX, P4, NET and LNG had no significant effects. Interestingly, in the three donor samples in which MPA reduced HIV-1 replication, the effects of MPA were also significant. Taken together, the data suggests that MPA increased HIV-1 replication in the majority of PBMC samples, while opposite effects were observed in a minority of donor samples. While these results suggest that there may be different responses upon MPA treatment, further repeats must be performed to validate the observations reported in this study.

Few studies have reported on the direct effects of progestogens on HIV-1 replication in PBMCs. In addition, of the studies that have reported on these effects, none have been performed under similar conditions. Huijbregts et al. (2013) found that in CD8+ depleted PBMCs or purified CD4+ T cells that had been differentially activated for 24 hours after 5 days, treatment with 1 µM DEX and MPA significantly increased HIV-1_{BaL} replication after 7 days post infection in all conditions except in CD4+ T cells activated with PHA (Huijbregts et al., 2013). Sampah et al. (2015) recently observed in un-stimulated CD8+ T cells, that MPA at concentrations similar to serum contraceptive concentrations, increased HIV-1 replication. Furthermore, the authors established that this increase in HIV-1 replication required the presence of CD14+ monocytes (Sampah et al., 2015). The results from the current study showed that MPA increased replication in the majority of PBMC donor samples. While it appears that the activation state of the different cells, as well as the different cell types may have important roles in HIV-1 replication, the data from the literature together with the data from this current study strongly suggest that MPA increases HIV-1 replication in the majority of PBMCs at near contraceptive serum concentrations. No other studies to date, have investigated the effects of P4, NET and LNG, in parallel, on HIV-1 replication in PBMCs. Thus the results from this study provide important insights into the direct effects of these progestogens on HIV-1 replication. Interestingly, the variable effects of P4 and LNG on HIV-1 replication, unlike the results for NET, suggest that these progestogens behave differently, despite their similar steroid receptor selectivity profiles (Africander et al., 2011; Stanczyk et al., 2013), further supporting the hypothesis that not all progestogens are the same (Hapgood et al., 2004).

Gene expression was also investigated in parallel with HIV-1 replication. The results from this study showed that in the MPA treated samples, HIV-1 replication correlated with IL6 mRNA levels. DEX had a similar mechanism of action to MPA on HIV-1 replication and IL6 mRNA expression, suggesting that

this mechanisms for both may be modulated, at least in part, by the GR. Interestingly the data from this study showed that after prolonged treatment (9 days), IL6 mRNA repression by MPA is lost. Future studies in the absence and presence of an IL6 neutralising antibody are needed to confirm the role of IL6 in modulating the effects of MPA on HIV-1 replication in PBMCs. Furthermore it will be of interest to determine the cell types involved in this mechanism. The model below (Figure 5.2), describes the possible mechanisms whereby MPA may modulate HIV-1 replication. In some donors, long term treatment may result in the activation and recruitment of NF-AT, which drives IL6 transcription. This may result in an increase in IL6 levels. Furthermore, long term treatment may shift the population of T cells present, to that of T reg cells, that are thought to be GC resistant (Tsitoura & Rothman, 2004). This may increase the expression of IL6, further increasing HIV-1 replication. In addition, MPA may dampen the CD8+ and pDC cell mediated immune response, potentially increasing the pool of replicating HIV-1 in CD4+ T cells (Huijbregts et al., 2014; Michel et al., 2015). It may also be important to determine the role of apoptosis in this system, and to determine which cell types are programmed for cell death. It may be that there is a shift to promote viral replication by increasing apoptosis in a different cell type. Using flow cytometry as in Tomasicchio et al. (2013), one could determine which cell types are programmed for cell death, as well as the effects on cytokine expression (Tomasicchio et al., 2013). These results would provide insight into the possible role of MPA on HIV-1 disease progression in women.

However a small subset of donors (3 out 10) maintained low mRNA levels of IL6 mRNA at day 9 with MPA, which correlated with low HIV-1 replication levels, compared to the vehicle control. It appeared that in some cases, MPA sensitivity was maintained throughout the time course, and HIV-1 viral replication was reduced. In this case, it may be that there is no increase in MEK/ERK signalling, that drives the activation of NF-AT transcription factors. As such, the GR, thought to bind as a monomer to NFκB (Van Bogaert et al., 2010; De Bosscher & Haegeman, 2009), may maintain its repressive function on gene expression, resulting in low levels of IL6 and subsequently low viral replication. These findings need to be confirmed by performing more repeats in PBMCs and identifying more donors, which maintain MPA responsiveness to IL6 gene repression. In addition, it may be informative to determine the mechanism in this population and identify possible targets for decreasing HIV-1 replication. Using flow cytometry, one could perform these experiments to identify cell type usage and population shift over time with treatment with MPA, looking specifically for a shift towards T reg cells (CD4+FoxP3+, CD4+CD25+ and CD4+IL10+) (Tsitoura & Rothman, 2004; Pandolfi et al., 2013; Chen et al., 2006; Nelson, 2004). In addition, to investigate transcription factor involvement ChIP assays could be performed and recruitment of NF-AT to the promoter region of IL6 could be determined, similar to experiments performed by Louw-du Toit et al. (2014a), Govender et al. (2014), Verhoog et al. (2011) and Hadley et al. (2011).

In the infection experiments, PBMCs were activated with PHA, and maintained in 30 U/ml IL2 for the duration of the study. IL2 is an important cytokine that is a key mediator in T cell maintenance and proliferation (Murphy et al., 2012; Kam et al., 1993). It has been found in several studies, that IL2 may lift the GR dependent apoptotic effects by DEX in PBMCs (Kam et al., 1993; Camargo et al., 2009; Vazquez-Tello et al., 2012). Additionally one study found that IL2 in combination with IL4 decreased GRα expression, that contributed to the diminished effects of DEX on cellular proliferation and apoptosis in PBMCs (Vazquez-Tello et al., 2012). Therefore, the effects of IL2 on MPA and DEX mediated gene transcription, via the GR, need to be determined. However, these effects were not observed in this study, as DEX significantly decreased cellular viability in PBMCs after 9 days in the presence of IL2. Since DEX and MPA significantly repressed IL6, IL8 and RANTES gene expression in the longer time course experiments (Fig. 3. 16), at days 2 and 3, it may be that the effects of IL2 in this system are time and or concentration dependent. Therefore, experiments using different amounts of IL2 with constant MPA and DEX concentrations in PBMCs should be performed, and changes in gene expression and apoptosis should be measured. The results of the experiments will give insight into the role of IL2 in changing the DEX and MPA effects on gene expression via the GR. Additionally the experiments should be performed in the absence of IL2 so that the effects of DEX and MPA can be compared to the results in the presence of IL2.

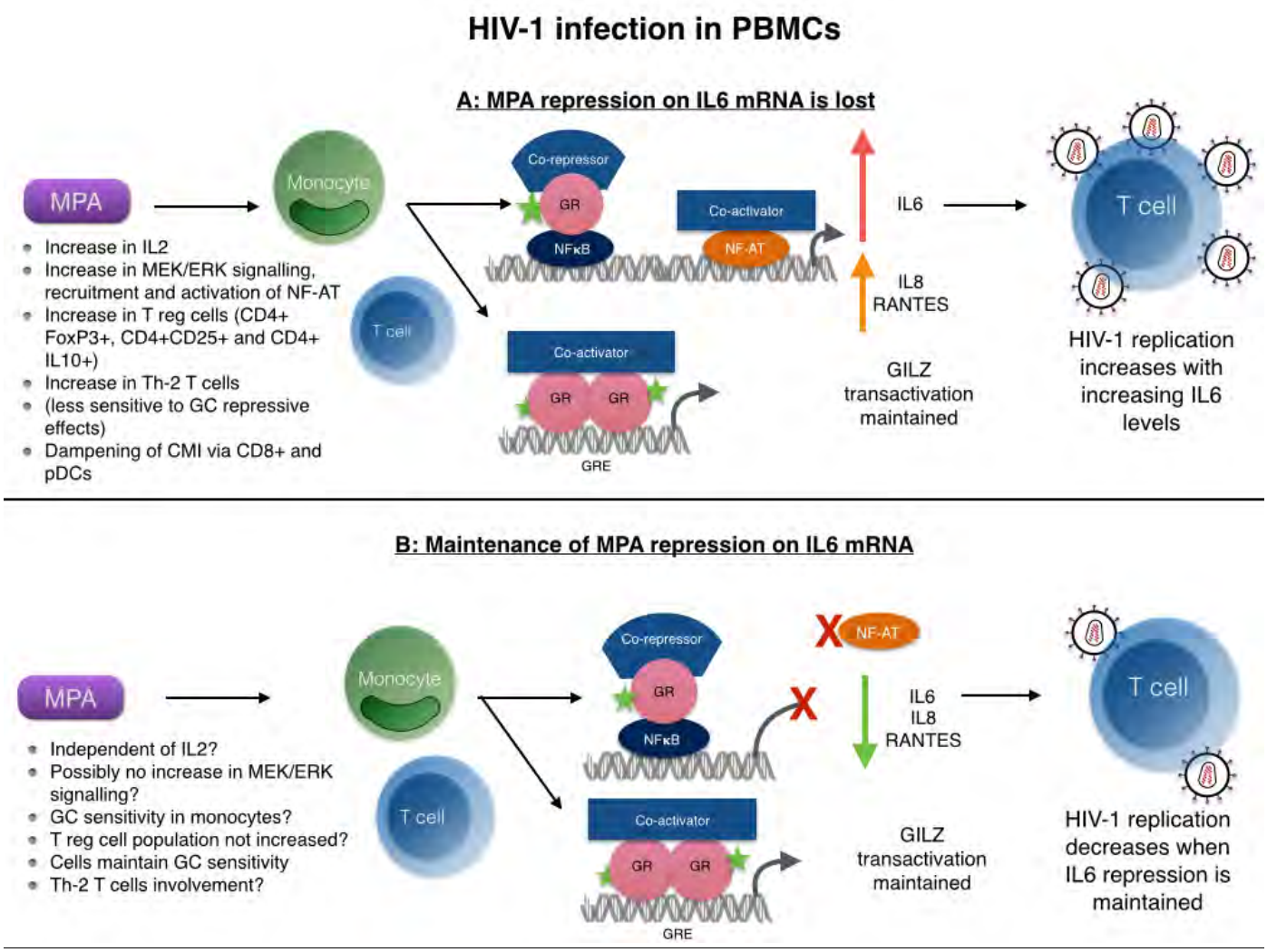

Figure 5.2: A proposed mechanism for the differential outcomes of HIV-1 replication in response to MPA treatment over a prolonged time course. (A) It could be that after prolonged MPA exposure, that glucocorticoid resistance develops through an increase in T reg cells and a shift from Th-1 to Th-2 cells (Chen et al., 2006; Goleva et al., 2002; Tsitoura & Rothman, 2004; Kaushic et al., 2003) that leads to a shift in transcription factor usage, such as NF-AT (Tsitoura & Rothman, 2004; Kam et al., 1993; Cruz-Topete & Cidlowski, 2015). This change in transcription factor use could lead to an increase in IL6 mRNA levels (Tsitoura & Rothman, 2004). When IL6 levels are pro-inflammatory, HIV-1 replication in target cells could increase (Birx et al., 1990; Rollenhagen & Asin, 2011). (B) In some cases, prolonged treatment does not result in a loss of GC sensitivity, suggesting that the IL2 dependent MEK/ERK signalling pathway is not always activated. Here, MPA, via the GR, is still able to decrease IL6, IL8 and RANTES gene expression while maintaining GILZ transactivation. When, IL6 mRNA expression levels are low, HIV-1 replication in target cells is reduced in PBMCS compared to basal.

MPA increases HIV-1 replication in the majority of donors, while NET has no or reduced effects on HIV-1 viral replication in cervical explants

Due to tissue sample constraints in obtaining primary cervical explants, only the effects of MPA and NET on HIV-1 replication were investigated. This is the first study to assess the direct effects of these progestogens in a cervical tissue model. In this study, MPA increased HIV-1 replication in the majority of ectocervical explants. However, the effect of MPA on HIV-1 replication was lost when pooling results from all donors. This may be due to the high inter-individual variation observed between the treatment groups, potentially masking or confounding the results. Indeed, it was observed that 5 out of 8 donor samples infected with HIV-$1_{pNL4.3}$ and 3 out of 6 donor samples infected with HIV-$1_{BaL\_Renilla}$, had increased HIV-1 replication profiles in the MPA treated samples, and that these were significantly different to the vehicle control when grouped together. Interestingly, the results from this study found that NET, unlike MPA, significantly reduced HIV-1 replication pooled data from all donor samples.

It could be that, in some cases, MPA increases HIV-1 infection by increasing the expression of CCR5 and CXCR4 on target cells (Chandra et al., 2013; Huijbregts et al., 2013; Sciaranghella et al., 2015; Goode et al., 2014), and/or by increasing cellular activation (Chandra et al., 2013; Huijbregts et al., 2013). In contrast, NET treatment has been found by others to have no significant immunomodulatory effects on DC and T cell function (Huijbregts et al., 2014; Huijbregts et al., 2013), and as such may not increase HIV-1 susceptibility in the FGT. Thus, it could be that the differences between MPA and NET may be due to their differential effects on target cell recruitment or activation. As such it could be that NET is a more acceptable contraceptive choice due to its limited effect on cell recruitment or activation, however this must be confirmed. Future research should therefore establish the effects of the progestogens on cell type activation, relative abundance of different cell types and relative co-receptor expression levels in the absence and presence of HIV-1 infection. These experiments may be difficult to perform, considering the effects of tissue digestion on receptor expression that may prevent adequate analysis using flow cytometry. It could be that select markers like CCR5, HLA-DR and CD4 be used in immunohistochemistry to determine the effects of the different progestogens on the relative expression levels of these markers. Furthermore, the effects of P4 and LNG on HIV-1 replication are not known, and should be assessed in the future.

Determination of the reasons underlying the differential inter-donor effects of MPA on HIV-1 replication in primary ectocervical explants was beyond the scope of this project. As such future research into the differential effects of MPA on HIV-1 replication in different donors should be elucidated. Some authors have proposed that young women are more susceptible to HIV-1 infection (Abdool Karim et al., 2010; Morrison et al., 2010; Morrison et al., 2012), and that this could be due to the state of inflammation and composition of the FGT (Roberts et al., 2012; Masson et al., 2014; Shrier et al., 2003). This may be why only modest effects on HIV-1 replication were observed in this current study, due to the greater age of the pre-menopausal women consented in this study (see Appendix C). However, the effect of age on

HIV-1 susceptibility or HIV-1 replication in women has not been directly proven. The differential effects between different donor samples by MPA on HIV-1 replication could also be due to the dynamic distribution of immune cells within the ectocervix. Some donors may have had a greater number of HIV-1 target cells and or greater relative expression levels of HIV-1 co-receptors compared to other donors. It could also be that the phase of menstrual cycle may also have an important role in the differences in HIV-1 replication between donors. A study by Saba et al. (2013) which found that explants from women in the luteal phase of the menstrual cycle were more permissible to HIV-1 infection (Saba et al., 2013). However, results from this current study suggests that the stage of the menstrual cycle does not affect the outcome of HIV-1 replication in samples treated with either MPA or NET, but may contribute to the likelihood of the sample becoming productively infected. It may therefore be of use to increase donor numbers, including more donors from different stages of the menstrual cycles and different ages, to further explore the effects of these inherent biological differences. It may be unrealistic to perform immunohistochemistry or flow cytometry on each donor sample to elucidate cell types and numbers per donor in these types of experiments. Grouping the samples according to phase of cycle, age or into groups of responses is the most appropriate way in which to measure the differential effect of MPA on HIV-1 replication as this study has done.

The effects of progestogens in PBMCs versus ectocervical explants on HIV-1 replication

Interestingly, the results from both PBMCs and primary cervical explants suggest that MPA increases HIV-1 replication in the majority of donor samples, while NET has no effect or reduced effects on HIV-1 replication. The results strongly suggest that MPA directly affects HIV-1 replication in both the systemic and in the local mucosa immune environments. The effects on HIV-$1_{BaL\text{-}Renilla}$ replication by MPA were more pronounced in PBMCs compared to the effects in primary cervical explants. These differences could be due to the cell type differences between these two multicellular systems. PBMCs used in the HIV-1 replication experiments were activated and most likely contain a greater relative amount of HIV-1 target cells compared to ectocervical tissue. However the differences in cell types, and the relative abundance of HIV-1 target cells between these two systems have yet to be fully elucidated. A recent study by Joag et al. (2014) found that viral entry was about three fold higher in cervical CD4+ T cells compared to their matched blood CD4+ T cell counter parts (Joag et al., 2014). In addition, the authors found that there may be shared host factors that influence viral entry in both CD4+ T cell populations (Joag et al., 2014). It may be of interest to isolate these cell types from matched PBMC and tissue sample donors and assess the effects of the different progestogens on HIV-1 replication. This may help to identify if there are host determinants that contribute to an increase in HIV-1 replication. Furthermore, this study found that there were differences in steroid receptor expression levels between PBMCs and primary cervical explants. It could be that the relative abundance of the different SRs could also play a role in the outcome in HIV-1 replication in PBMCs and primary ectocervical explants. Despite these differences, the results from this study show that

treatment with MPA increases HIV-1 replication in the majority of donors in both PBMCs and ectocervical explants. Further, the results show that after short term treatment, MPA significantly represses key pro-inflammatory genes, while after long term incubation, repression of these genes is lost in both PBMCs and ectocervical explants. Taken together the results suggest that MPA may exert similar effects on similar cell types (like T cells and dendritic cells) found in both PBMCs and ectocervical explants that may lead to an increase in HIV-1 replication via a common mechanism.

The role of the SRs in HIV-1 replication in PBMCs and primary ectocervical explants

While the results found that MPA increased HIV-1 replication in the majority of PBMC and cervical explant samples, the study did not directly investigate the role of the GR in mediating these differential effects in these different cellular systems. However the results from this study indirectly suggest that the GR may have an important role in modulating the effects of MPA on HIV-1 replication. In PBMCs, MPA, like DEX, increased HIV-1 replication that correlated with increased IL6 mRNA levels. Further, results from this study found that PBMCs expressed detectable levels of GR protein and ER, MR and GR mRNA levels. Thus the results suggest that the changes in both HIV-1 replication and gene expression most likely involve the GR, since progestogens exert their responses via SRs. Similarly, MPA increased HIV-1 replication in the majority of explant samples. While the ectocervical explants detectably express AR, ER, PR and GR protein levels and all the SRs at an mRNA level, the data in the explants suggest that MPA may act predominantly via the GR. In addition, the results from this study found that PR and ER levels were significantly different in the different phases of the menstrual cycle. Saba et al. (2013) found that explants in the luteal phase were more permissible to HIV-1 infection and had higher viral titres compared to samples in the follicular phase (Saba et al., 2013). Taken together, the data suggests that SRs, particularly, the GR could have an important role in HIV-1 infection and replication in PBMCs and cervical explants. Therefore, the next step is to elucidate the role of the GR in HIV-1 replication in PBMCs and explants. Using the selective antagonist RU486, one could determine the role of the GR in mediating this response. The role of the GR may be difficult to elucidate in primary ectocervical explants, due to the presence of both the PR and the GR within this system. Therefore it may be necessary to separate and purify the different cell types using different cell sorting and tissue digestion techniques. Single cell types may be more permissible to mechanistic research questions, and allow one to elucidate the role of the GR on HIV-1 replication in select cell types. Furthermore, the use of specific agonists and antagonists for the different steroid receptors may help to identify which SRs are important in modulating the effects of HIV-1 replication with the different progestogens.

The relationship between inflammation and HIV-1

A direct causal link between inflammation and HIV-1 replication was not investigated in the current study. However the results from this study have shown that the effects of MPA on select pro-inflammatory genes change over time. Furthermore, the results in PBMCs show that the effects of

gene expression by the different progestogens in the presence of HIV-1 after 9 days were similar to the effects of the progestogens in the absence of HIV-1. This result strongly suggests that HIV-1 infection does not significantly contribute to the effects on pro-inflammatory cytokine expression by the different progestogens. However, the results do suggest that changes in expression of immunomodulatory genes a short time after exposure to relatively high MPA concentrations, such as shortly after injection, may contribute to an increase in HIV-1 replication in PBMCs. Furthermore, the results suggest that in MPA treated PBMCs, IL6 mRNA levels may contribute to HIV-1 replication after 9 days post infection. The effects of MPA and NET on HIV-1 replication and gene expression in ectocervical explants were not fully elucidated, but the data suggest that MPA does not significantly increase IL6 mRNA expression, after 12 days, as seen in the PBMCs.

Whether the state of inflammation in the female genital tract is important in directly determining the susceptibility to HIV-1 infection remains to be determined (Wira et al., 2010; Wira et al., 2014; Wira et al., 2015; Shattock et al., 2008; Miller & Shattock, 2003). Few studies have assessed the link between pro-inflammation and HIV-1 susceptibility. Clinical studies have used correlation analysis to suggest links, but it should be noted that these do not prove a causal relationship. Morrison et al. (2014) found that an increase in RANTES, and a decrease SLPI correlated with HIV-1 seroconversion in women (Morrison et al., 2014). Masson et al. (2014) found that women with identified sexually transmitted infections (STIs) had high concentrations of pro-inflammatory cytokines in the CVL compared to the CVL of women with no infections (Masson et al., 2014). Some evidence for causal relationships has been obtained in *ex vivo* studies, and suggests that exogenous IL8 (Lane et al., 2001) and TNF-α (de Jong et al., 2008) may increase HIV-1 replication in primary cells. It has also been observed in latently infected monocytes and T cell lines, that TNF-α can stimulate HIV-1 replication (Griffin et al., 1989; Griffin et al., 1991; Lane et al., 1999). However there are some contrary reports with regards to a link between inflammation and HIV-1 susceptibility, from both clinical and *ex vivo* data. One study found that lower levels of IL6 and IL10 were associated with an increased risk in HIV-1 infection (Lehman et al., 2014). Another study found that IL8 added exogenously before infection with HIV-1 in ectocervical tissue explants, decreased initial HIV-1 reverse transcription and viral integration (Rollenhagen & Asin, 2010). In addition, Lane et al. (1999) found that TNF-α dose dependently decreased HIV-1 replication in freshly isolated peripheral blood monocytes, through a mechanism involving the increase in RANTES expression (Lane et al., 1999). Taken together the different studies indicate that the pro-inflammatory molecules may increase or decrease HIV-1 replication, and suggest that the role of these pro-inflammatory molecules on HIV-1 susceptibility and infection may be complex and time-dependent. The results from this study did not show any significant changes in IL6 and IL8 mRNA levels with MPA or NET treatment in the presence of HIV-1 after 12 days. However, it could be that IL6 and IL8 mRNA levels have a role in HIV-1 susceptibility and initial infection. MPA, unlike NET, significantly repressed IL6 and IL8 mRNA levels after 48 hours in ectocervical explants. It could be

that select repression of key immunomodulatory genes by MPA after short term exposure could lead to an increase in HIV-1 susceptibility by dampening the initial immune response (Huijbregts et al., 2014; Quispe-Calla et al., 2015). However the role of IL6 and IL8 on HIV-1 susceptibility are yet to be fully elucidated.

In order to understand the role of inflammation on HIV-1 replication in the FGT, one could perform add back experiments. Here supernatants collected from explant samples treated with the different progestogens would be harvested at select time points. Thereafter, the supernatants could be added to an infected indicator cell line and changes in HIV-1 replication could be monitored. In parallel, a Luminex assay could be performed to determine the changes in pro-inflammatory gene expression. This experiment would elucidate whether the different progestogens differentially affect pro-inflammatory gene expression that may favour HIV-1 replication. In addition, this experiment could be repeated to assess the effects on initial HIV-1 infection, whereby the supernatant from the explants could be added to an uninfected indicator cell line. After incubation with the supernatant, the indicator cells lines could be infected with HIV-1, and changes in HIV-1 replication could be monitored. This experiment would elucidate whether the different progestogens differentially affect select pro-inflammatory gene expression that may differentially affect HIV-1 target cells to increase initial HIV-1 infection. Furthermore, the role of progestogens in the presence of the endogenous glucocorticoid, cortisol should be investigated. The effect of endogenous cortisol on the responses observed on gene expression and HIV-1 infection by the different progestins also remains to be investigated.

Future research should also investigate the role of individual cell types, as it could also be that inflammation selectively activates and recruits target cells to different and localised regions in the local female genital tract to increase the susceptibility to infection (de Jong et al., 2008). In a recent review by Shey et al. (2015), the authors suggest that dendritic cells play an important role in facilitating or preventing HIV-1 infection by driving inflammation in the genital tract (Shey et al., 2015).Thus using DCs isolated from primary ectocervical tissue (Rodriguez-Garcia et al., 2013; Wira et al., 2014; Wira et al., 2015; Rodriguez-Garcia et al., 2013), one could stimulate with MPA before infecting with HIV-1 and subsequently co-culture the pDCs with PBMCs or an indicator T cell line and measure the differences in HIV-1 replication (Buffa et al., 2009). It has also been shown that Langerhan cells, a type of DC, are important in early infection in the FGT (Kawamura et al., 2000; Kawamura et al., 2004; Matsuzawa et al., 2014; Blauvelt et al., 2001; Sugaya et al., 2004), and the effects of progestogens on this cell type should be explored. Furthermore, recent reports suggest that MPA increases transcytosis in primary epithelial cells (Ferreira et al., 2014). However, more research must be performed into comparing these effects with other progestins and the natural ligand P4 at concentrations that mimic serum concentrations. Recently, Ayehunie et al. (2015) developed a vaginal epithelial tissue model (EpiVaginal) that is responsive to E2 and P4 treatment, and expresses PR-B, which is under E2 control. Microarray analysis showed that this model expresses genes involved in the immune response, epithelial

differentiation and wound healing (Ayehunie et al., 2015). Thus it could be useful to perform select experiments in this model to determine the effects of MPA, P4, NET and LNG on epithelial thickness and gene regulation.

While it has been proposed that inflammation may play a critical role in the susceptibility to disease in the FGT (Masson et al., 2015; Masson et al., 2014), the cytokine profiles from plasma did not show any significant differences or associations with STIs (Masson et al., 2014; Masson et al., 2015). Thus, how inflammation in the systemic system may relate to or indicate an increased susceptibility in STIs remains unknown. In a recent study by Sciaranghella et al. (2015), the authors found that women on DMPA or using an LNG intrauterine device had increased expression levels of CCR5 in CD4+ T cells in their PBMCs compared to women on no hormonal contraception and oral contraceptives. Interestingly, LNG had greater effects on T cell expression and CCR5 expression levels in this study (Sciaranghella et al., 2015). Thus the effects of MPA on CCR5 and CXCR4 expression and cellular activation, through an increase in HLA-DR and CD68+ receptors (Camargo et al., 2009), are important to elucidate. Using flow cytometry, one will be able to determine how MPA at serum contraceptive concentrations, changes the activation state of PBMCs. Interestingly, a recent study found that MPA increased HIV-1 infection in non-activated PBMCs, and that this increase required CD14+ monocytes (Sampah et al., 2015), suggesting that the state of activation is not the only mechanism by which MPA increases HIV-1 replication. The results from the current study found that MPA increased HIV-1 replication increase correlated with IL6 mRNA levels in PBMCs. Further, the results in this study found that IL6 was predominantly expressed in CD14+ monocytes after 48 hours. Thus it could be that MPA, after prolonged exposure, increases IL6 mRNA levels in CD14+ monocytes that could contribute to an increase in HIV-1 replication in other HIV-1 target cells. Therefore future research should focus on the changes in IL6 mRNA or protein levels in CD14+ monocytes after prolonged exposure to MPA and determine whether these changes affect HIV-1 replication in HIV-1 target cells.

Taken together, the data reported in this study as well as others suggest that pro-inflammation is not the only mechanism whereby susceptibility to HIV-1 infection is increased, nor does it appear to be a reliable predictor. Rather, it appears to be a more dynamic interplay between which cytokines or chemokines are regulated and the recruitment of target cells to the target sites in response to MPA treatment. It also appears to be driven in a compartmental, time, and concentration dependent manner. What is not known is the effect of DMPA on early HIV-1 infection events, and whether long term treatment with DMPA could lead to a pro-inflammatory state in the FGT. This area should be focused upon in future experiments, as it is pivotal in the understanding of how contraceptives may increase HIV-1 acquisition in women. The infection experiments were performed on activated PBMCs in this current study. It may therefore be of interest to perform the infection experiments in the absence and presence of activation in matched donors so that the effects of MPA on HIV-1 replication in activated and non-activated PBMCs can be determined. It may be that while pro-inflammation in the FGT is an indicator of disease susceptibility and disease progression; it

may not be a good indicator of disease susceptibility and or disease progression in the systemic immune system.

Cervical explants are useful models but have limitations

The use of *ex vivo* cervical explants has provided researchers with key insights into which cell are target cells of HIV-1 infection in the FGT (Hladik et al., 2007; Greenhead et al., 2000). Furthermore, cervical explants have been used to assess the effects of HIV-1 inhibitory compounds on HIV-1 replication (Fletcher et al., 2006; Fletcher et al., 2008 Dodou et al., 2014; Harman et al., 2012). However the methodology has some important limitations.

The results from this study found significant differences in gene expression with different progestogens after 48 hours in four explant donor samples, with triplicate replicates. Additionally, HIV-1 replication studies found significantly different effects in HIV-1 replication after 10 days post infection in 5 – 8 donor samples, each in triplicate. These results show that even with relatively few donors, statistically significant differences of $p < 0.05$ to $p < 0.0001$ can be measured. However, the large inter- and intra- donor variability in the results with ectocervical explants suggests that for some experiments where the effects are different for different donors or responses are small, the n-value would need to be substantially increased in order to measure statistically significant differences. One way to decrease the experimental error in these experiments would be to improve the uniformity of the explant tissues pieces used in replicates. In this study, a punch was used in order to obtain similar pieces of tissue for each replicate treatment. However, the depth of the tissue varied; as such more stroma may have been present in one sample compared to another from the same donor. Since distribution of immune cells is dynamic (Chandra et al., 2013; Pudney et al., 2005; Wira et al., 2015), it could be that the depth of the tissue could be a contributing factor to why high intra-individual variation was observed in the HIV-1 replication experiments. This problem could be improved by increasing the replicate number or by more uniform and time consuming cutting of the tissue pieces or weighing. Both of these strategies, however, have other disadvantages such as increased and variable time of preparation and hence potentially increased loss of tissue integrity and loss of reproducibility. Although weighing each sample to account for tissue size may lead to lower variability, it may not be feasible to weigh each sample, especially if sample sizes are large.

Inter-donor variability may be as the result of several variables. These include the differences in age, menstrual phase, contraceptive use, medication use, the presence of infections and or the presence of unique microflora (Anderson et al., 2010). Only women who were pre-menopausal and undergoing a hysterectomy for benign reasons were consented for this study. Additionally, hormone levels, HSV, HIV-1 and HPV status were determined for each donor. While these controls greatly contributed to the study and to the analysis thereof, not all differences could be accounted or controlled for. The age of the donor is also particularly important in HIV-1 studies. Some observational studies have observed

that young women on MPA may be more susceptible to HIV-1 infection (Leclerc et al., 2008; Morrison et al., 2010; Morrison et al., 2012). However, the likelihood of a young woman undergoing a hysterectomy is low, and thus the use of tissue explants may be restricted to donors from older women. The donors in this study were not on contraception at the time of surgery. Thus observing the differences in gene expression or HIV-1 replication in explants from women on contraception was not possible. It may be that the only way to observe differences in MPA versus NET in young women, is to enrol young women in a study, place them on different contraceptive arms and acquire biopsy, CVL and blood samples, similar to what several studies have done (Huijbregts et al., 2013; Huijbregts et al., 2014; Michel et al., 2015). However, the ethical considerations, cohort management and the costs involved in acquiring these samples may be prohibitive in some environments. While it may be that cervical explants are from older donors, it is a useful approach in which to analyse the direct effects of multiple progestogens on gene expression and HIV-1 replication, given that a relatively large amount of tissue is obtained, compared to biopsies. Further categorising the explants received could be a useful approach to reduce variability. Immunohistochemistry or immunofluorescence techniques could be used to determine basal levels of select cell types (CD4+ T cells, Langerhan cells, macrophages) or steroid receptor profiles of a donor that may be useful as a means to group and characterise responses. Furthermore, one could expand the profile of the donor by acquiring information on inflammation state and drug use, which may greatly help to group donors into response types.

Another of the challenges and limitations of explant studies is establishing conditions and obtaining productive infection. For the HIV-1 replication studies, it would be important to include controls such as an HIV-1 viral replication or entry inhibitor (such as Lamivudine or Maraviroc) to confirm productive viral infection, similar to the methods used by Saba et al. (2013). A pilot study was performed in the current study with the R5 HIV-1 viral entry inhibitor, Maraviroc. The inhibitor was added 15 minutes prior to infection at a final concentration of 5 µg/ml. The data indicated, that while HIV-1 viral replication decreased over time, initial viral replication levels at day 3 post infection were the same as the control (appendix E, Fig. E.2.4). This may be due to too short a pre-exposure time to Maraviroc or too low a concentration of inhibitor. Further experiments are needed to determine the optimal conditions for these experiments, so that these compounds may be used as appropriate controls.

A limit of explant methodology is achieving a true polarised tissue environment in which to study HIV-1 transmission. Polarisation of the tissue involves orientating the tissue epithelial side up on a transwell system and providing structure such that HIV-1 entry is limited to the epithelial surface of the explant (Anderson et al., 2010; Merbah et al. 2011; Dezzutti et al., 2012). However, due to the rate of tissue deterioration and subsequent changes in cellular permeability (Anderson et al., 2010), these methods have been used with limited success. Additionally, recruitment of target cells from nearby vessels or tissue sites are absent in an isolated tissue explant. Due to these limitations associated with transmission methodology, transmission was not investigated in this study. While polarisation may be

difficult to achieve, another consideration for these infection experiments is the use of a non-polarised transwell system or collagen raft to better mimic the tissue environment. Several studies have used non-polarised transwell system to identify targets cells in early viral infection (Hladik et al., 2007; Dezzutti & Hladik, 2012) and viral penetration of the cervical epithelium (Dezzutti et al., 2001; Carias et al., 2013). In addition, it has been observed, that the tissue structure is maintained better using the raft system (reviewed in Merbah et al., 2011). Thus, it could be of benefit to repeat these experiments in a transwell system or on a collagen raft (Merbah et al., 2011), that may better mimic the primary environment.

Another important consideration in explant methodology is the viability of the tissue over time. It was found that tissue viability did not significantly vary over time (see Appendix A.6.1 B). However, when looking at the MTT viability of each donor over time, there were distinct differences in viability over time between donors (Appendix A.6.1 A). In some donors, viability dramatically decreased, while in others there was no change or a slight increase in viability. These differences could be due to the different tissue sizes used for each assay that could contribute to these differential effects in viability. Another caveat in the viability assay used in this study, is that it gives a measure of total cellular viability, which may mask changes in viability of individual cell types. This is one of the first studies to report on tissue viability at the start and end of an infection experiment. While the results of this study found that that there was no difference in viability over time, it is an important factor to monitor. In addition, only fresh tissue was used in all experiments. Initial pilot experiments found that frozen tissue had an ~80% reduction in viability compared to its fresh tissue counterpart. Interestingly, RNA quality was greatly reduced in explant samples after 12 days incubation. It could be that while viability of the tissue may not greatly affect HIV-1 replication, it could contribute to the quality of the RNA isolated. Given the high susceptibility of RNA to degradation, it may be that measuring secreted cytokines and chemokines in the supernatants is the only feasible method for determination of gene expression after 12 days.

Taken together, explants are a useful model in which to study the direct effects of progestogens in tissue of the female genital tract. However, donor variability, sample acquisition, culture set up, length of incubation time, tissue uniformity and viability are important factors to consider in the experimental design and in the subsequent outcomes of the experiment.

NET may be a better choice of injectable contraceptive for women in high risk areas

Importantly, this study suggests that NET does not result in an increase in HIV-1 replication. While MPA significantly increased HIV-1 replication in most donor samples, it may be that the finding that NET is better than MPA is more important. In this study NET had little to no impact on immune gene expression and in both PBMCs and primary ectocervical explants as well as no to reduced levels of HIV-1 replication in PBMCs and primary ectocervical explants. It could be that NET is able to convert

to estrogen, and that this may offer protective effects (Kuhnz et al., 1997). It has been suggested that the effects of MPA on immune function and epithelial integrity could be due to the fact that MPA induces hypoestrodial levels in women (Borgdorff et al., 2015; Morrison et al., 2015; van de Wijgert et al., 2013). With a reduction in estrogen levels, barrier function and proliferation in the local genital mucosa are reduced more so than women in the luteal phase. Thus, the difference between MPA and NET could be due to the different levels of E2 *in vivo*. It will thus be of interest if E2 levels could be assessed in the *ex vivo* explant experiments to confirm if there is an increase in E2 levels in the NET treated samples over time, and will further support what has been suggested in the literature.

Additionally, most observational studies that have reported on NET use, have reported no significant effects on HIV-1 susceptibility, with only one study finding significance (reviewed in Polis et al., 2014; Morrison et al., 2015), while recent meta-analyses have suggested that MPA may increase HIV-1 acquisition (Ralph et al., 2015; Morrison et al., 2015). In a recent study reporting on the effects of MPA on transmission and acquisition, the authors observed that MPA did not increase transmission or acquisition (Wall et al., 2014b; Wall et al., 2014a; Wall et al., 2015). Wall et al. (2015) suggest that good counselling and condom use in this cohort may have contributed to the low rates observed in the study, but also encourage increasing the contraceptive method mix used in high risk areas (Wall et al., 2015). Additionally, in an observational analysis comparing the effects of MPA and NET usage on HIV-1 risk in women, Nouguchi et al. (2015) observed that women on DMPA compared to women on NET had an increased risk of HIV-1, with an aHR of 4.76 (95% CI 2.15 – 10.52) (Noguchi et al., 2015).

The issue of contraceptive use in high risk areas has reached a critical point. Comment pieces by Hapgood (2013) and Colvin and Harrison (2015), suggest that the debate over the need for a trial may be unnecessary and inhibitory (Hapgood, 2013; Colvin & Harrison, 2015). Further Colvin and Harrison (2015) suggest that a more holistic approach where both biological, clinical and basic scientific research are all needed to address the use of DMPA, so that the most informed decision can be made (Colvin & Harrison, 2015). As such the findings reported in this study and others, together with observational data, suggest that MPA should be reconsidered as a choice of contraception in the contraceptive method mix in high risk areas. However, there are more ethical considerations that need to be taken into account if policy makers are to make these decisions. Haddad et al. (2015) recently reviewed these considerations in accordance with the Baum framework for ethical considerations, and stated that policymakers need to determine the benefit of removing MPA from the useable pool contraceptives versus the harm that may follow in the reduction of effective and available contraceptives especially in areas where contraceptive use is already limited (Haddad et al., 2015). Indeed, Ralph et al. (2015) stated in their meta-analysis that removing MPA without a suitable replacement (Ralph et al., 2015) will not be beneficial. The authors also state that contraceptive use should be re-evaluated in the context of graphical location and site-specific need (Ralph et al., 2015).

A recent review article by Halpern et al. (2015) sought to address the current limitation on longer acting progestins in the contraceptive method mix, by highlighting potential new candidates using new delivery technologies for development. However the authors highlighted that these new technologies and formulations must take into consideration the limitations of the current long acting progestins (Halpern et al., 2015), so that contraceptives with the lowest risk profile are taken forward in development. It has also been found that MPA administered at a lower dose subcutaneously (104 mg) had better efficacy compared to MPA administered at a higher dose intramuscularly (150 mg), while maintaining lower MPA serum concentrations in women (Shelton & Halpern, 2014). This may not be an adequate option however, as the peak serum concentrations are still similar to that of the intramuscularly delivered DMPA (Shelton & Halpern, 2014). Additionally, several studies have shown that pre-exposure prophylactic (PrEP) treatment in conjunction with MPA does not diminish the efficacy of PrEP, nor MPA efficacy (Heffron et al., 2014). However, PrEP is not yet available to women in these areas, and as such other alternatives are needed in the interim, nor has a lower dose formulation of MPA been approved for contraceptive use. Lastly, while a trial has been approved assessing the differences between DMPA, LNG (intrauterine device) and the copper T (Rees, 2014), this may take a few years before results are forthcoming (Ralph et al., 2015; Westhoff & Winikoff, 2014), further supporting the need for basic research on this topic to help inform contraceptive choice in the interim.

Concluding remarks

This study, together with recent findings in the literature, suggest that MPA use changes the immunomodulatory landscape at both the systemic and local immune environment, increasing HIV-1 susceptibility and potentially disease progression. Further, this study suggests that MPA increases HIV-1 viral replication in PBMCs, which positively correlated with an increase in IL6 mRNA expression levels, while MPA increases or decreases HIV-1 replication in primary cervical explants via an unknown mechanism. LNG, used in oral contraceptives and intrauterine devices, had no effect on gene expression or HIV-1 replication in PBMCs at high concentrations, however studies in explant systems are yet to be performed. However of importance, is that NET has little to no effect on immune gene expression, does not result in an increase HIV-1 viral replication in PBMCs, and appears to have the least variable effects in primary cervical explants, significantly reducing viral replication. Comment pieces by Hapgood (2013) and Jones and van de Wijgert (2015) suggest that women in high risk areas be encouraged to use NET-EN over MPA, due to the lower reported risk profile of NET on HIV-1 susceptibility compared to MPA, as well as their similar efficacy in pregnancy rates (Jones & van de Wijgert, 2015; Hapgood, 2013). While several commentaries caution against removing MPA from the contraceptive pool (Jain, 2013; Jain, 2012; Haddad et al., 2015; Ralph et al., 2015; Morrison et al., 2015), NET, rather than MPA, may be a preferable choice of injectable contraceptive in regions of high HIV-1 risk, until better long acting and lower dose progestins and counselling systems are made available.

Chapter 6

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Appendix A

Supplementary Data

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A.1. Luminex PBMC Data

Figure A.1.1 shows the supplementary Luminex data to accompany those results presented in Chapter 3 (Fig. 3.5), which showed strong dose dependent repressive effects for DEX treatment more so than MPA treatment on IL-1ra, VEGF, IL7, IL12 (p70) and TNFα relative protein levels. Figure A.1.1 shows that DEX, unlike MPA, has significant effects on IL-10 (Fig. A.1.1 B), IL2 (Fig. A.1.1 C), IFN-γ (Fig. A.1.1 D), Eotaxin (Fig. A.1.1 E), GM-SCF (Fig. A.1.1 F), IL4 (Fig. A.1.1 G) and FGF basic (Fig. A.1.1 H) protein levels compared to the vehicle control. While both DEX and MPA significantly reduce IL-1β protein expression levels compared to the control (Fig. A.1.1 A). Furthermore, the results show that P4 and NET have no effect on any of the genes (Fig. A.1.1).

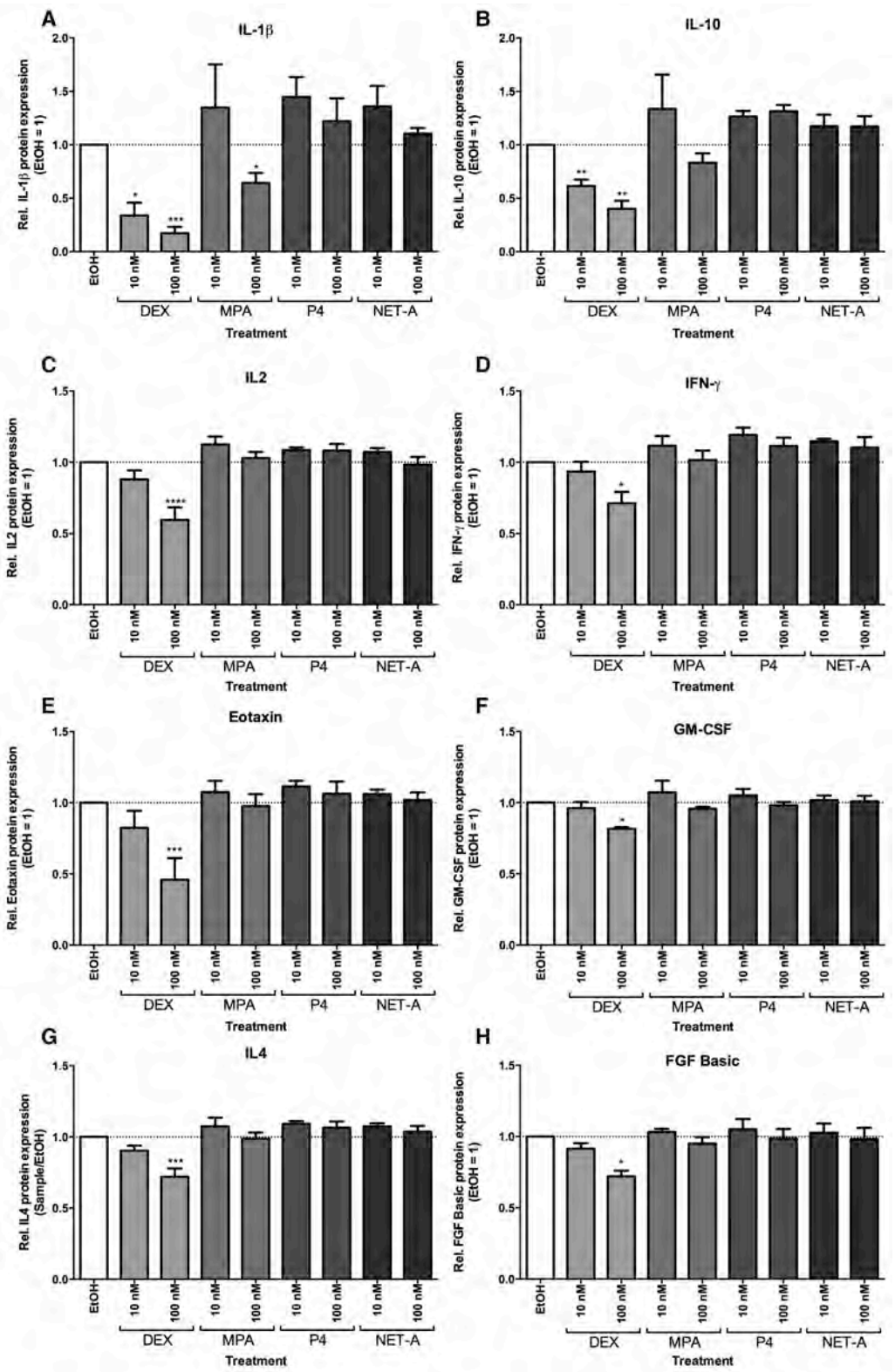


Figure A.1.1: DEX more so than MPA, dose-dependently regulates select cytokine and chemokine basal protein expression levels in primary PBMCs. PBMCs were stimulated with increasing amounts of DEX, MPA, P4, NET-A or vehicle (0,1% EtOH) for 48 hrs. Supernatants were harvested and processed for differential protein expression using the Bio-Rad 27-plex Human cytokine and chemokine panel (Bio-Rad, Germany) on a Bio-plex 200 (Bio-Rad, Germany). Relative protein expression levels of IL-1β (A), IL-10 (B), IL-2 (C), IFN-ɣ (D), Eotaxin (E), GM-CSF (F), IL4 (G) and FGF Basic (H) levels were normalised to vehicle control (EtOH) set to 1. Histograms represent pooled data from four independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a post hoc Dunnett's test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with *, **, *** denoting $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

A.2. Gating Strategies for flow cytometry

Figure A.2.1 and A.2.2 show the gating strategies and the scatter plots for IL6 and GILZ protein expression used in the flow cytometry data reported in Fig. 3.6.

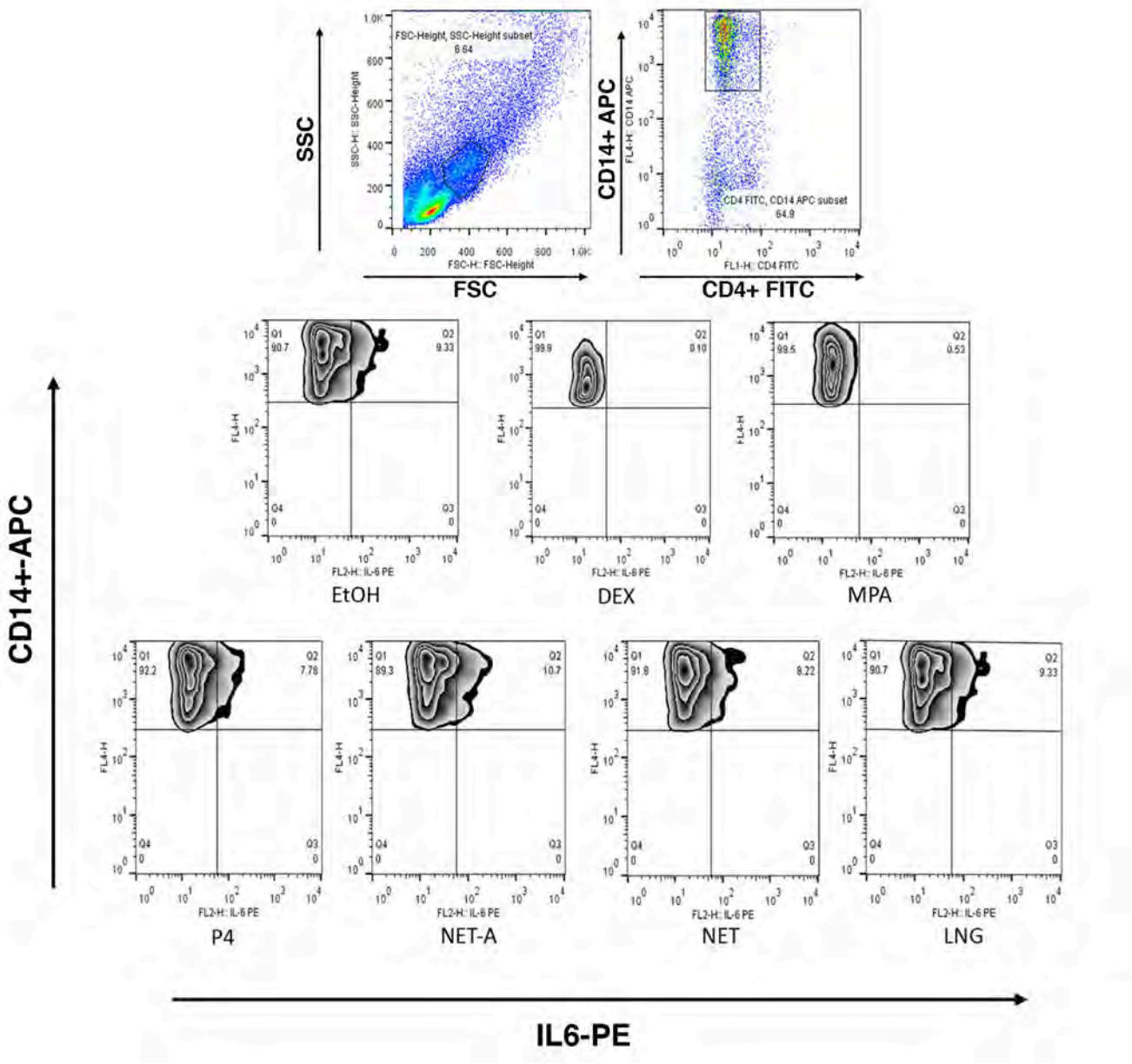


Figure A.2.1: Gating strategy for IL6 detection in CD14$^+$ monocytes. Cells were treated with 100 nM DEX, 1 µM MPA, 1 µM P4, 10 µM NET-A, 10 µM NET, 10 µM LNG or vehicle control (EtOH) for 48 hrs. Cells were stained with anti-CD4, anti-CD14 and 7-AAD, followed by intracellular detection of IL6. Above is the gating strategy and representative zebra plots of the vehicle control (EtOH) and the ligands listed above. Data was acquired on a FACS calibur system (BD Biosciences) and analysed using Flo-Jo software (Tree Star Inc., San Carlos, CA, USA).

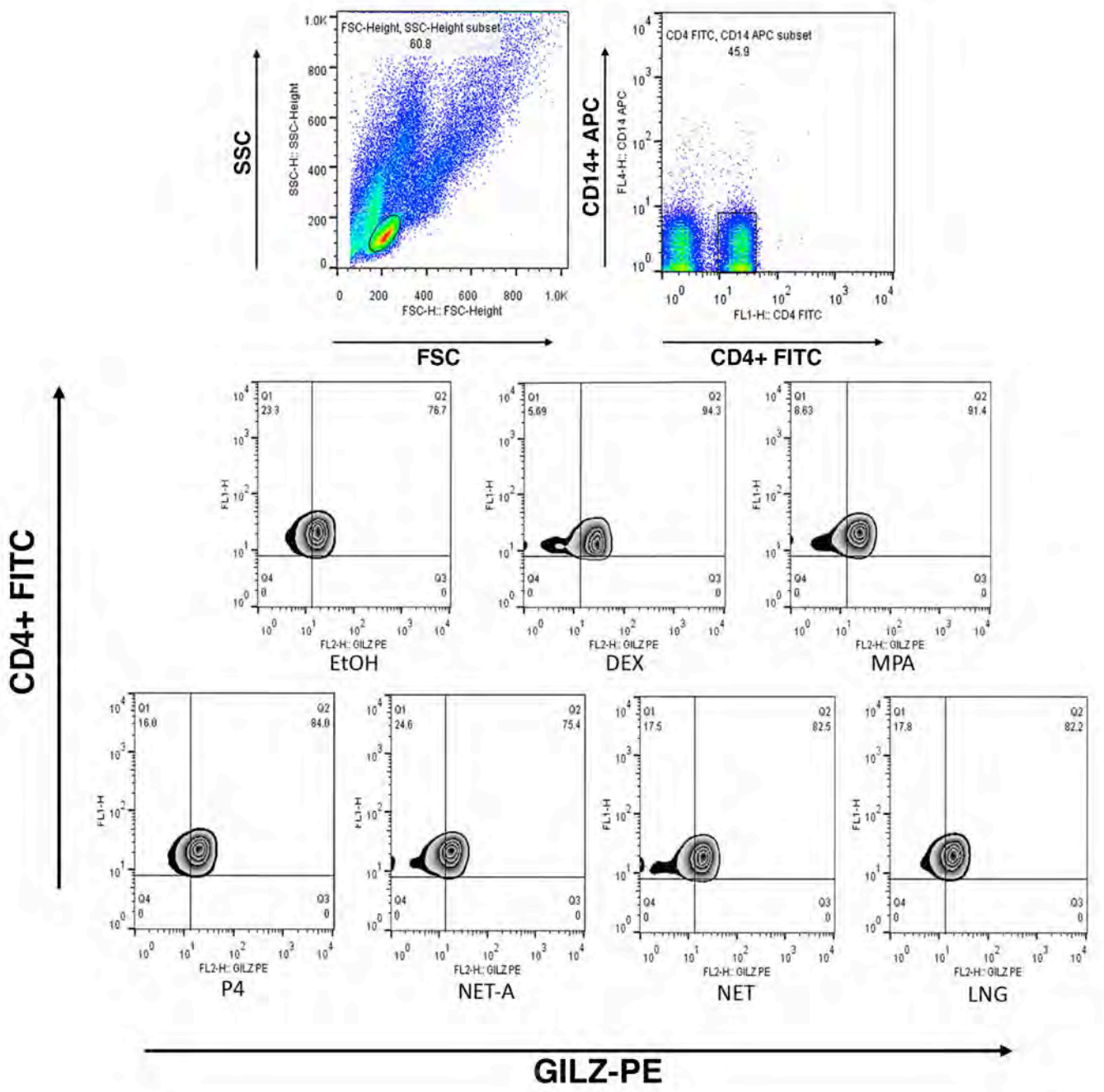


Figure A.2.2: Gating strategy for GILZ detection in CD4$^+$ T-cells. Cells were treated with 100 nM DEX, 1 µM MPA, 1 µM P4, 10 µM NET-A, 10 µM NET, 10 µM LNG or vehicle control (EtOH) for 48 hrs. Cells were stained with anti-CD4, anti-CD14 and 7-AAD, followed by intracellular detection of GILZ. Above is the gating strategy and representative zebra plots of the vehicle control (EtOH) and the ligands listed above. Data was acquired on a FACS calibur system (BD Biosciences) and analysed using Flo-Jo software (Tree Star Inc., San Carlos, CA, USA).

A.3. HIV-1 replication and relative IL6 mRNA expression levels in DEX treated PBMCs

It was found in chapter 3 that MPA increased HIV-1 replication in PBMCs from most donors, and that this increase correlated with an increase in IL6 mRNA levels in those samples. To further support that these effects observed in the MPA treated samples were glucocorticoid-like, more so than for the other progestogens, IL6 mRNA levels were matched with their HIV-1 replication data in each treatment group. A Pearsons r correlation was performed for each of these treatment groups. For samples treated with CORT, P4, NET and LNG HIV-1 replication levels did not correlate with IL6 mRNA levels in their respective treatment groups. However, DEX, positively correlated with relative infection levels in the DEX treated samples, with a significance of p=0.0381 (Fig. A.3.1 A). In addition, the data indicate that the relative infection levels were higher in the high IL6 mRNA expression group than in the low IL6 mRNA expression group (Fig. A.3.1 B).

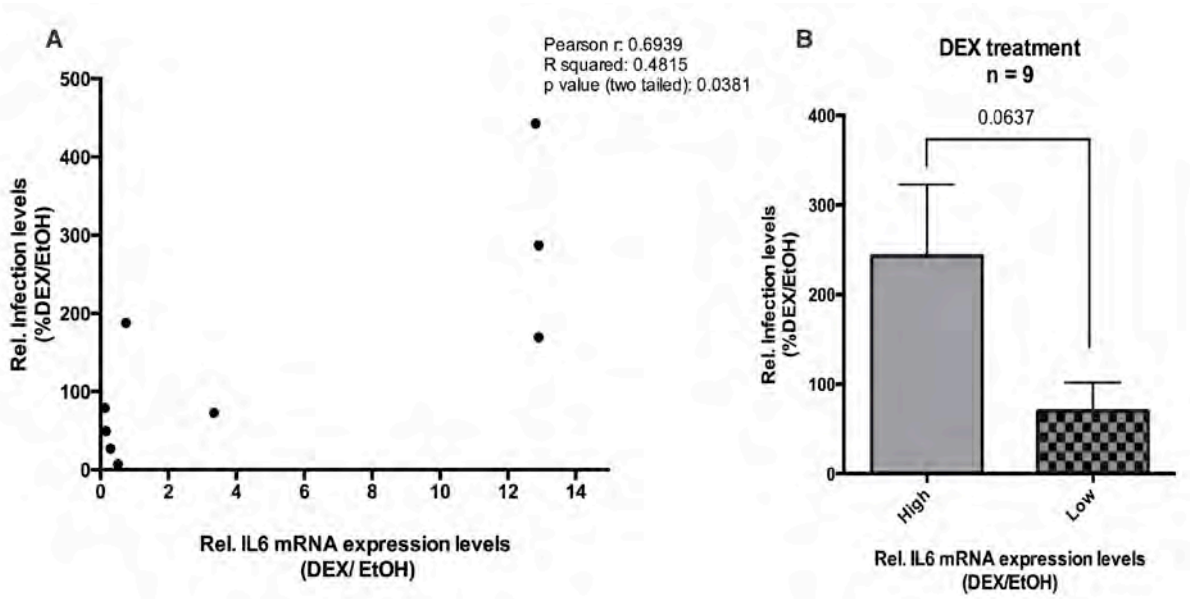


Figure A.3.1: Higher DEX infection levels positively correlate with higher IL6 mRNA infection levels. PHA activated PBMCs were treated as described in the legend of Fig. 3.11. (A) Relative DEX infection levels, normalised to vehicle treatment (EtOH), were plotted against matching relative IL6 mRNA expression levels, normalised to vehicle treatment (EtOH). Relative IL6 mRNA transcript levels were determined by comparing C(t) values to a standard curve derived from a serial dilution of pooled cDNA (from the experimental repeats) with a known concentration. An XY scatter plot represents 10 individuals with mean infection levels on the Y-axis and IL6 relative mRNA expression on the X-axis. A Pearsons r correlation analysis (comparing r for X vs. Every Y data set) was performed with a two-tailed post-test t-test analysis. (B) DEX infection levels were grouped according to low IL6 mRNA expression levels or high IL6 mRNA expression levels. The histogram is representative of 4 -5 biological repeats per bar plotted as mean ± SEM. Statistical significance was determined by an unpaired student t-tests comparing the two groups, with p = 0.0637.

A.4. Gene expression data separated according to high and low relative HIV-1 infection levels in MPA treated PBMCs

In chapter 3, it was found that the results for PBMCs treated with MPA and infected with HIV-1 could be grouped into two groups. In some samples, HIV-1 replication was significantly higher with MPA treatment as compared to the control group (Fig. 3.11). Additionally, it was found that HIV-1 replication in this MPA treated group significantly correlated with higher IL6 mRNA levels compared to the control group (Fig 3.14). As such, the mRNA expression levels were separated. Figure A.4.1 shows the effects of the different ligands on IL6 mRNA levels again (as seen in chapter 3) as well as IL8, RANTES and GILZ mRNA expression levels in this group. The results show that while IL6 mRNA levels are significantly upregulated with CORT and MPA in the presence of HIV-1 (Fig. A.4.1 A), IL8 and RANTES levels are not (Fig. A.4.1 B and C). Transactivation of GILZ by CORT, DEX and MPA is maintained in the absence and presence of HIV-1 (Fig. A.4.1 D). Furthermore, P4, NET and LNG had no effect on the relative expression levels of these genes in the absence and presence of HIV-1 (Fig A.4.1).

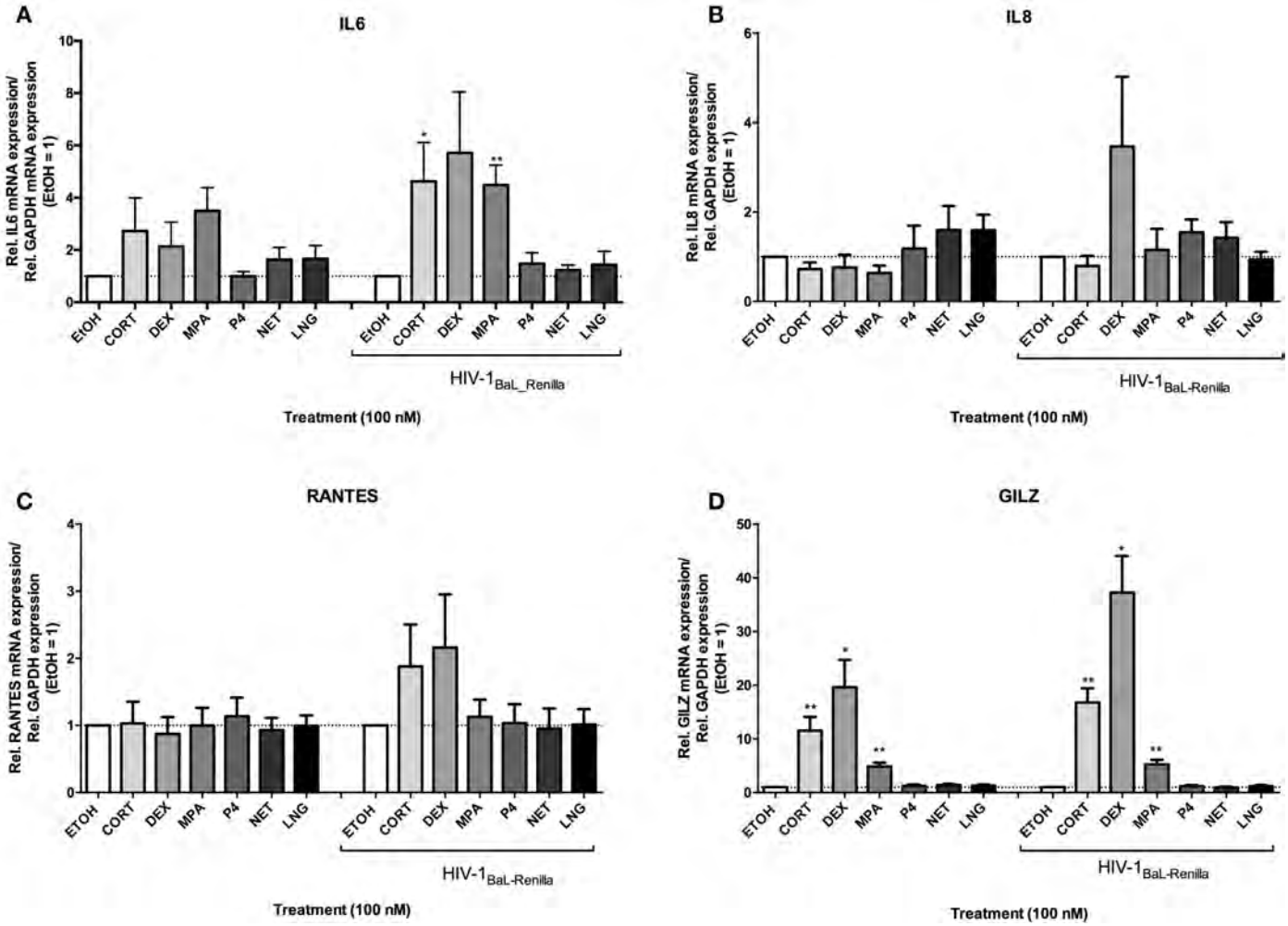


Figure A.4.1: Gene expression in donor samples where MPA increased HIV-1 infection in primary PBMCs. PHA activated PBMCs were pre-treated with 0.01% (*v/v*) 100 nM Cortisol (CORT), DEX, MPA, P4, NET or LNG, with a vehicle control (EtOH), for 48 hours. Pre-treated PBMCs were infected with 10 IU/ml HIV-$1_{BaL\text{-}Renilla}$ IMC for 2 hours and media with the respective ligands was replaced every second day. PBMCs were harvested at 7 days post infection in TriReagent® and subsequently processed for RNA. 250 ng RNA was reversed transcribed to cDNA (Roche Applied Science, South Africa), where it was used in subsequent real time qPCR as template to determine the differential expression levels. Relative mRNA expression levels of IL 6 (A), IL 8 (B), RANTES (C) and GIL Z (D) were normalised to GAPDH mRNA expression levels. Relative fold change in mRNA levels was determined by setting the vehicle treatment (EtOH) to 1. Histograms represent pooled data from seven independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by a non-parametric Wilcoxon Rank Test comparing each sample to the vehicle control (EtOH) set to 1, with * and ** denoting $p<0.05$, and $p<0.01$ respectively.

Additionally, the results in chapter 3 found that in the MPA treated samples where HIV-1 replication was low, IL6 mRNA levels were reduced or lower compared to the control group (Fig 3.15). Figure A.4.2 shows the changes in gene expression with the different ligands in the presence and absence of HIV-1 infection for this group. Fig A.4.2 shows the results for IL6 mRNA levels again (as seen in Fig 3.15), as well as the results for IL8, RANTES and GILZ mRNA levels. The data indicates that IL6 mRNA levels decreased with CORT, DEX, and similar to basal for MPA in the presence of HIV-1 (Fig. A.4.2 A). Similarly, IL8 mRNA levels appear to be reduced with CORT and DEX in the presence of HIV-1, while MPA like P4, NET and

LNG has no effect on IL8 mRNA gene expression in the presence of HIV-1. RANTES mRNA levels were not regulated in the presence or absence by CORT, DEX, P4, NET or LNG. However, MPA in the presence of HIV-1 significantly increased RANTES mRNA levels (Fig. 4.2 C). Transactivation of GILZ by CORT, DEX and MPA is maintained in the absence and presence of HIV-1; however this effect was not significant (Fig. A.4.2 D).

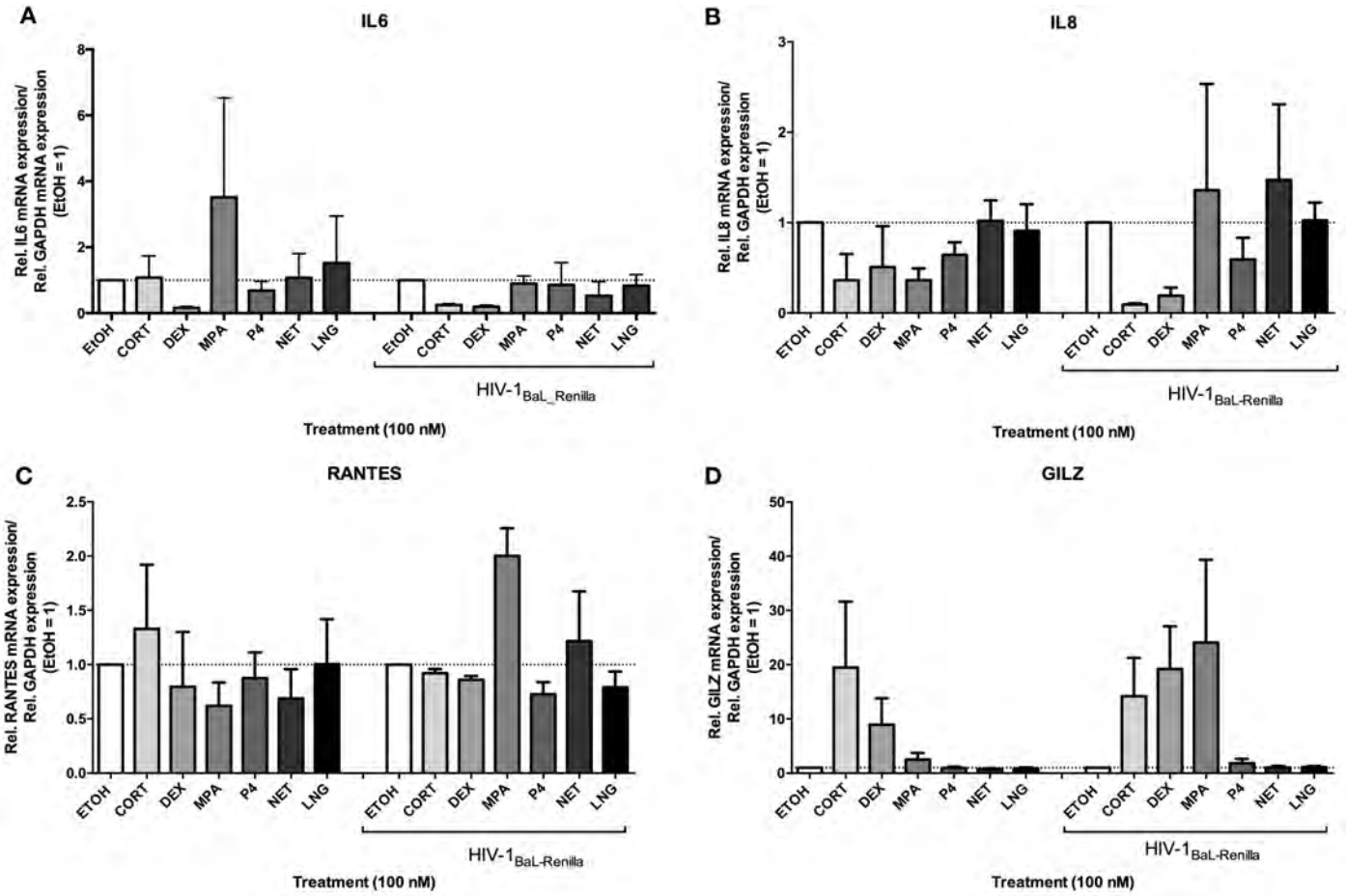
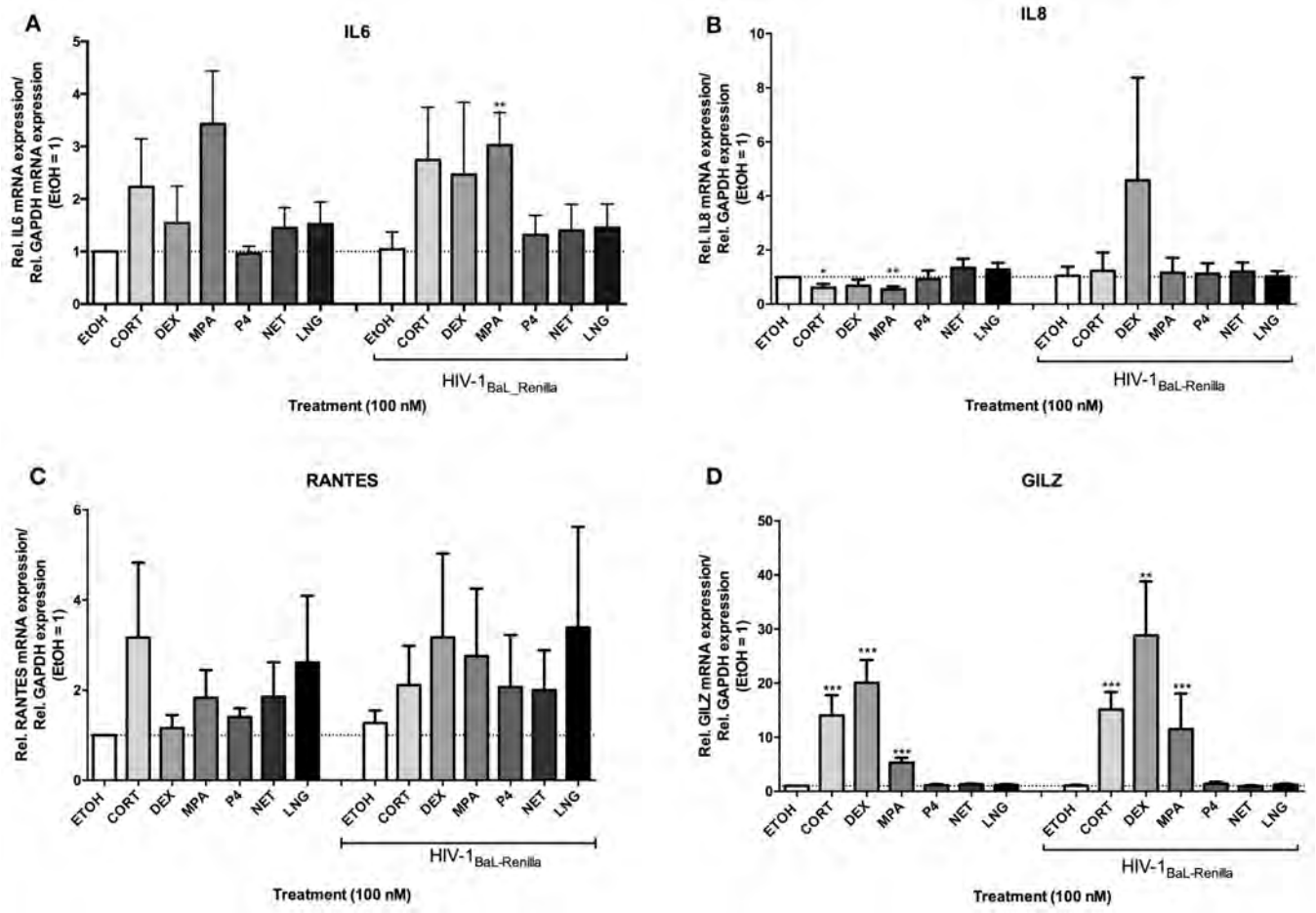


Figure A.4.2: Gene expression in donors samples where MPA decreased HIV-1 infection in primary PBMCs. PHA activated PBMCs were pre-treated with 0.01% (*v/v*) 100 nM Cortisol (CORT), DEX, MPA, P4, NET or LNG, with a vehicle control (EtOH), for 48 hours. Pre-treated PBMCs were infected with 10 IU/ml HIV-$1_{BaL\text{-}Renilla}$ IMC for 2 hours and media with the respective ligands was replaced every second day. PBMCs were harvested at 7 days post infection in TriReagent® and subsequently processed for RNA. 250 ng RNA was reversed transcribed to cDNA (Roche Applied Science, South Africa), where it was used in subsequent real time qPCR as template to determine the differential expression levels. Relative mRNA expression levels of IL 6 (A), IL 8 (B), RANTES (C) and GIL Z (D) were normalised to GAPDH mRNA expression levels. Relative fold change in mRNA levels was determined by setting the vehicle treatment (EtOH) to 1. Histograms represent pooled data from three independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by a non-parametric Wilcoxon Rank Test comparing each sample to the vehicle control (EtOH) set to 1.

A.5. Basal Expression levels of IL6, IL8, RANTES and GILZ in the presence of HIV-1 or over time

In Chapter 3, gene expression was determined by normalising each treatment group to the vehicle control (EtOH) set to 1. While these data are useful in presenting the effects of the different progestogens on gene expression, it does not give an indication on the changes in basal transcription levels in the presence of HIV-$1_{BaL\_Renilla}$ or indicate if basal mRNA expression levels change over time. As such the following gene expression data from Figures 3.12 and 3.16 were reanalysed and re-plotted so that changes in basal transcription levels in the control groups could be determined. For Figure 3.12 gene expression was plotted relative to the vehicle control in the uninfected group, so that changes in basal transcription in the presence of HIV-$1_{BaL\_Renilla}$ could be evaluated. Similarly in Figure 3.16, data was analysed such that gene expression was relative to the day 2 EtOH control so that changes in basal transcription levels over time could be evaluated.

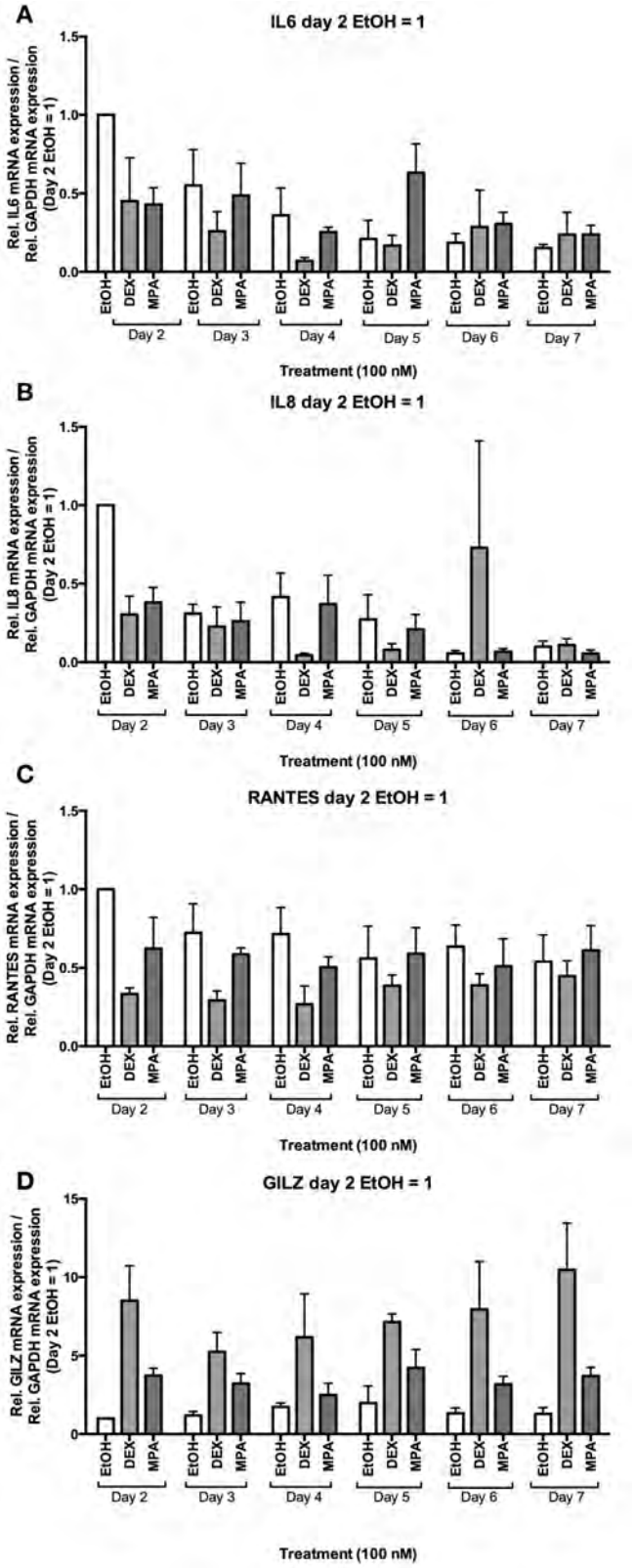
In Figure A.5.1, it is evident that basal mRNA levels of IL6 (A), IL8 (B), RANTES (C) or GILZ (D) in the control EtOH groups did not change in the presence of HIV-$1_{BaL\_Renilla}$.



<u>Figure A.5.1:</u> Gene expression in donors samples where MPA decreased HIV-1 infection in primary PBMCs. PHA activated PBMCs were pre-treated with 0.01% (*v/v*) 100 nM Cortisol (CORT), DEX, MPA, P4, NET or LNG, with a vehicle control (EtOH), for 48 hours. Pre-treated PBMCs were infected with 10 IU/ml HIV-$1_{BaL\text{-}Renilla}$ IMC for 2 hours and media with the respective ligands was replaced every second day. PBMCs were harvested at 7 days post infection in TriReagent® and subsequently processed for RNA. 250 ng RNA was reversed transcribed to cDNA (Roche Applied Science, South Africa), where it was used in subsequent real time qPCR as template to determine the differential expression levels. Relative mRNA expression levels of IL 6 (A), IL 8 (B), RANTES (C) and GIL Z (D) were normalised to GAPDH mRNA expression levels. Relative fold change in mRNA levels was determined by setting the vehicle treatment (EtOH) in the uninfected group to 1. Histograms represent pooled data from three independent experiments from female donors plotted as mean ± SEM. Statistical significance was determined by a non-parametric Wilcoxon Rank Test comparing each sample to the vehicle control (EtOH) set to 1, with ** and *** denoting $p<0.01$, and $p<0.001$ respectively.

In Figure A.5.2, it is evident that IL6 mRNA expression levels decrease over time (Fig A.5.2 A). Similarly IL8 mRNA expression levels decrease substantially over the time course (Fig. A.5.2 B), while RANTES basal mRNA expression levels (EtOH) moderately decrease over time (Fig. A.5.2 C). Interestingly, it appears that basal GILZ mRNA expression levels do not change over time (Fig. A.5.2 D).

Furthermore the data suggests that while basal IL6 and IL8 mRNA levels decrease over time, DEX and MPA treated expression levels do not decrease in expression levels to the same extent as the basal conditions (Fig A.5.2 A and B).



<u>Figure 3.16:</u> DEX and MPA-mediated repressive effects on select genes is lost over time, while transactivation effects are maintained. PHA activated PBMCs were pre-treated with 100 nM DEX, MPA or 0.1% (*v/v*) vehicle control (EtOH) for 7 days with half media exchange at day 3 and 6. Samples were harvested every day from day 2 to day 7 for RNA isolation. 250 ng RNA was reverse transcribed to cDNA and used as template in subsequent real time qPCR. Relative mRNA expression levels of IL6 (A) and IL8 (B) were normalised to GAPDH mRNA expression levels. Relative fold change in expression was determined by setting vehicle treatment (EtOH) at day 2 to 1. Histograms represent pooled data from four independent experiments from four female donors plotted as mean ± SEM. Statistical significance was determined by one-way ANOVA with a Dunnett's post-test or unpaired student t-tests comparing each sample to the vehicle control (EtOH) with *, **, *** and **** denoting $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ respectively

A.6. MTT Viability Assay in Explants

In chapter 4, primary cervical tissue was obtained from women who had undergone hysterectomies for benign reasons. For each sample, an MTT viability assay was performed to determine the viability of the tissue upon arrival. Additionally, for the infection experiments in which incubation times were longer than 2 days, MTT viability assays were also performed at the end of the experiment (Fig A.5.1). This was done to compare the viability of the sample at the start of the experiment to the viability at end of the experiment. The cervical tissue obtained from the pathologist varied greatly in size. Thus for some of the infection experiments, an MTT assay was not performed at the end of the experiment (Prog 0049 and Prog 0051), as there was no available piece of tissue (Fig A.6.1 A). When pooling the MTT data, there was no difference between the viability of the donor tissue at the start of the experiment compared to the viability of the donor tissue at the end of the experiment (Fig. A.6.1 B). Interestingly, in two donors (Prog 0028 and Prot 0003) viability was greater at the end as compared to the start of the experiment. This could be due to the fact that the MTT viability assay is performed on a different piece of explant tissue at the start and at the end of the experiment. Since sample sizes varied in this study, it could be that larger explant pieces produced higher levels of the purple formazan that could contribute to the variability of the viability measurements in this assay.

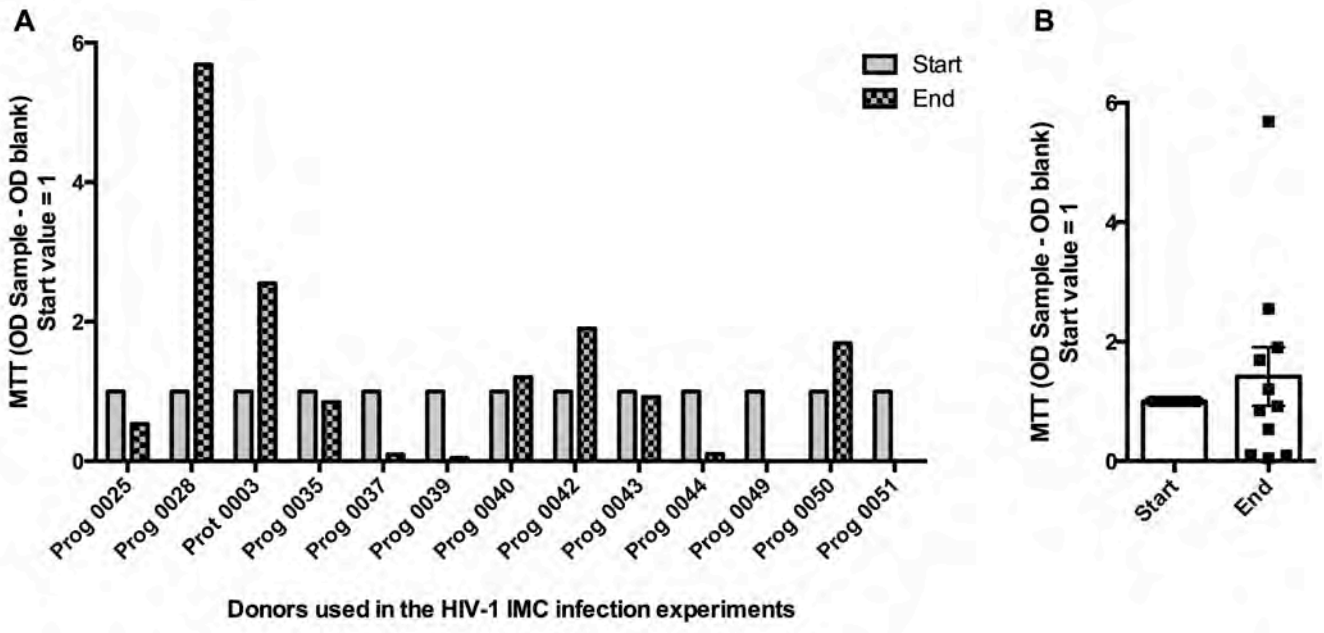


Figure A.6.1: Viability of tissue used in the infection studies. Where possible, MTT assays were performed at the start (solid bar) and at the end (checked bar) of the experiment. 5 mg/ml MTT in phenol red free RPMI was added at a final concentration of 10% of the culture volume. Tissues were incubated for 2 hours at 37°C. The tissue was subsequently removed and measured on a scale, and placed in 200 µl 0.1N HCl in isopropanol overnight at -20°C. 100 µl of the sample was measured at 520 nM, with a background measure at 620 nM in a spectrophotometer (Thermo Scientific). MTT values were measured and divided by the weight of the tissue. (A) For each donor, MTT values at the end of the experiment were normalised to the MTT value obtained at the start of the experiment. (B) shows the average MTT values obtained at the start and end of the pooled infection experiments.

Appendix B

Repeated measure two way ANOVA analysis for ectocervical explant HIV-1 infection time course analysis

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Introduction

In order to statistically analyse the effects of MPA and NET on HIV-1 infectivity in explants compared to the vehicle treatment, a repeated measures two way ANOVA was performed. In these analyses, only matched samples with matched time points can be used. As such data from days 3, 7 and 10 post infection were used in the analysis for all treatment groups. Results from the analysis of ectocervical explants infected with either HIV-$1_{BaL\_Renilla}$ or HIV-$1_{pNL4.3}$ were either pooled to represent the entire data set, or grouped together for those donor samples that indicated an increase in infection, or a decrease in infection, with MPA treatment compared to the vehicle control. Statistical significance of difference for time, treatment or an interaction was determined using two way ANOVA, with a Tukey's multiple comparison test to determine the statistical significance between treatment groups at the selected time points.

Table B1: Pooled repeats (n = 6) of ectocervical explants infected with HIV-$1_{BaL\_Renilla}$

Source of Variation	% of total variation	P value	P value summary	Significant?
Time	68,64	< 0,0001	****	Yes
Treatment	1,149	0,2116	ns	No
Interaction: Time x Treatment	0,3529	0,3671	ns	No

Tukey's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary
Day 3 post infection				
EtOH vs. MPA	-4,963	-24,75 to 14,82	No	ns
EtOH vs. NET	1,816	-17,97 to 21,60	No	ns
MPA vs. NET	6,779	-13,01 to 26,56	No	ns
Day 7 Post Infection				
EtOH vs. MPA	-7,905	-27,69 to 11,88	No	ns
EtOH vs. NET	17,68	-2,099 to 37,47	No	ns
MPA vs. NET	25,59	5,806 to 45,37	Yes	*
Day 10 Post Infection				
EtOH vs. MPA	2,121	-17,66 to 21,90	No	ns
EtOH vs. NET	19,31	-0,4693 to 39,10	No	ns
MPA vs. NET	17,19	-2,590 to 36,98	No	ns

Table B2: Pooled repeats (n = 7) of ectocervical explants infected with HIV-$1_{pNL4.3}$

Source of Variation	% of total variation	P value	P value summary	Significant?
Time	60,01	< 0,0001	****	Yes
Treatment	7,524	0,0564	ns	No
Interaction: Time x Treatment	1,303	0,0025	**	Yes

Tukey's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary
Day 3 post infection				
EtOH vs. MPA	0,6049	-13,71 to 14,92	No	ns
EtOH vs. NET	15,65	1,342 to 29,97	Yes	*
MPA vs. NET	15,05	0,7372 to 29,36	Yes	*
Day 7 Post Infection				
EtOH vs. MPA	-0,8907	-15,20 to 13,42	No	ns
EtOH vs. NET	33,45	19,14 to 47,76	Yes	****
MPA vs. NET	34,34	20,03 to 48,65	Yes	****
Day 10 Post Infection				
EtOH vs. MPA	-0,4432	-14,76 to 13,87	No	ns
EtOH vs. NET	48,22	33,91 to 62,53	Yes	****
MPA vs. NET	48,67	34,35 to 62,98	Yes	****

Table B3: Pooled repeats (n = 3) of ectocervical explants where MPA treated samples had increased infection levels compared to the vehicle control for samples infected with HIV-$1_{BaL\_Renilla}$

Source of Variation	% of total variation	P value	P value summary	Significant?
Time	84,56	0,0005	***	Yes
Treatment	5,677	0,1105	ns	No
Interaction: Time x Treatment	1,136	0,0244	*	Yes

Tukey's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary
Day 3 post infection				
EtOH vs. MPA	-32,1	-53,65 to -10,55	Yes	**
EtOH vs. NET	-10,22	-31,77 to 11,33	No	ns
MPA vs. NET	21,88	0,3262 to 43,43	Yes	*
Day 7 Post Infection				
EtOH vs. MPA	-36,97	-58,52 to -15,42	Yes	**
EtOH vs. NET	7,533	-14,02 to 29,08	No	ns
MPA vs. NET	44,5	22,95 to 66,05	Yes	***
Day 10 Post Infection				
EtOH vs. MPA	-26,8	-48,35 to -5,250	Yes	*
EtOH vs. NET	33,2	11,65 to 54,75	Yes	**
MPA vs. NET	60	38,45 to 81,55	Yes	***

Table B4: Pooled repeats (n = 3) of ectocervical explants where MPA treated samples had decreased infection levels compared to the vehicle control for samples infected with HIV-$1_{BaL\_Renilla}$

Source of Variation	% of total variation	P value	P value summary	Significant?
Time	66,49	0,009	**	Yes
Treatment	1,053	0,6235	ns	No
Interaction: Time x Treatment	0,6438	0,284	ns	No

Tukey's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary
Day 3 post infection				
EtOH vs. MPA	7,06	-11,88 to 26,00	No	ns
EtOH vs. NET	4,743	-14,20 to 23,68	No	ns
MPA vs. NET	-2,317	-21,26 to 16,62	No	ns
Day 7 Post Infection				
EtOH vs. MPA	8,463	-10,48 to 27,40	No	ns
EtOH vs. NET	12,63	-6,308 to 31,57	No	ns
MPA vs. NET	4,17	-14,77 to 23,11	No	ns
Day 10 Post Infection				
EtOH vs. MPA	20,56	1,614 to 39,50	Yes	*
EtOH vs. NET	3,333	-15,61 to 22,27	No	ns
MPA vs. NET	-17,22	-36,16 to 1,719	No	ns

Table B5: Pooled repeats (n = 5) of ectocervical explants where MPA treated samples had increased infection levels compared to the vehicle control for samples infected with HIV-$1_{pNL4.3}$

Source of Variation	% of total variation	P value	P value summary	Significant?
Time	65,56	< 0,0001	****	Yes
Treatment	9,887	0,0228	*	Yes
Interaction: Time x Treatment	1,91	< 0,0001	****	Yes

Tukey's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary
Day 3 post infection				
EtOH vs. MPA	-9,652	19,30 to -0,00335	Yes	*
EtOH vs. NET	7,98	-1,669 to 17,63	No	ns
MPA vs. NET	17,63	7,983 to 27,28	Yes	***
Day 7 Post Infection				
EtOH vs. MPA	-28,92	-38,57 to -19,27	Yes	****
EtOH vs. NET	20,7	11,05 to 30,35	Yes	***
MPA vs. NET	49,62	39,97 to 59,27	Yes	****
Day 10 Post Infection				
EtOH vs. MPA	-37,3	-46,95 to -27,65	Yes	****
EtOH vs. NET	25,64	15,99 to 35,29	Yes	****
MPA vs. NET	62,94	53,29 to 72,59	Yes	****

Table B6: Pooled repeats (n = 3) of ectocervical explants where MPA treated samples had decreased infection levels compared to the vehicle control for samples infected with HIV-$1_{pNL4.3}$*

Source of Variation	% of total variation	P value	P value summary	Significant?
Time	65,56	< 0,0001	****	Yes
Treatment	9,887	0,0228	*	Yes
Interaction: Time x Treatment	1,91	< 0,0001	****	Yes

Tukey's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary
Sidak's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary
EtOH - MPA				
Day 3 post infection	16,24	-20,93 to 53,41	No	ns
Day 7 Post Infection	32,3	-4,867 to 69,47	No	ns
Day 10 Post Infection	43	5,833 to 80,17	Yes	*

*For this grouping, only two out of the three experiment groups included treatment with NET, as such for this analysis the NET group was excluded

Appendix C

Donor Information

Patient Number	HIV	HPV	HSV-1	HSV-2	Age	LH (IU/L)	FSH (IU/L)	E2 (pmol/L)	P4 (nmol/L)	Stage of menstrual cycle	On Contraception	Type of Contraceptive	How long since contraception?	Type of experiments performed on tissue
Prog 0001	_	_	_	_	45	3	3.2	274.6	9	Luteal	No	N/A	N/A	SR western
Prog 0002	_	_	+	_	43	8.6	11.9	367.6	1.4	Follicular	No	N/A	Never Taken	SR Western
Prog 0005	_	_	+	+	43	5.9	2.8	504.5	30.3	Luteal	No	N/A	N/A	SR western
Prog 0006	_	_	+	+	37	4.0	4.3	318.4	9.1	Follicular	No	N/A	N/A	SR western
Prog 0007	_	_	+	_	41	6.2	3.5	929.2	1.6	Follicular	No	N/A	N/A	SR Western
Prog 0008	_	_	+	_	42	2.3	3.7	280.6	11.1	Follicular	No	N/A	N/A	SR western
Prog 0010	_	_	+	_	45	34.3	34.8	18.4	0.7	Perimenopausal	No	N/A	Unknown	Progestins + Glucocorticoids 48 hr
Prog 0011	_	_	+	_	49	7.9	6.0	1325	1.6	Ovulatory	No	N/A	Unknown	Progestins + Glucocorticoids 48 hr, SR western
Prog 0012	_	_	+	+	50	1.7	3.7	306.8	43.1	Luteal	No	N/A	Unknown	SR western
Prog 0013	_	_	+	+	45	1.5	4.3	237.6	8.6	Luteal	No	N/A	Unknown	SR western
Prog 0015	_	_	+	+	48	24.7	33.9	197.7	0.9	Perimenopausal	No	N/A	Unknown	Progestins + Glucocorticoids 48 hr
Prog 0021	_	_	+	_	47	8.8	14.0	194.1	0.8	Follicular	No	N/A	Unknown	SR western
Prog 0023	_	_	+	_	37	13.3	4.3	2110	2.0	Follicular	No	N/A	Unknown	SR western
Prog0024	_	_	+	+	45	1.8	1.9	1016	52.9	Luteal	No	N/A	Unknown	Progestins + Glucocorticoids 48 hr, SR western
Prog0025	_	_	+	+	47	12.6	17.5	152.7	0.5	Follicular	No	Depo	Discontinued 1 year prior to surgery	100 nM progestins + BaL infection; SR western

Patient Number	HIV	HPV	HSV-1	HSV-2	Age	LH (IU/l)	FSH (IU/l)	E2 (pmol/l)	P4 (nmol/l)	Stage of menstrual cycle	On Contraception	Type of Contraceptive	How long since contraception?	Type of experiments performed on tissue
Prog0026	_	_	+	_	35	7.4	5.7	197.4	0.7	Follicular	No	N/A	N/A	MPA dose response, 100 nM progestins fo endocervix; SR western
Prog 0027	_	_	+	_	36	2.4	2.8	341.7	7.3	Luteal	No	N/A	N/A	MPA dose response, 100 nM progestins fo endocervix, SR western
Prog 0028	_	_	+	_	45	5.4	4	1169	0.2	Follicular	No	N/A	N/A	MPA dose response, 100 nM progestins of endocervix, SR western
Prog 0032	_	_	+	_	45	0.1	0.1	18.4	0.4	Perimenopausal	No	N/A	N/A	100 nM progestins of endocervix
Prog 0034			+	_	41	4.1	2.6	343.1	18.8	Luteal	No	Sterilisation	N/A	SR western
Prog 0035	_	_	+	_	50	4.1	2.6	343.1	18.8	Luteal	No	Sterilisation	N/A	100 nM progestins + Infection with pNL4.3
Prog 0037	_	_	+	_	46	35.7	63.8	39.3	0.5	Perimenopausal	No	N/A	N/A	100 nM progestins + Infection with pNL4.3
Prog 0039	_	_	+	_	49	3.8	2.8	408.6	12	Luteal	No	Sterilisation	N/A	100 nM progestins + Infection with pNL4.3, SR western
Prog 0040	_	_	+	_	43	3.1	7.2	125.9	0.3	Follicular	No	N/A	N/A	100 nM progestins + Infection with pNL4.3
Prog 0041	_	_	+	_	52	78.3	70.2	36.8	0.2	Perimenopausal	No	N/A	N/A	100 nM progestins + Infection with pNL4.3
Prog 0042	_	_	+	+	44	10.4	6.1	230.4	1.6	Follicular	Yes	DepoProvera	Discontinued 1 month prior to suergery	100 nM progestins + Infection with pNL4.3 and Bal
Prog 0043	_	_	+	_	43	27.5	9.8	2501.0	0.9	Ovulatory	No	N/A	N/A	100 nM progestins + Infection with pNL4.3 and Bal, SR western
Prog 0044	_	_	+	_	48	6.3	6.1	537.1	0.3	Follicular	No	N/A	N/A	100 nM progestins + Infection with pNL4.3 and Bal
Prog 0045	_	_	+	+	50	4.9	9.1	113.6	1.4	Follicular	No	N/A	N/A	100 nM progestins + Infection with pNL4.3 and Bal
Prog 0047	_	_	+	_	47	6.2	3.7	517.9	6.1	Luteal	No	N/A	N/A	SR Western

Patient Number	HIV	HPV	HSV-1	HSV-2	Age	LH (IU/L)	FSH (IU/L)	E2 (pmol/L)	P4 (nmol/L)	Stage of menstrual cycle	On Contraception	Type of Contraceptive	How long since contraception?	Type of experiments performed on tissue
Prog 0049	_	_	+	_	44	3	5.9	84.2	0.5	Follicular	No	N/A	N/A	100 nM progestins + Infection with pNL4.3 and Bal
Prog 0050	_	_	_	_	38	5.6	6.3	476.3	0.9	Follicular	No	N/A	N/A	100 nM progestins + Infection with pNL4.3; SR western
Prog 0051	_	_	+	+	41	0.6	3.3	212.4	0.5	Luteal	No	N/A	N/A	100 nM progestins + Infection with pNL4.3
Prog 0052	_	_	+	_	40	8.9	8.8	141.8	0.9	Follicular	No	N/A	N/A	100 nM progestins + BaL infection
PROT 0001	_	_	+	+	43	0,6	1,9	370,5	32,1	Luteal	No	Sterilisation	N/A	Progestins + Glucocorticoids 48 hr
PROT 0003	_	_	+	+	47	5	8,7	83,7	9,1	Follicular	No	N/A	10 years (triphasal), 20 years ago (injectable)	100 nM progestins + BaL infection

Appendix D

Buffers and Solutions

D 1: Growth Media for *E. coli* 220

Luria Broth (LB): 220

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1 X TRIS buffered saline (TBS): 222

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D 1: Growth Media for *E. coli*

Luria Broth (LB):

1% (*w/v*) tryptone (Merck, South Africa)
0.5% (*w/v*) yeast extract (Merck, South Africa)
1% (*w/v*) NaCl (Merck, South Africa)

Containing 100 µg/ml ampicillin or 100 µg/ml ampicillin and 100 µg/ml carbenicillin for IMC propagation

For LB-agar plates add 1.5% agar (Merck, South Africa)

D 2: DNA gel electrophoresis

50 X TAE buffer for gel electrophoresis:

2.0 M TRIS (Sigma-Aldrich, South Africa)
0.05 M EDTA (Merck, South Africa)

pH 8.2 - 8.4 (at 25°C)

Use glacial acetic acid (Merck, South Africa) to pH buffer.
Autoclave.

Prepare 1 X TAE from stock with dH_2O.

1 X TAE gel with agarose:

For 100 ml gel:

Dissolve appropriate amount of agarose (SeaChem) into 100 ml 1 X TAE and bring to the boil

For plasmid DNA: 0.5% agarose
For PCR product visualisation: 1.5% agarose

Once cooled, add 1 µl of 10 mg/ml EtBr per 100 ml 1 X TAE - agarose

Mix well and pour into gel casting set.

D 3: RNA isolation

DEPC-treated H_2O:

1 ml diethyl pyrocarbonate (DEPC; Sigma-Aldrich)
in 1 L dH_2O (1:1000 dilution)
Incubate 2 h at 37°C, autoclave twice to inactivate DEPC

10X Morpholinopropanesulfonic acid (MOPS) buffer:

0.2 M	MOPS (Sigma-Aldrich)
0.05 M	sodium acetate (Merck)
0.01M	EDTA, pH 8.0 (Merck)

Adjust pH to 7.0 with 10 M NaOH

Denaturing formaldehyde gel mix (1% 100 ml):

Dissolve 1 g agarose (SeaChem) in 70 ml DEPC-treated H_2O and bring to boil.
Add 10 ml 10X MOPS buffer and 20 ml formaldehyde (Merck, South Africa) in fume hood.
Mix well and pour.

RNA sample loading buffer:

1.8 ml	DEPC H_2O
0.8 ml	Bromophenol blue solution (saturated) (Merck, South Africa)
1 ml	glycerol (Merck, South Africa)
1.5 ml	10x MOPS
2.6 ml	formaldehyde (Merck, South Africa)
7.3 ml	formamide (Merck, South Africa)

Add 2.5 µl 10 mg/ml ethidium bromide (Sigma-Aldrich, South Africa) per 1 ml RNA sample loading buffer just before use.

RNA electrophoresis buffer (500 ml):

50 ml	1x MOPS
14 ml	formaldehyde
436 ml	DEPC-treated water

D 4: Western blot analysis

1 X TAPS lysis buffer:

0.1 M	TAPS, pH 9.5 (1 M stock in water, pH to 9.5 at 25°C, filter sterilise)
1 mM	PMSF (0.1 M stock in isopropanol)
5 µg/ml	Leupeptin (5 mg/ml stock in H_2O)
2 µg/ml	Aproptinin (2 mg/ml stock in H_2O)
1 X	completeMini protease inhibitor tablet (1 tablet/ 10 ml buffer; Roche Applied Science, South Africa)

Vortex, until dissolved and store at 4°C.
To be made fresh for each isolation, with PMSF added last.

5X SDS sample buffer:

100 mM	TRIS-HCl, pH 6.8
5% (*w/v*)	SDS (Sigma-Aldrich, South Africa)
20% (*v/v*)	glycerol
2% (*v/v*)	β-mercaptoethanol (Merck, South Africa)
0.1% (*w/v*)	bromophenol blue

1X Running buffer:

25 mM	TRIS-HCl, pH 6.8
250 mM	glycine
0.1% (*w/v*)	SDS

Transfer buffer:

25 mM	TRIS-HCl, pH 6.8
200 mM	glycine (Merck, South Africa)
10% (*v/v*)	methanol (Merck, South Africa)

1 X TRIS buffered saline (TBS):

50 mM	TRIS-HCl, pH 6.8
150 mM	NaCl (Merck)

TBS-Tween (TBS-T):

50 mM	TRIS-HCl, pH 6.8
150 mM	NaCl
0.1% (*v/v*)	Tween-20 (Merck, South Africa)

Stripping buffer:

100 mM	β-mercaptoethanol
2% (*w/v*)	SDS
62.5 mM	TRIS-HCl, pH 6.7

Appendix E

Optimisation results used to determine the methodology employed in this study

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E.2: RNA isolation and infection optimisation experiments in primary cervical explants 227

E.1: Infection optimisation experiments in PBMCS

In chapter 3 it was necessary to establish optimal infection conditions in PBMCs. The first optimisation step was to determine whether the use of IL2 was necessary for the infection experiments. In Fig. E.1.1 it was evident that IL2 was needed to detect p24 levels in PBMCs over time. In samples infected without IL2, p24 values in the virus control (VC) and EtOH HIV samples were low and similar to the mock infected conditions (cells only: CO and EtOH). In contrast, PBMCs infected in the presence of IL2 had much higher detectable p24 levels. Additionally, p24 levels appeared to increase over time in the presence of IL2.

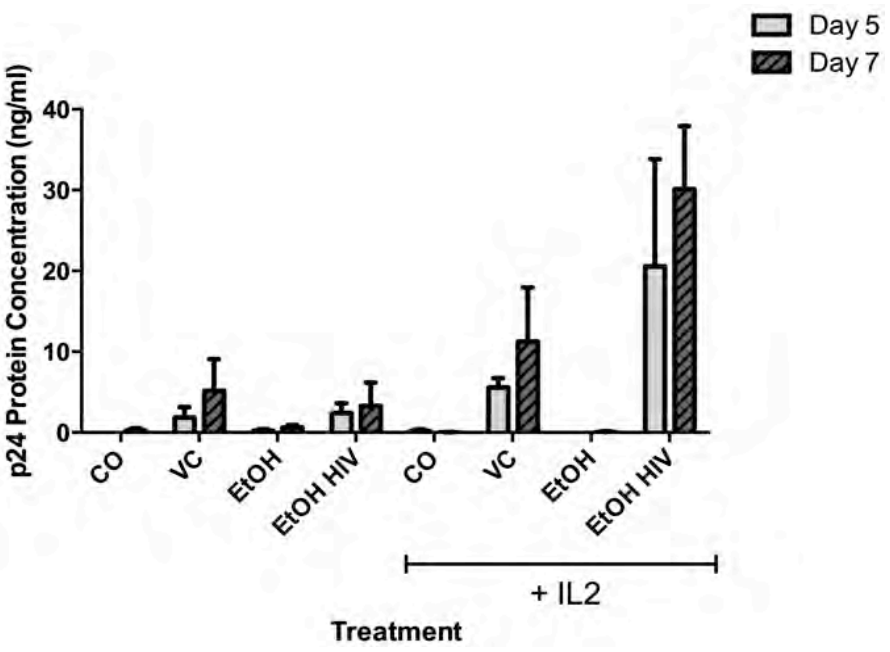


Figure E.1.1 Higher p24 expression levels were detected via p24 ELISA in PBMCs incubated with 30 U/ml IL2. PBMCs were activated with PHA for one day and subsequently cultured in the presence or absence of 30 U/ml IL2 for the duration of the experiment. PBMCs (± IL2, with +IL2 conditions indicated with a bracket in the Fig) were either left untreated [cells only (CO)], or incubated with the vehicle control (EtOH) for 48 hours. Thereafter, PBMCs were either infected with 10 IU/ml HIV-$1_{BaL\_Renilla}$ or mock infected with a media control for 2 hours. PBMCs that were untreated and subsequently infected with HIV-$1_{BaL\_Renilla}$ are shown as the virus control (VC). PBMCs stimulated under control conditions (EtOH) and subsequently infected with HIV-$1_{BaL\_Renilla}$ are denoted as "EtOH HIV". PBMCs infected with the mock control (media), remained labeled as either "CO" or "EtOH". After infection, PBMCs were washed 4 times with 1 X PBS and media with the respective ligands was added. Media was replaced every second day. PBMCs were harvested at days 5 and 7 days post infection and p24 expression levels were measured by p24 ELISA. Histograms show pooled results of three independent experiments, with three donors and plotted as mean ± SEM. CO: cells only; VC: virus control.

Given the prohibitively high cost of purchasing of highly sensitive p24 ELISAs; the use of HIV-$1_{BaL\text{-}Renilla}$ IMC which allows one to detect HIV-1 replication levels via luminescence, was investigated. In addition PBMC viability over time was investigated to determine the time point with minimal loss of viability but sufficient time for HIV-1 replication to be detected. In Figure E.1.2 A, it was evident that cell viability did not change over time, and that 0.01% EtOH had no effect compared to no treatment (CO), in the absence of HIV-1 infection. In addtion in samples infected with HIV-1, EtOH HIV samples were similar to the virus control samples (VC). Furthermore, PBMC viability was the similar in all conditions. Interestingly, when comparing the results for the p24 ELISA to those from the renilla assay, it was observed that the p24 assay results had higher background levels in the uninfected group compared to the uninfected renilla group (Fig. E.1.2 B and C). Furthermore, in the renilla assay, an increase in luminescence was observed over time (Fig. E.1.2 C) in the infected group, compared to the p24 ELISA assay (Fig.E.1.2 B). These differences could be due to the fact that the luminescence is measured from the cells, whereas the p24 ELISA measures secreted p24 levels in the supernatant, which may be more variable. Therefore for the infection experiments in PBMCs, luminescence was measured using the renilla assay at 7 days post infection.

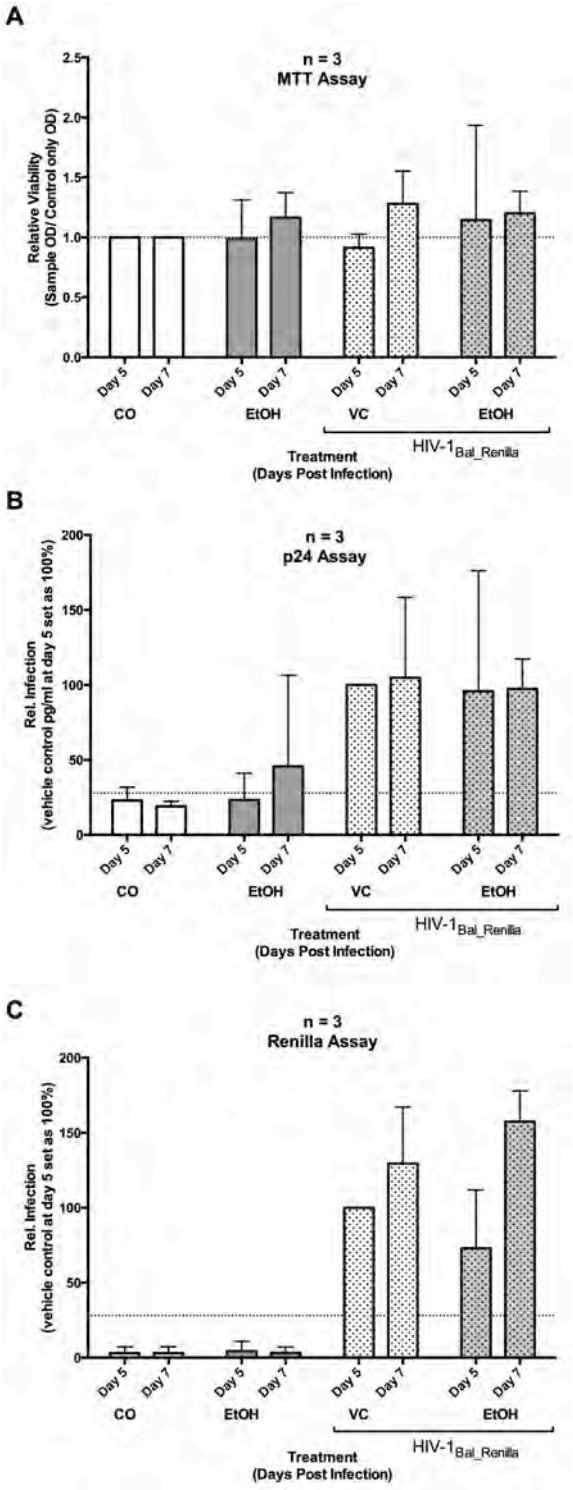


Figure E.1.2: Cell viability did not greatly change between days 5 and 7 post infection in PBMCs. Moreover, relative infection levels detected via luminscence were less variable than p24 levels measured by p24 ELISA in PBMCs. PHA activated PBMCs were either pre-treated with 0.01% (*v/v*) EtOH or left untreated (CO) for 48 hours. Pre-treated PBMCs were either infected with 10 IU/ml HIV-$1_{BaL\_Renilla}$ IMC (VC and EtOH) or a mock control for 2 hours (CO and EtOH), washed 4 times with 1 X PBS and media with the respective ligands was added. Media was replaced every second day. PBMCs were harvested at days 5 and 7 post infection and MTT viability, p24 expression levels and luciferase activity were measured. (A) MTT activity was detected with a multiplate reader (Thermo Scientific) using a MTT cell viability kit (Sigma-Aldrich, South Africa). Values were normalised to vehicle which was set to 1. (B) PBMCs were harvested at days 5 and 7 days post infection and p24 expression levels were measured by p24 ELISA or (C) Renilla luciferase was measured with the Promega Renilla-Glo™ kit using a luminometer (Modulus Microplate, Glomax, Promega). P24 values and renilla values from B and C were normalised against their corresponding MTT values, and normalised to the virus control (VC) at day 5 post infection set to 100%. Histograms (A-C) show pooled results of three independent experiments, with three donors and plotted as mean ± SEM. CO: cells only, VC: virus control.

E.2: RNA isolation and infection optimisation experiments in primary cervical explants

It was important to establish RNA isolation conditions in primary cervical explants for the work undertaken in chapter 4. Initially RNA isolation kits were used to optimise RNA isolation conditions from explant samples after 48 hours of culture. Several RNA isolation kits, namely the Qiagen AllPrep Mini Kit RNA/DNA/Protein, Qiagen RNeasy Nano Kit, The Bioline RNA Mini Kit and the Machery Nagel XS-RNA isolation kit. As a comparison, TriReagent® was used. OD readings (including 260/280 and 260/230 ratios) and RNA formaldehyde gels were used to assess the quantity and quality of the total RNA isolated by these different methods. Surprisingly, TriReagent® was the best RNA isolation method used as judged by both yield and integrity.

Interestingly, no kit produced sufficient RNA yields for subsequent downstream applications (see Table E.2). RNA yields as determined from the OD/ml measurements were low, and were insufficient for analysis on an RNA formaldehyde gel (less that 150 ng). Interestingly, TriReagent® produced the best yields of RNA (Table E.2) and quality. When assessing the quality of the TriReagent® isolated RNA from explant tissue after 48 hours of culture on an RNA formaldehyde gel, the 28S and 18S ribosomal bands were relatively sharp. Additionally there was minimal smearing on the gel (Fig. E.2.1 A), suggesting that the quality of the RNA was acceptable. Furthermore 260/280 ratios were between 1.8 – 2.0 and 260/230 ratios were between 1.4 – 1.8, suggesting that there was some protein, phenol and/or salt contaiminants using this isolation method.

Similarly protein isolation was optimised (Table E.2). The Qiagen AllPrep Kit produced the highest quantity of protein as assessed by western blotting. Additionally protein isolation using the Qiagen AllPrep kit was the most consistent out of all the other methods tested. This method was used in Fig 4.1 in chapter 4. Some of the protein isolation methods included the resuspension of the protein pellet in a sample application buffer, as such these samples could not be quantitated using the Bradford assay.

It is also known that one can isolate protein from the phenol fraction left in the TriReagent® RNA isolation method. This method was used and the results can be seen in chapter 4, Fig 4.5. However, this method was inconsistent in both quality and quantity of protein. Some samples had no protein precipitate. In addtion, some samples appeared degraded on the SDS-PAGE gel.

Table E.2: Quantity and yield of RNA and protein using different commercially available kits or reagents.

Kit	RNA Isolation	Protein Isolation
Qiagen AllPrep Kit	Poor (3 - 50 ng yield in 25 µl)	Best (200 - 300 µg yield)
Machery Nagel TriPrep Kit	Poor (0 – 10 ng yield in 20 µl)	Good
Machery Nagel XS RNA isolation	Poor (0 - 20 ng yield in 20 µl)	N/A
TriReagent® (Sigma-Aldrich)	Best (400 - 2000 ng yield in 10 µl)	Good
Bioline RNA Mini Kit	Worst (no RNA eluted)	N/A

When isolating RNA, using the TriReagent® method, from samples that had been incubated for 14 days, RNA quality was greatly reduced as assessed using RNA formaldehyde gels. As seen in Fig E.2.1, RNA from samples that had been incubated for 48 hours with different ligands produced good quality RNA (Fig. E.2.1 A), while RNA isolated from samples after 12 days incubation with ligands did not (Fig. E.2.1 B). RNA in this sample set appeared degraded, with 28S and 18S ribosomal bands indistinct. Additionally samples in Fig. E.2.1 B had high levels of smearing, indicating the low quality of the RNA isolated.

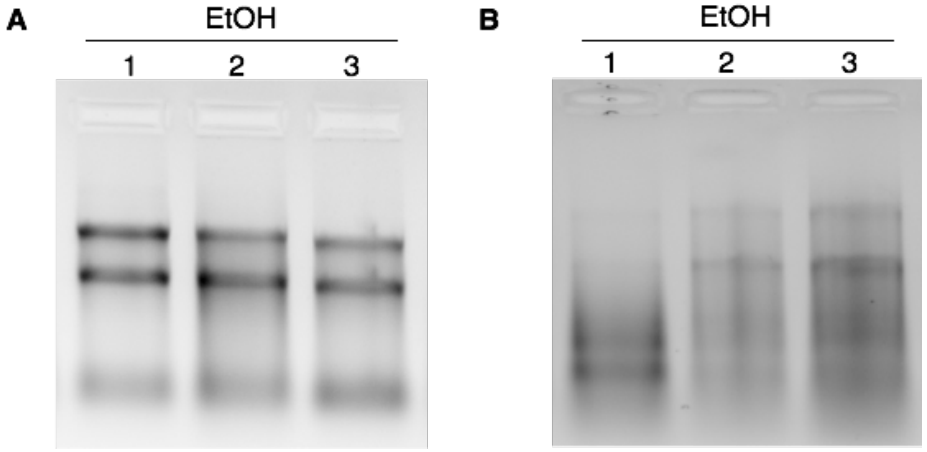


Figure E.2.1: RNA isolation from primary ectocervical explants using TriReagent® produces the best possible quality RNA from samples after 48 hours, while RNA quality is significantly reduced after 12 day incubation with ligands. (A) 250 ng RNA obtained from ectocervical explants after 48 hours incubation with 0.1% EtOH and (B) 250 ng RNA obtained from ectocervical explants after 12 days incubation with 0.1% EtOH was electrophoresed for 30 minutes at 65 V on a A 1% agarose RNA formaldehyde gel. Gels were visualised on a Agarose gels were visualised under 256 nM UV light in a Syngene, G:Box (Vacutec, England) and images acquired using GeneSnap from synGene, version 7.08 (SynGene, England). (A) Lanes 1,2 and 3 are individual explant samples from the same donor treated with EtOH for 2 days. (B) Lanes 1,2 and 3 are individual explant samples from a different donor treated with EtOH for 12 days.

One of the challenges experienced in this project was the optimisation of the infection experiments with primary cervical explants. In order to acquire the data for chapter 4, it was necessary to establish the conditions. The technique was learned at Imperial College, UK in 2011, and subsequently set up in South Africa. Due to the lower titre virus produced for the duration of this project, infecting each tissue explant individually was not feasible. As such, conditions were adapted from Arkeylan et al (2013) so that a triplicate treatment group was infected in one 24 well for 2 hours. Figure E.2.2 A and B shows the results using two donors, with three explant samples used per condition. In each experiment, triplicate groups were either mock infected (NV control), infected individually, or infected in a group of three in a 24 well plate (as described in chapter 2) with HIV-$1_{BaL\text{-}Renilla}$. Virus replication levels were assessed by measuring p24 HIV-1 protein levels in the supernatant. The results from these pilot experiements suggest that the group infection method results in higher levels of p24 levels produced (Fig. E.2.2 A and B). In addition, the data from one donor indicated that infecting the samples in a group appeared to reduce the error within a group (Fig. E.2.2 B). Furthermore, the infection levels were similar between the two methods, suggesting that infecting in a group did not change the trend in the infection levels over time as compared to infecting individually. Thus the group infection method was used for all infection experiments in chapter 4.

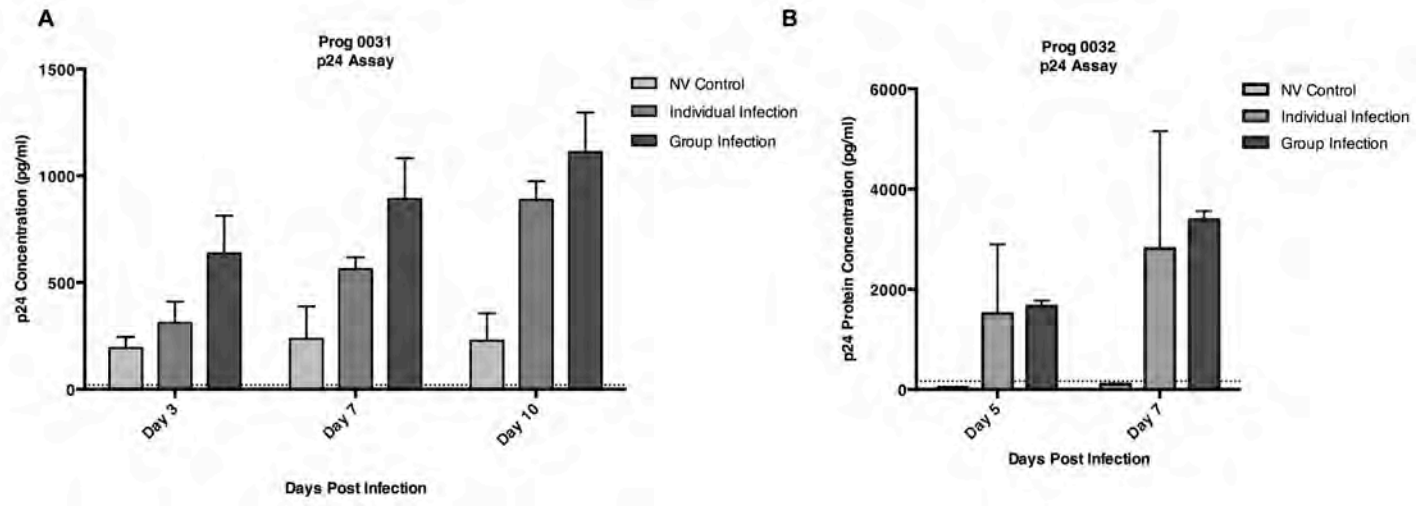
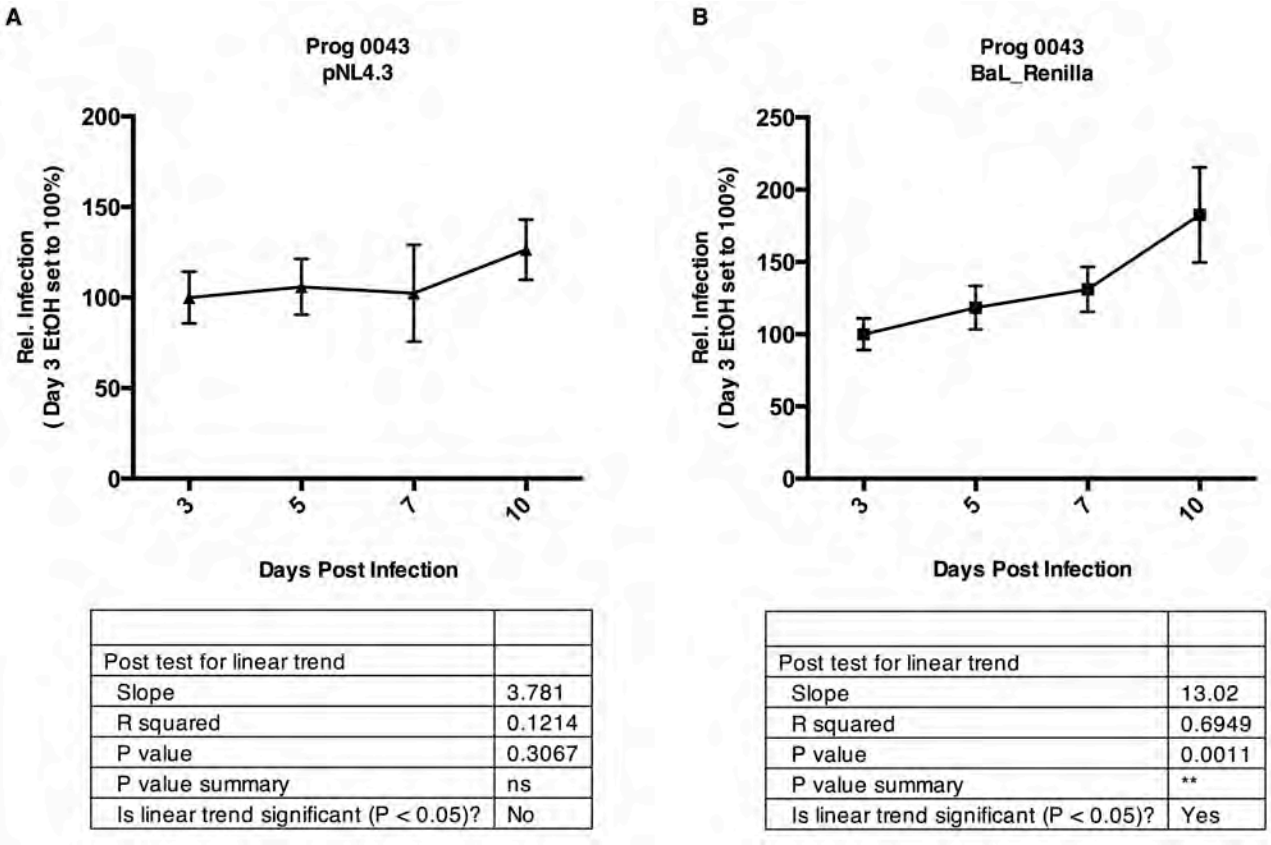


Figure E.2.2: Primary cervical explants were infected using different methods. (A) and (B) Ectocervical explants in triplicate, were either infected individually or placed in a well of a 24-well plate and infected with 500 IU/ml HIV-$1_{BaL\_Renilla}$ for 2 hours. Additionally a no virus control (NV) was included. Explants were cultured for a further 7 days and infection was measured by p24 ELISA (Innogenetics, Belgium). The histograms are representative of absolute p24 values (pg/ml). Histograms show pooled results of one independent experiments plotted as mean ± SD.

Since a virus inhibitor control was not included in every experiment, it was important to establish whether productive infection occured. As such, it was thought that a good indication of productive infection would be if the infection levels of the vehicle control increased significantly over time. Supernatants were harvested at days 3, 5, 7 and 10 post infection from the explant samples. Thereafter, p24 levels were measured using a p24 ELISA. In order to assess the effects of MPA and NET on HIV-1 replication, p24 expression levels were made relative to the vehicle control (EtOH), with the EtOH values set to 100% relative infection at day 3 post infection. The relative expression values of the vehicle control were then analysed using a one-way ANOVA with a linear post-test, to determine if HIV-1 replication under the control conditions increased over time. Fig. E.2.3 shows the results from one such experiment. In Fig. E.2.3 A, Prog 0043 was infected with HIV-$1_{pNL4.3}$ IMC and the relative infection levels were subsequently assessed using the post-test analysis. It was observed in this experiment that the infection levels did not significantly increase over time, with a p value = 0.3067. As such this experiment was excluded from all subsequent analyses. In Fig E.2.3 B, Prog 0043 was infected with HIV-$1_{BaL\_Renilla}$ IMC. In this sample, the linear trend post-test indicated that the values significantly increased over time (p = 0.0011), as such this experiment was included in all analyses.



Post test for linear trend	
Slope	3.781
R squared	0.1214
P value	0.3067
P value summary	ns
Is linear trend significant (P < 0.05)?	No

Post test for linear trend	
Slope	13.02
R squared	0.6949
P value	0.0011
P value summary	**
Is linear trend significant (P < 0.05)?	Yes

Figure E.2.3: Productive infection in primary cervical explants was determined using a one-way ANOVA with a post-test for a linear trend. Ectocervical explants were processed as described in Fig 4.8 and 4.9. The relative infection levels of the vehicle control were assessed for a linear trend as a correlator of productive infection. (A) The relative infection levels in the vehicle control of donor, Prog 0043, infected with HIV-$1_{pNL4.3}$ IMC did not increase over time. The table below decribes the slope, R squared value and p value of the curve. (B) The relative infection levels in the vehicle control of donor, Prog 0043, infected with HIV-$1_{BaL\_Renilla}$ IMC increases over time, and that this increase is significant. The table below the graph decribes the slope, R squared value and p value of the curve. The XY plots are representative of the mean +/- SD of triplicates from one donor. A one-way ANOVA with a post-test for a linear trend was performed in Prism 6 for Mac OS X (GraphPad Software, version 6).

One pilot experiment using the R5 HIV-1 entry inhibitor, Maraviroc (MVC), observed that HIV-1 replication decreased over time in the MVC treated tissue explant compared to the vehicle control (EtOH) (Fig. E.2.4). However pre-incubation time with 5 µg/ml MVC did not reduce initial virus infection levels compared to the control (Fig E.2.4, day 3). It could be that a longer pre-incubation time or a higher concentration of MVC are needed. The data from this pilot experiment suggests that the increase in HIV-$1_{BaL\_Renilla}$ replication in the vehicle control (EtOH) is productive (Fig. 2.4) compared to MVC in which HIV-1 levels decrease over time.

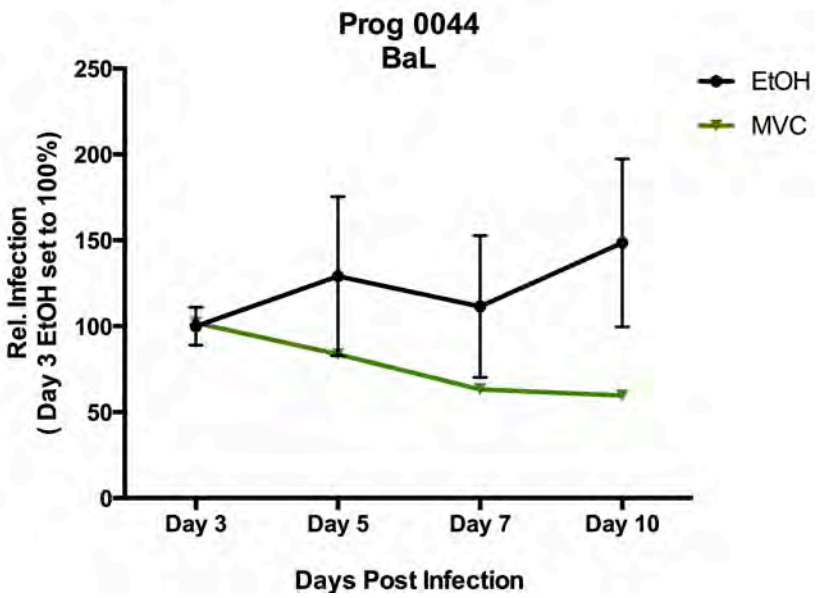


Figure E.2.4: Pilot study with MVC to determine productive infection in primary cervical explants. Ectocervical explants were processed as described in Fig 4.8. The XY plots are representative of the mean +/- SD of triplicates in the vehicle control (EtOH) or the mean of one sample in the MVC treated group from one donor.

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PLOS ONE

The Injectable-Only Contraceptive Medroxyprogesterone Acetate, Unlike Norethisterone Acetate and Progesterone, Regulates Inflammatory Genes in Endocervical Cells via the Glucocorticoid Receptor

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Abstract

Clinical studies suggest that the injectable contraceptive medroxyprogesterone acetate (MPA) increases susceptibility to infections such as HIV-1, unlike the injectable contraceptive norethisterone enanthate (NET-EN). We investigated the differential effects, molecular mechanism of action and steroid receptor involvement in gene expression by MPA as compared to NET and progesterone (P4) in the End1/E6E7 cell line model for the endocervical epithelium, a key point of entry for pathogens in the female genital mucosa. MPA, unlike NET-acetate (NET-A) and P4, increases mRNA expression of the anti-inflammatory GILZ and IκBα genes. Similarly, MPA unlike NET-A, decreases mRNA expression of the pro-inflammatory IL-6, IL-8 and RANTES genes, and IL-6 and IL-8 protein levels. The predominant steroid receptor expressed in the End1/E6E7 and primary endocervical epithelial cells is the glucocorticoid receptor (GR), and GR knockdown experiments show that the anti-inflammatory effects of MPA are mediated by the GR. Chromatin-immunoprecipitation results suggest that MPA, unlike NET-A and P4, represses pro-inflammatory cytokine gene expression in cervical epithelial cells via a mechanism involving recruitment of the GR to cytokine gene promoters, like the GR agonist dexamethasone. This is at least in part consistent with direct effects on transcription, without a requirement for new protein synthesis. Dose response analysis shows that MPA has a potency of ~24 nM for transactivation of the anti-inflammatory GILZ gene and ~4–20 nM for repression of the pro-inflammatory genes, suggesting that these effects are likely to be relevant at injectable contraceptive doses of MPA. These findings suggest that in the context of the genital mucosa, these GR-mediated glucocorticoid-like effects of MPA in cervical epithelial cells are likely to play a critical role in discriminating between the effects on inflammation caused by different progestins and P4 and hence susceptibility to genital infections, given the predominant expression of the GR in primary endocervical epithelial cells.

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Introduction

A central issue in women's health in developing countries is choice of contraceptive with minimal effects on susceptibility to infectious diseases, in particular to human immunodeficiency virus (HIV)-1 acquisition via the female reproductive tract (FRT). Epithelial cells lining the FRT are the first line of defence against pathogens and serve not only as a physical barrier but also express a wide variety of immune mediators aiding in both innate and adaptive immunity [1–3]. Interleukin (IL)-6, IL-8 and regulated-upon-activation-normal-T-cell-expressed-and-secreted (RANTES) are expressed in both primary and immortalised vaginal and cervical epithelial cells [4–6]. In particular, the simple columnar epithelial cells of the endocervix constitutively express IL-6, IL-8, and RANTES genes [5], with the endocervical cells being more active in cytokine secretion than the ectocervical cells [7,8]. Pathogens such as herpes simplex virus (HSV), human papillomavirus (HPV), and HIV have been shown to infect epithelial cells of the FRT and the process is affected by treatment with hormones such as progesterone (P4) [9,10].

Several reports suggest that endogenous steroid hormone levels and synthetic progestins used in contraception, influence susceptibility and disease predisposition to many genital tract infections (reviewed in [2,11]). Treatment of animals and humans with P4 or synthetic progestins has been reported to increase susceptibility to viral and bacterial infections [12–16]. Consistent with these findings, the progestin medroxyprogesterone acetate (MPA) is used as an immuno-compromising agent to induce viral infectivity in mice [17]. Furthermore, a prospective cohort study reported that injectable contraceptive users are more susceptible to both chlamydia and gonococcal infections than oral contraceptive users

[15]. MPA, administered for contraception as Depo-MPA (DMPA) or Depo-Provera, is a 150 mg three-monthly intramuscular injection used by millions of women worldwide, particularly in Sub-Saharan Africa with high HIV-1 incidence and prevalence [18,19]. Norethisterone enanthate (NET-EN) is a 200 mg two-monthly injectable with less widespread use than MPA, although its usage is high in some regions of South Africa [20]. In most studies the adjusted hazard ratio for HIV-1 acquisition by DMPA, or injectable contraceptives users where the majority of women are on MPA, is higher than that associated with no contraception or oral contraception [18,21–30]. While only a few studies have investigated the risks associated with the use of injectable NET-EN on HIV-1 acquisition, none have shown a significant association with HIV-1 acquisition [18,27,30]. In addition, increases in both HIV-1 and HSV shedding have been reported with MPA [31,32], as well as the presence of more viral variants and higher viral loads in DMPA users infected with HIV than non-users [33]. The mechanisms whereby endogenous P4 and synthetic progestins affect pathogen entry and transmission in the FRT are not well understood, but may involve modulation of the immune response both at the systemic level and at the genital mucosa. Understanding the relative effects of MPA vs NET on genital mucosal immune function is extremely important for choice of contraception, especially for developing countries where injectable contraception usage is high. For example, at the Kwazulu-Natal site in South Africa for the CAPRISA microbicide trial, about 80% of the women investigated were on injectable progestin-only (DMPA or NET-EN), as compared to 15% on oral contraceptives [34]. HIV prevalence among young women in the general population in southern Africa is highest (about 25%) in the 20–24 age group and the ratio of the prevalence of HIV infection among women relative to men shows that these women are approximately 3.3 times more likely to be infected with HIV than young men in this region [35].

Clinical research on the effects of contraceptives on HIV-1 acquisition, transmission and disease progression has been hampered by a lack of understanding of the molecular mechanisms of action of the progestin components of contraceptives and a lack of appreciation of the differences between progestins, which cannot be considered to act as a single class of compounds regarding their side-effect profiles [36–38]. Although MPA and NET elicit similar progestational effects to P4 [39,40], differences in biological effects mediated via steroid receptors other than the progesterone receptor (PR) could be expected [36–38], and have been demonstrated for the GR [41]. Synthetic progestins were designed to mimic the actions of the natural ligand P4 but with better bioavailability [38]. Both NET-EN and NET-A are metabolised to the active molecule NET, as well as other metabolites, unlike MPA, which is itself the active compound [42]. Progestins were also designed to be potent, high affinity PR agonists. However, many progestins bind to other members of the steroid receptor family, including the GR, the androgen receptor (AR) and mineralocorticoid receptor (MR) [43], thereby exhibiting off-target effects via these receptors [37,38]. It has been shown that MPA has a higher relative binding affinity compared to NET-A and P4 for the human GR (relative binding affinity % of 79.1, 0.88 and 5.57 for MPA, NET-A and P4, respectively) [41] and unlike NET-A and P4, acts as a potent partial to full GR agonist for both transactivation and transrepression [41,44]. The GR, a ligand activated steroid receptor, has potent anti-inflammatory and immunosuppressive activity [45]. Consistent with this idea, we have previously shown that MPA, at doses in the range found in serum of contraceptive users, represses expression of mRNA and protein levels of the pro-inflammatory cytokine IL-6 and the chemokine IL-8, in mouse fibroblast cells, most likely via the GR [46]. Similarly, Bamberger *et al.* showed that MPA represses IL-2, IL-1, and IL-6 protein expression in normal human lymphocytes, most likely via the GR [47]. Thus it is possible that MPA used as contraceptive modulates immune function and inflammation, and hence responses to pathogens, by changes in cytokine gene expression, particularly in the genital mucosa. A key question that remains to be investigated is what the effect is of different synthetic progestins as compared to P4 on cytokine gene expression and immune function in the FRT. These are likely to vary since we have previously shown that MPA, compared to NET-A and P4, elicit very different effects on IL-8 promoter expression in HEK293 cells, mediated via the GR [41], as well as exhibit differential effects in several steps of the GR pathway [44]. In support of an immunosuppressive role of MPA in increasing HIV-1 pathogenesis, MPA was recently shown to have immunosuppressive effects on key regulators of cellular and humoral immunity and increased HIV-1 replication in activated peripheral blood mononuclear cells (PBMCs) *ex vivo* [48,49]. The Hel laboratory also showed that women using DMPA displayed lower levels of IFNα in plasma and genital secretions compared to controls with no hormonal contraception, consistent with an immunosuppressive effect of DMPA *in vivo* [48,49]. A possible mechanism for differential effects of progestins and P4 on HIV-1 acquisition may include differential effects on inflammation in the FRT. However the direct effects of MPA, as compared to NET and P4, on expression of inflammatory markers in endocervical cells, the prime site for HIV-1 acquisition, have not been previously investigated. Using a human immortalised endocervical (End1/E6E7) epithelial cell line [50] as a model for the mucosal surface of the endocervix, as well as the HeLa cervical cell line, the present study aimed to determine the relative effects, molecular mechanisms and steroid receptor involvement of MPA, P4 and NET-A in expression of key inflammatory response genes.

Materials and Methods

Antibodies and Compounds

The following primary antibodies were obtained from Santa Cruz Biotechnology Inc., USA; GR(H-300): sc-8992, PR(C-20) (which detects PRA and B isoforms): sc-539, AR(441): sc-7305, GAPDH(0411): sc-47724, MR(MCR, H300): sc-11412, ERα(MC-20): sc-542. The flotillin-1 (610820) antibody was purchased from BD Transduction Laboratories (USA). The following secondary antibodies were obtained from Santa Cruz Biotechnology Inc., USA; anti-mouse: sc-2005, anti-goat: sc-2350 (used as IgG for the ChIP assay) and anti-rabbit: sc-2313. The ligands dexamethasone (DEX), MPA, P4, NET-A, NET, aldosterone (ALD) and mibolerone (MIB) were obtained from Sigma-Aldrich (South Africa). Human tumour necrosis factor α (TNFα) was obtained from Celtic Diagnostics (South Africa). Protease inhibitor cocktail tablets (EDTA-free) (cat #04693159001) were obtained from Roche (South Africa). Cycloheximide (CHX) was purchased from Sigma-Aldrich (South Africa).

Cell Culture

Human epithelial cervical cancer cells (HeLa) purchased from America Type Culture Collection (ATCC, USA) were cultured in 75 cm^2 flasks (Greiner Bio-one International, Austria) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 10% (v/v) foetal bovine serum (Highveld Biological, South Africa) 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco, Invitrogen, UK). End1/E6E7 (human endocervical cells immortalized with human papillomavirus E6/E7 [54] were obtained from Dr Fichorova, OB/GYN Depart-

ment, Brigham & Women's Hospital, Boston, USA. The End1/E6E7 cells were cultured in 75 cm^2 flasks (Greiner Bio-one International, Austria) in keratinocyte serum-free medium (kersfm; Sigma-Aldrich, South Africa) supplemented with keratinocyte growth supplement, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Invitrogen, UK). All cells were maintained at 37°C in a 5% CO_2 incubator. Cells were passaged with 0.25% trypsin/0.1% EDTA in PBS (Highveld Biological, South Africa). Trypsinization was terminated with neutralization medium [DMEM (Sigma-Aldrich, South Africa), 10% (v/v) calf serum (Highveld Biological, South Africa), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Invitrogen, UK)]. The cell lines were regularly tested for mycoplasma infection by means of Hoechst staining [55], and only mycoplasma-negative cells were used in experiments.

Plasmids

pcDNA3 (empty vector) plasmid was obtained from Invitrogen, while the pcDNA3-hGR (GR) plasmid was a gift from Prof. D.W. Ray (Centre for Molecular Medicine, School of Clinical and Laboratory Sciences, University of Manchester, UK [56]. pMT-PR-B (PR) was obtained from Prof. S. Okret (Karolinska Institute, Sweden) [51]. pRS-hMR (MR) expression plasmid was obtained from Prof. R.M. Evans (University of California, USA) [52]. pSV-hAR (AR) was a kind gift from Prof.F. Classens (Catholic University of Leuven, Belgium) [53]. pSG5-hER (ER) was obtained from Prof.F. Gannon (EMBL, Germany) [54].

RNA Isolation and Quantitative Real Time PCR (qRT-PCR)

Total RNA was isolated from cells using Tri-reagent (Sigma-Aldrich, South Africa) according to the manufacturer's instructions, and RNA (500 ng) was reverse transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, South Africa) according to the manufacturer's instructions. RT-PCR was performed using the Rotor-gene, RG-3000A (Corbett Research, South Africa) according to the manufacturer's instructions using the Sensi-Mix SYBR Green I system (Celtic Diagnostics, South Africa). The specific primer sets used were as follows; for GILZ (cat #QT00091035, Qiagen, South Africa), for IκBα, 5′-ACTCGTTCCTGCACTTGGCC-3′ (forward primer) and 5′-TGCTCACAGGCAAGGTGTAG-3′ (reverse primer), for IL-6, 5′-TCTCCACAAGCGCCTTCG-3′ (forward primer) and 5′-CTCAGGGCTGAGATGCCG-3′ (reverse primer), for IL-8, 5′-TGCCAAGGAGTGCTAAAG-3′ (forward primer) and 5′-CTCCACAACCCTCTGCAC-3′ (reverse primer), for RANTES 5′-TACCATGAAGGTCTCCGC-3′ (forward primer) and 5′-GACAAAGACGACTGCTGG-3′ (reverse primer), for GAPDH 5′-TGAACGGGAAGCTCACTGG-3′ (forward primer) and 5′-CCACCACCCTGTTGCTGTA-3′ (reverse primer). Relative transcript levels were calculated with the method described by Pfaffl*et al* 2001 and were normalized to relative GAPDH transcript levels [55].

Western Blotting

For the steroid receptor controls, COS-1 cells were seeded into 12-well plates (Greiner bio-one, Cellstar, Austria) at a density of 25×10^4 cells/well. The next day the cells were transfected with 1 μg/well of empty vector, GR, AR or PR and 2 μg/well of MR or ER using FuGENE 6 (Roche Diagnostics, South Africa). After 24 hrs, the cells were washed once with PBS and lysed with 50 μl 2X SDS sample buffer (5 X SDS sample buffer: 100 mM TRIS-HCL pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% β-mercaptoethanol and 0.1% (w/v) bromophenol-blue) and boiled for 10 min at 100°C. In addition, lysates were prepared from End1/E6E7 and HeLa cells seeded into 12-well plates at a density of 35×10^4 cells/well and 15×10^4 cells/well, respectively. Equivalent amounts of protein were loaded on either a 6% or 8% SDS-PAGE before being transferred onto a Hybond-ECL nitrocellulose membrane (Amersham, South Africa) using the Mini Protean III blotting system (Bio-Rad, South Africa). Blots were probed for anti-GR (1:4000), anti-PR (1:1000), anti-AR (1:1000), anti-ER (1:500), anti-MR (1:1000), anti-GAPDH (1:20 000) at 4°C overnight. Blots were washed 3 times with TBS containing 0.1% Tween for 5 mins each and subsequently incubated with horseradish peroxidise (HRP)-conjugated goat anti-rabbit (1:10000) or goat anti-mouse (1:5000) secondary at room temperature for 1 hr. Protein detection was performed using ECL substrate (Thermo Scientific, South Africa) with visualization on X-ray hyperfilm (Amersham, South Africa). Bands on the X-ray film were quantified using AlphaEaseFC software version 3.1.2 (Alpha Innotech Corporation).

GR Knockdown by Small Interference RNA (siRNA)

GR knockdown was performed as previously described [56], but briefly End1/E6E7 cells were seeded in 12-well plates at a density of 35×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transfected with 10 nM validated GR HS_NR3C1_5 (cat #SI02654757) (Qiagen, South Africa) siRNA directed against the human GR or validated non-silencing scrambled sequence control (NSC) siRNA (cat #1027310) (Qiagen, South Africa) using HiPerfect transfection reagent (Qiagen, South Africa) as per the manufacturer's instructions. Cells were incubated for 48 hrs before being treated for 24 hrs with 100 nM test compounds. RNA was then harvested and mRNA levels were analysed by qRT-PCR, as described above. To verify the protein knockdown, cells were transfected in parallel and analysed by Western blotting as described above.

Luminex

Supernatants were collected from the siRNA experiments prior to cell harvest. Thereafter, cytokine protein levels were measured using a luminex assay kit according to the manufacturer's protocol (Bio-rad, South Africa).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed as described in Verhoog *et al* 2011 with modifications [56]. HeLa cells were plated at 3×10^6 cells per dish in 15 cm dishes and grown for 24 hrs in full DMEM, before changing to phenol red-free DMEM (Sigma-Aldrich, South Africa) for an additional 24 hrs. Thereafter, the cells were incubated with serum-free, phenol-free DMEM for 2 hrs, before treatment with 100 nM DEX, MPA, P4 and NET-A for 1 hr. Cells were crosslinked for 10 min at 37°C with1% formaldehyde and the reaction was stopped with 0.1 mM glycine for 5 min, shaking at room temperature. Cells were scraped in PBS, pelleted by centrifugation and resuspended in 500 μl nuclear lysis buffer (1% SDS, 50 mMTRIS-HCL, pH 8.0, 10 mM EDTA, 1x protease inhibitor cocktail), before sonication. For immunoprecipitation, 100 μg DNA was pre-cleared with protein A/G agarose beads (sc-2003, Santa Cruz Biotechnology, USA) for 1 hr, rotating at 4°C, before incubating with 2 μg anti-GR (H300) (Santa Cruz Biotechnology, USA) or 2 μg anti-goat (Santa Cruz Biotechnology, USA), as IgG control, overnight on a rotator at 4°C. The following day, the complex was precipitated with protein A/G agarose beads for 6 hrs at 4°C, before being washed sequentially with 1 ml each of wash buffer I, II and III [57], followed by three washes with 1 ml TE buffer [10 mMTris pH 8, 0.1 mM EDTA]. Proteins were eluted from the beads by addition of 300 μl elution

buffer (1% SDS, 100 nM $NaHCO_3$), before the addition of 300 nM NaCl and incubation at 65°C overnight to reverse crosslinks. The following day 15 nM EDTA, 125 nM TRIS-HCL pH 6.5 and 20 µg proteinase K (Roche, South Africa) were added and samples were then incubated at 45°C for 1 hr. DNA was purified using PCR cleanup columns (Qiagen, South Africa). Real time qRT-PCR was performed on a Corbett Rotorgene, using the Sensimix (Quantace, South Africa), which measures SYBR Green fluorescence. ChIP primers used: for IL-6 5′-TCTACAA-CAGCCGCTCACAG-3′ (forward primer) and 5′- AGCGTTC-CAGTTAATTTGTATTTGT-3′ (reverse primer), for IL-8 5′-GGGCCATCAGTTGCAAAT-3′ (forward primer) and 5′-TTCCTTCCGGTG GTTTCTTC-3′ (reverse primer).

Primary Cervical Epithelial Cells

Primary cervical epithelial cells (VEN-100) were bought from Mat Tek Corporation (USA). Delivery time was 5 days. Upon arrival, the cells were incubated overnight in VEN-100-MM medium (Mat Tek Corporation, USA) at 37°C in a 5% CO2 incubator. The following day cell viability was determined using the Thiazolyl Blue Tetrazolium Bromide (MTT) cell determination kit (cat #CGD1, Sigma-Aldrich, South Africa) according to the manufacturer's instructions [58]. At this time, some cells were washed with PBS and either lysed with a N-[Tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid (TAPS) buffer (0.1 M TAPS, pH 9.5) on ice (to perform Western blotting as above) or with TRIzol® (for RNA isolation, cDNA synthesis and qRT-PCR as described above). Having established the viability of the cells, the majority of the VEN-100s were incubated in VEN-100-ASY-HCF hydrocortisone free assay medium (Mat Tek Corporation, USA) and 100 nM test compound for 48 hours, before performing an additional MTT assay.

Results

MPA, but not NET-A, Acts like a Full to Partial GR Agonist for Upregulation of anti-Inflammatory and Downregulation of Pro-inflammatory mRNAs

We investigated the effects of the synthetic progestins on the expression of GR regulated inflammatory genes in the End1/E6E7 endocervical epithelial cell line as well as the HeLa cervical epithelial cell line. These cell lines were chosen as the model systems for this study due to the ability to perform mechanistic studies using current methodology. Furthermore, the End1/E6E7 cell line displays similar morphological and immunocytochemical properties to those of primary endocervical epithelial cells [50]. The genes investigated were chosen based on their established mechanism of regulation via the GR, and their constitutive expression in endocervical epithelial cells [7]. GILZ and IκBα are anti-inflammatory genes that are upregulated by glucocorticoids (GCs) such as DEX, while IL-6, IL-8 and RANTES are pro-inflammatory genes that are downregulated by DEX [59,60]. The GILZ and IκBα genes contain multiple glucocorticoid response elements (GREs) and are commonly referred to as GR transactivation genes [61,62]. The IL-6 and IL-8 gene promoters have binding sites for transcription factors that include activator protein-1 (AP-1) and nuclear factor κB (NFκB) [63], and these genes are transrepressed by the liganded GR via tethering mechanisms [64]. Cells were treated with P4, MPA and NET-A, as well as the GR agonist DEX for 24 hrs. Thereafter, cytokine gene mRNA was measured by real time qRT-PCR. As expected the GR synthetic agonist DEX upregulated both GILZ and IκBα mRNA in both the cell lines (Figure 1). In addition, MPA upregulated GILZ and IκBα mRNA in both End1/E6E7 and HeLa cell lines (Figure 1). P4 and NET-A have no effect on the expression of GILZ or IκBα mRNA in either of the cell lines (Figure 1). Figure 2 A and B show that DEX and MPA, unlike NET-A and P4, repress both IL-6 and IL-8 mRNA levels, respectively, in the End1/E6E7 cell line. Interestingly, RANTES mRNA levels are repressed by DEX, MPA and P4 (Figure 2 C). The regulation of IL-6 mRNA levels by the ligands in the HeLa cells (Figure 2 D) is similar to the End1/E6E7 cells (Figure 2 A), where both DEX and MPA repress IL-6 mRNA levels. Furthermore, it appears that NET-A upregulates IL-6 mRNA levels in the HeLa cells (Figure 2 D). Similar to the End1/E6E7 cell line, DEX appears to repress IL-8 mRNA levels in the HeLa cells (Figure 2 E). However, unlike the End1/E6E7 cell line, at the 24 hr time point MPA does not appear to effect IL-8 mRNA expression in the HeLa cells (Figure 2 E). Interestingly though, at a 4 hr time point both DEX and MPA repress IL-8 gene expression in the HeLa cell line (Figure 2 H). It appears that NET-A and P4 upregulate IL8 mRNA levels in the HeLa cells (Figure 2 E). In contrast to the End1/E6E7 cells, the DEX, MPA and P4 repression of RANTES mRNA levels does not occur in the HeLa cells (Figure 2 F), although this could be due to low basal levels of RANTES in HeLa cells (as indicated by real time qRT-PCR Ct values, data not shown). Interestingly, it appears that NET-A upregulates RANTES mRNA levels (Figure 2 F). Taken together, these results show that MPA acts like the GR agonist DEX in upregulating GILZ and IκBα anti-inflammatory gene and generally downregulating IL-6, IL-8 and RANTES pro-inflammatory gene mRNA levels, unlike P4 and NET-A, with some exceptions. The results also suggest cell-specific, gene-specific and temporal differences in the regulation of some of the genes in response to the ligands, such as undetectable repression of IL-8 and RANTES mRNA by MPA in HeLa cells at 24 hrs, but similar repression of IL-8 in HeLa cells at 4 hrs compared to IL-8 in End1/E6E7 cells at 24 hrs. In addition, some experiments show repression by P4 of RANTES in the End1/E6E7 cells at 24 hrs, unlike in HeLa cells. Furthermore, it appears that regulation of mRNA levels by the ligands may be time dependent (Figure2). The experiments in Figure 1 and 2 were performed in the absence of induction of the cytokine/chemokine genes with a pro-inflammatory ligand, since these genes are constitutively expressed in cervical epithelial cells. Experiments performed in the presence of TNFα, to mimic infection, showed a similar % repression of the pro-inflammatory genes with DEX and MPA, unlike P4 and NET (Figure S1). All further experiments were performed in the absence of TNFα.

MPA Regulation of Inflammatory Gene mRNA Levels is dose- and Time-dependent

Having shown that MPA acts like a GR agonist in regulating mRNA levels of inflammatory genes, it was next determined if this regulation is dose- and/or time-dependent. End1/E6E7 cells were treated with increasing concentrations of the ligands for 4 hr and 24 hrs, respectively, followed by qRT-PCR analysis. Figure 3 A and B show that both DEX and MPA increase GILZ mRNA levels in a dose-dependent manner, whileP4 and NET-A appear to have no effect on GILZ gene expression at any of the concentrations or time points. It also appears that the maximal response for MPA and DEX regulation of GILZ mRNA levels does not change between 4 and 24 hours. DEX and MPA, unlike P4 and NET-A, repress IL-6 mRNA levels in a dose-dependent mannerat both 4 hrs and 24 hrs (Figure 3 C and D). However, MPA appears to show a greater maximal repression of IL-6 mRNA levels at 24 hrs than at 4 hrs, acting like a partial agonist at 4 hrs, but a full agonist at 24 hrs. Interestingly, it appears that

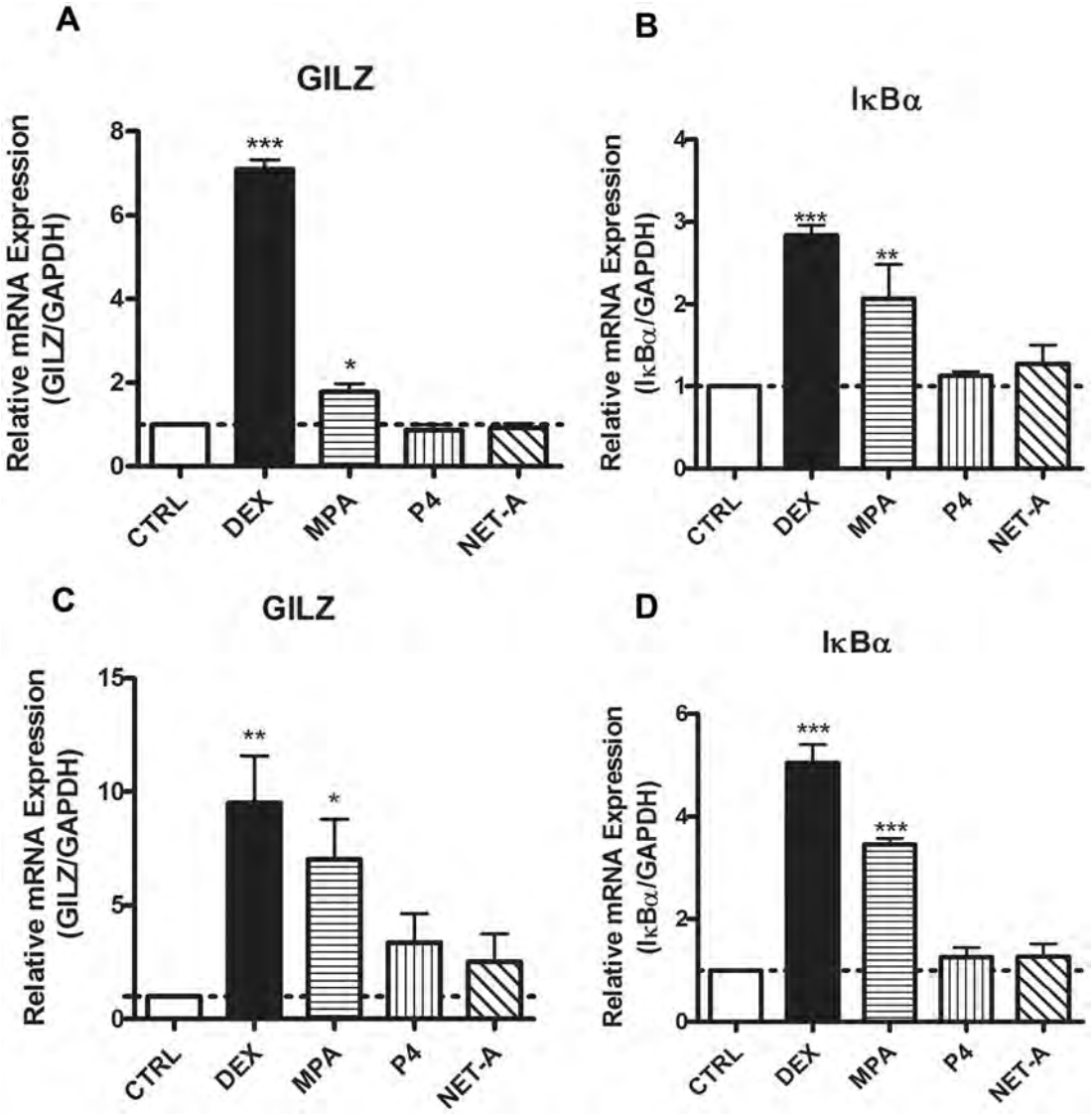

Figure 1. MPA, but not NET-A or P4, acts like a partial GR agonist for upregulation of anti-inflammatory mRNAs. (**A and B**) End1/E6E7 cells were treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). (**C and D**) HeLa cells were treated for 24 hrs with 100 nM DEX, 1 μM MPA, 10 μM P4, 10 μM NET-A or vehicle (ethanol) (CTRL). Thereafter the cells were harvested; total RNA was isolated and reverse-transcribed. Relative GILZ and IκBα gene expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalized to basal activity (CTRL) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean ±SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with Dunnett post-test. Statistical significance is denoted by *, ** or *** to indicate $P<0.05$, $P<0.001$ or $P<0.0001$, respectively.
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1 μM NET-A may upregulate IL-6 mRNA at 24 hrs. Figure 3 E and F show that IL-8 mRNA levels are also dose-dependently repressed by DEX and MPA at both 4 hrs and 24 hrs, with the MPA dose-dependent repression of IL-8 being more robust at 24 hrs. In addition, P4 and NET-A appear to be upregulating IL-8 at 4 hrs only. No repression of RANTES mRNA is apparent at 4 hrs (Figure 3 G). However, at 24 hrs RANTES mRNA levels are repressed by DEX and MPA in a dose-dependent manner, while NET-A and P4 appear to show some partial agonist activity (10–20%) for repression at high concentrations (Figure 3 H). MPA appears to have a potency (EC_{50}) of ~24 nM for transactivation of GILZ and a potency of ~21, 4 and 5 nM for repression of IL-6, IL-8 and RANTES mRNA, respectively at 24 hrs.

The GR is the Predominant Steroid Receptor Protein Detected in Cervical Cell Lines and Primary Cervical Epithelial Cells

Given the differential steroid receptor selectivity of MPA, NET and P4, we next investigated whether the GR, AR, PR, MR or ERα are expressed in these cell lines, with a view to determination of steroid receptor involvement in the differential gene expression responses. Cell lysates were prepared and the steroid receptor mRNA and protein levels were detected by qRT- PCR and Western blotting, respectively. The Western blot and PCR screen show that the End1/E6E7 cells express only endogenous GR mRNA and protein, respectively (Figure 4 A and B). According to the PCR screen (Figure 4 A), HeLa cells express endogenous GR, AR and MR mRNA. However the Western blot (Figure 4 B)

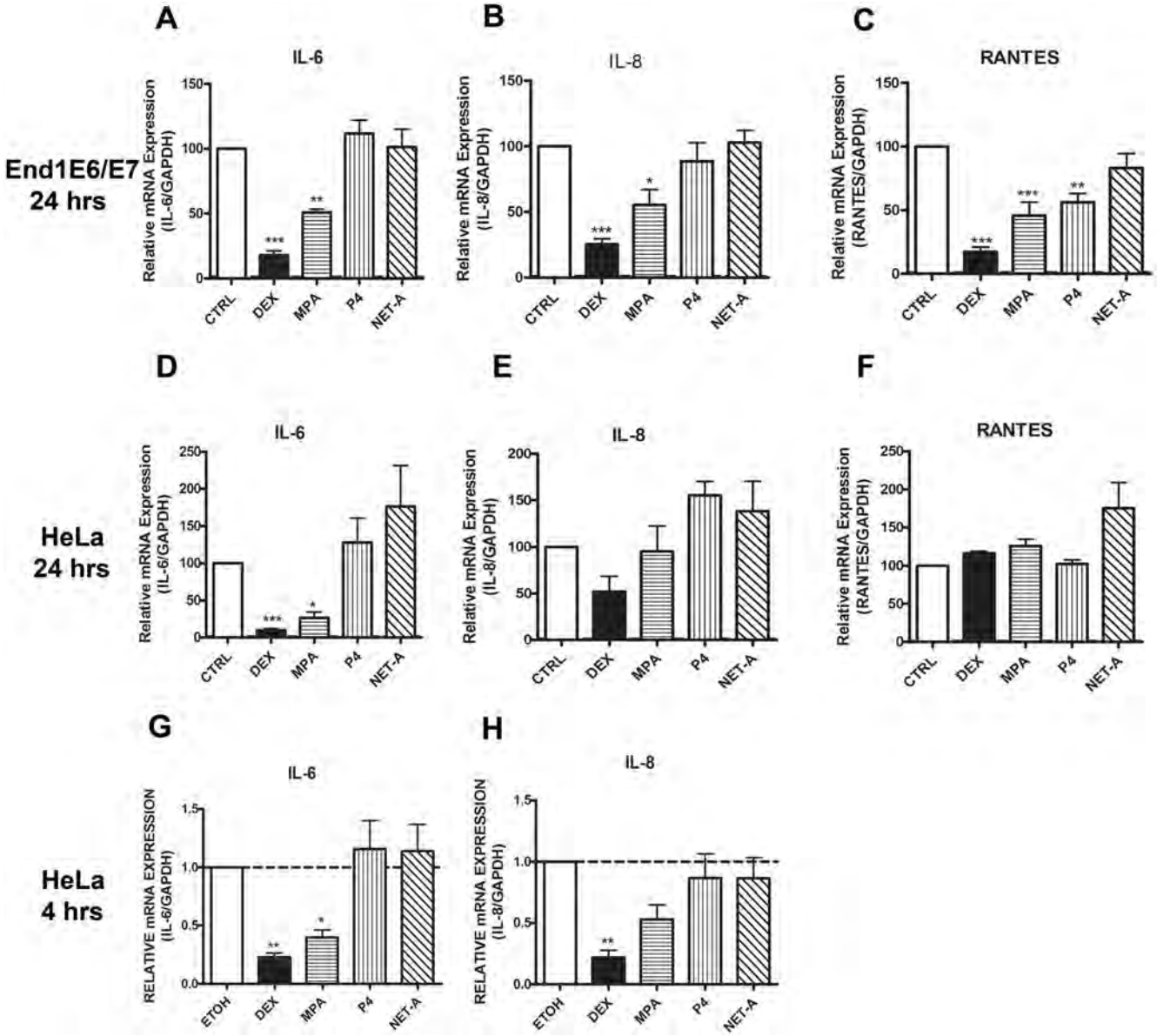

Figure 2. MPA, but not NET-A, acts like a full/partial GR agonist for repression of pro-inflammatory mRNAs. (A–C) End1/E6E7 cells were treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). (**D–H**) HeLa cells were treated for 24 hrs (**D–F**) or 4 hrs (**G–H**) with 100 nM DEX, 1 μM MPA, 10 μM P4, 10 μM NET-A or vehicle (ethanol) (CTRL). Thereafter the cells were harvested, total RNA was isolated and reverse-transcribed. Relative IL-6, IL-8 and RANTES gene expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalized to basal activity (CTRL) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean ± SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with Dunnett post-test. Statistical significance is denoted by *, ** or *** to indicate $P<0.05$, $P<0.001$ or $P<0.0001$, respectively.
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reveals that in HeLa cells only endogenous GR protein is detectable. Although it appears that the HeLa and End1/E6E7 cells express MR protein (Figure 4 B), this is a non-specific band that also appears in the negative control. Since the End1/E6E7 cells do not express detectable MR mRNA it is highly unlikely that the cells express MR protein. However, it is possible that the HeLa cells do express low levels of the AR and MR that are beyond the detection level of the Western blots. It was therefore determined if the repression of cytokine genes in this cell line could be mediated via the AR and MR. HeLa cells were treated with the GR, AR and MR specific agonists (DEX, mibolerone and aldosterone, respectively) and cytokine gene expression was measured by qRT-PCR. IL-6 and IL-8 gene expression was measured since it was established above that RANTES is not regulated by the ligands of interest in the HeLa cells. Figure 4 C shows that only DEX represses IL-6 gene expression, while it appears that aldosterone, and possibly and mibolerone, upregulate IL-6 mRNA expression. In addition, it appears that DEX represses IL-8, while aldosterone, and possibly mibolerone, upregulate IL-8 gene expression (Figure 4 D). These results indicate that DEX- and MPA-mediated repression of the cytokine genes in HeLa cells is likely to occur via the GR. Additionally, since only GR mRNA and protein were

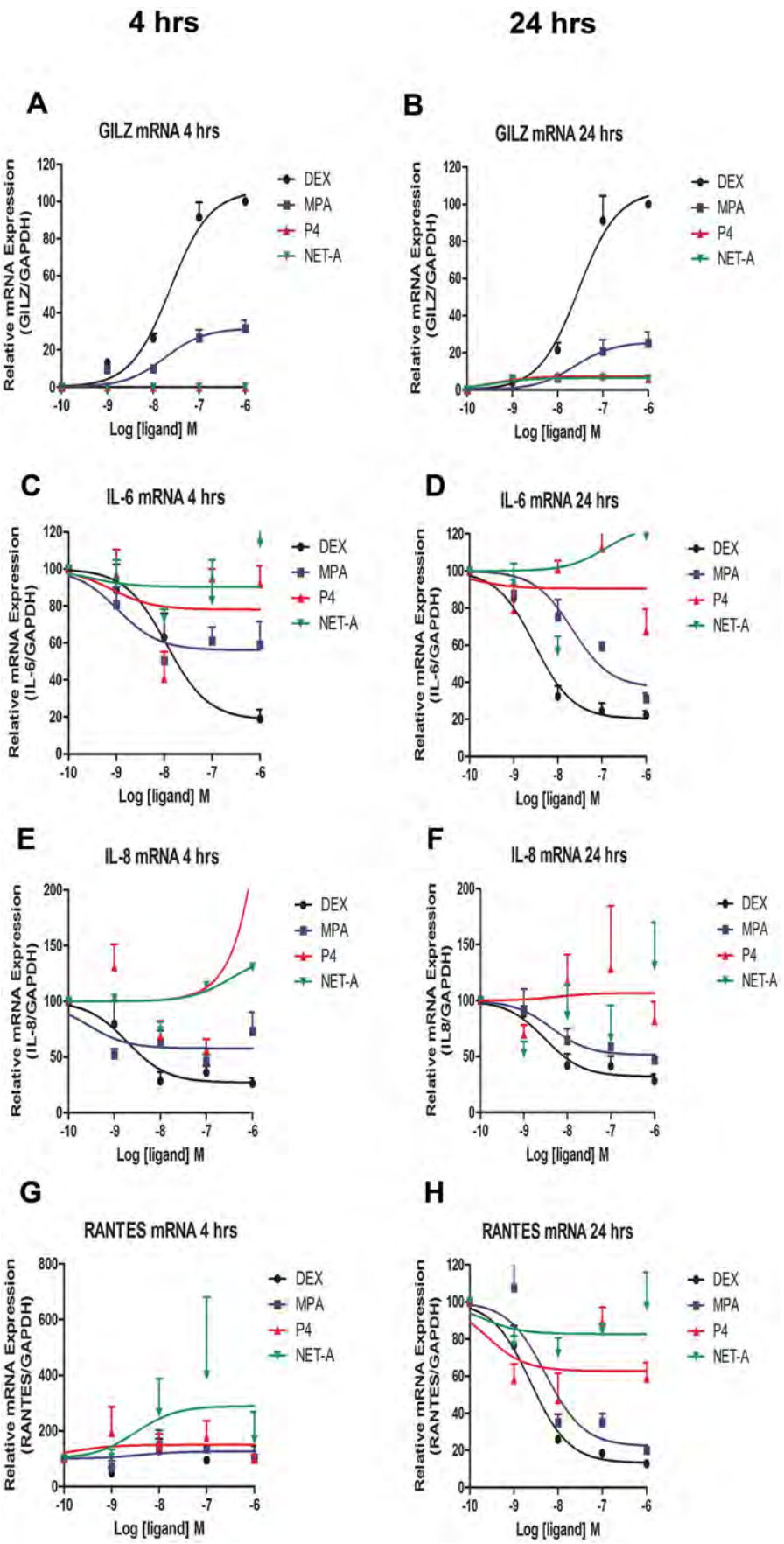
4 hrs
24 hrs
A
GILZ mRNA 4 hrs
B
GILZ mRNA 24 hrs
C
IL-6 mRNA 4 hrs
D
IL-6 mRNA 24 hrs
E
IL-8 mRNA 4 hrs
F
IL-8 mRNA 24 hrs
G
RANTES mRNA 4 hrs
H
RANTES mRNA 24 hrs
Relative mRNA Expression (GILZ/GAPDH)
Relative mRNA Expression (IL-6/GAPDH)
Relative mRNA Expression (IL-8/GAPDH)
Relative mRNA Expression (IL8/GAPDH)
Relative mRNA Expression (RANTES/GAPDH)
Log [ligand] M
DEX
MPA
P4
NET-A

Figure 3. MPA-mediated regulation of inflammatory gene mRNA levels is dose- and time-dependent. End1/E6E7 cells were treated with increasing amounts (1 nM, 10 nM, 100 nM and 1 μM) of DEX, MPA, P4 or NET-A, or vehicle (ethanol) (CTRL) for 4 and 24 hrs, respectively. Thereafter, the cells were harvested, total RNA was isolated and reverse-transcribed. Relative (**A, B**) GILZ, (**C, D**) IL-6, (**E, F**) IL-8 and (**G, H**) RANTES gene expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative GILZ gene expression was normalized to 1 μM DEX set to 100% in order to obtain % partial agonist activity. Relative IL-6, IL-8 and RANTES expression was normalized to basal activity (CTRL) set to 100 in order to obtain % repression. For IL-6 and RANTES mRNA, statistically significant repression with MPA relative to control was found at 10 nM, 100 nM and 1 μM. The 1 μM data point for P4 on IL-8 4 hrs (**E**) is 231% and is not displayed due to the y-axis scale. Graphs represent pooled results of at least three independent experiments and are plotted as mean ± SEM.
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detected in the End1/E6E7 cells, these results indicate that in both the cell lines, the DEX and MPA regulation of expression of the inflammatory genes is most likely mediated via the GR.

Whether the steroid receptor expression profile in the cervical epithelial cell lines mimics that of primary cervical epithelial cells is unknown. We investigated the steroid receptor content in commercially available primary endocervical cells (VEN-100; bio-engineered multilayer of primary cells) by PCR and Western blot. While we could detect GR, MR, AR and ERα mRNA (Figure5 A), the only steroid receptor protein we detected in the primary cells was the GR (Figure 5 B). No PR mRNA or protein was detected in the cell lines or primary cells, despite positive controls showing that PR expression can be detected by these methods. Thus despite the finding that some MR, AR or ERα mRNA was detected in some of the cells lines or primary cells, the only steroid receptor protein detected in any of the models was the GR, suggesting that the GR is the predominant steroid receptor mediating responses to MPA in both the cervical epithelial cell lines and primary endocervical cells.

Regulation of Inflammatory Gene mRNA Levels by DEX and MPA is Mediated by the GR and is Mimicked at the Protein Level for IL-6 and IL-8

In order to provide direct proof that the GR is involved in the regulation of the inflammatory genes in response to the synthetic progestin MPA, GR knockdown experiments were performed in the End1/E6E7 cell line. Reduction of GR protein in these cells was verified by Western blotting (Figure 6 A and 6B). As expected DEX and MPA upregulated GILZ mRNA, while P4, NET-A and NET did not (Figure 6 C). Notably, NET was included in this experiment as a control to exclude the possibility that the acetate form (NET-A) would regulate the genes differently. However, it is shown that NET-A acts similarly to NET. Both the DEX- and MPA-induced upregulation of GILZ mRNA is diminished when GR is knocked down. Figure 6 D shows that DEX upregulates IκBα mRNA levels and this induction is repressed when GR is knocked down. Here the MPA induction of IκBα is not significant, possibly due to the blunting of the response in the NSC knockdown conditions, and therefore a loss of induction is not apparent with the knockdown. Western blotting revealed that, unlike for the mRNA levels, DEX, MPA and NET-A all significantly increased total IκBα protein levels (Figure S2). Protein levels could not be determined for GILZ due to the unavailability of a suitable antibody. As expected DEX and MPA repress IL-6 mRNA levels (Figure 7 A), which is lifted when the GR is knocked down. In addition, DEX-mediated repression is also evident on IL-6 protein levels (Figure 7 B). Consistent with the mRNA data, MPA appears to repressIL-6 protein levels and the repression is lifted in the knockdown. Interestingly P4 also appears to repress IL-6 protein levels, although significance could not be established. Similarly, a significant difference is observed for both DEX and MPA responses upon GR knockdown for IL-8 mRNA levels (Figure 7 C). Figure 7 D shows that DEX and MPA also appear to repress IL-8 protein levels, while GR knockdown appears to lift this repression. RANTES mRNA levels are shown in Figure 7 E to be repressed by both DEX and MPA, but not by P4 or NET-A, in a GR-dependent manner. We were unable to detect secreted RANTES protein, possibly due to its instability in the medium (data not shown). Gene expression studies could not be performed with the primary cells since they did not maintain cell viability for the long periods of time required for the assessment (Figure S3). Taken together, these results show that DEX- and MPA-mediated regulation of the inflammatory gene mRNA levels is mediated via the GR in the endocervical cell line. This GR dependence is mimicked at the protein level for DEX and appears to also be mimicked at the protein level for MPA, for IL-6 and IL-8.

We have previously shown in COS-1 cells that the most potent GR ligands result in the most rapid GR degradation, with a good correlation shown between ligand-selective GR half-life and transactivation and transrepression efficacy [65]. Consistent with these results, we show that in the End1/E6E7 cells, MPA results in GR turnover typical for a relatively potent GR partial agonist, unlike NET-A and P4 (Figure S4).

Inhibition of Protein Synthesis Supports a Mechanism of Direct Regulation by the GR of the Inflammatory Genes

In order to investigate whether the GR is directly or indirectly involved in the regulation of these genes, cycloheximide (CHX; *de novo* protein synthesis inhibitor) experiments were performed in the End1/E6E7 cells to determine whether the GR-mediated regulation of the mRNA levels requires new protein synthesis [66]. Figure 8 A shows that the addition of CHX only partially dampens the DEX while ablating the MPA induction of GILZ mRNA. However, the effects of all the ligands on IκBα mRNA levels were unchanged by CHX (Figure 8 B). These results suggest that upregulation of GILZ mRNA levels is only partially dependent on transactivation by the GR and it is also in part dependent on synthesis of another protein. IκBα mRNA upregulation, however, appears independent of new protein synthesis, suggesting that the mechanism predominantly involves direct transactivation by the GR of the IκBα gene. Figure 8 C shows that DEX, but not MPA-mediated repression of RANTES is partially lifted by treatment with CHX, suggesting a mechanism at least partially involving transrepression of these promoters by the GR. In contrast, both DEX- and MPA-mediated repression of IL-6 are independent of new protein synthesis, as they are not affected by CHX treatment (Figure 8 D). A similar trend is observed for DEX on the IL8 promoter (Figure 8 E), although for this gene the results for MPA were inconclusive. To confirm that the CHX inhibited *de novo* protein synthesis, End1/E6E7 cells were pre-treated with CHX and then treated with DEX (in the presence of CHX) for 24 hrs, thereafter cell lysates were prepared and Western blotting was performed. lκBα protein levels were used as a positive control to show that the concentration of CHX used was sufficient to prevent new protein synthesis (Figure 8 F and G). In summary, we demonstrate that under conditions where CHX is shown to inhibit new protein synthesis, all the anti-inflammatory and pro-inflam-

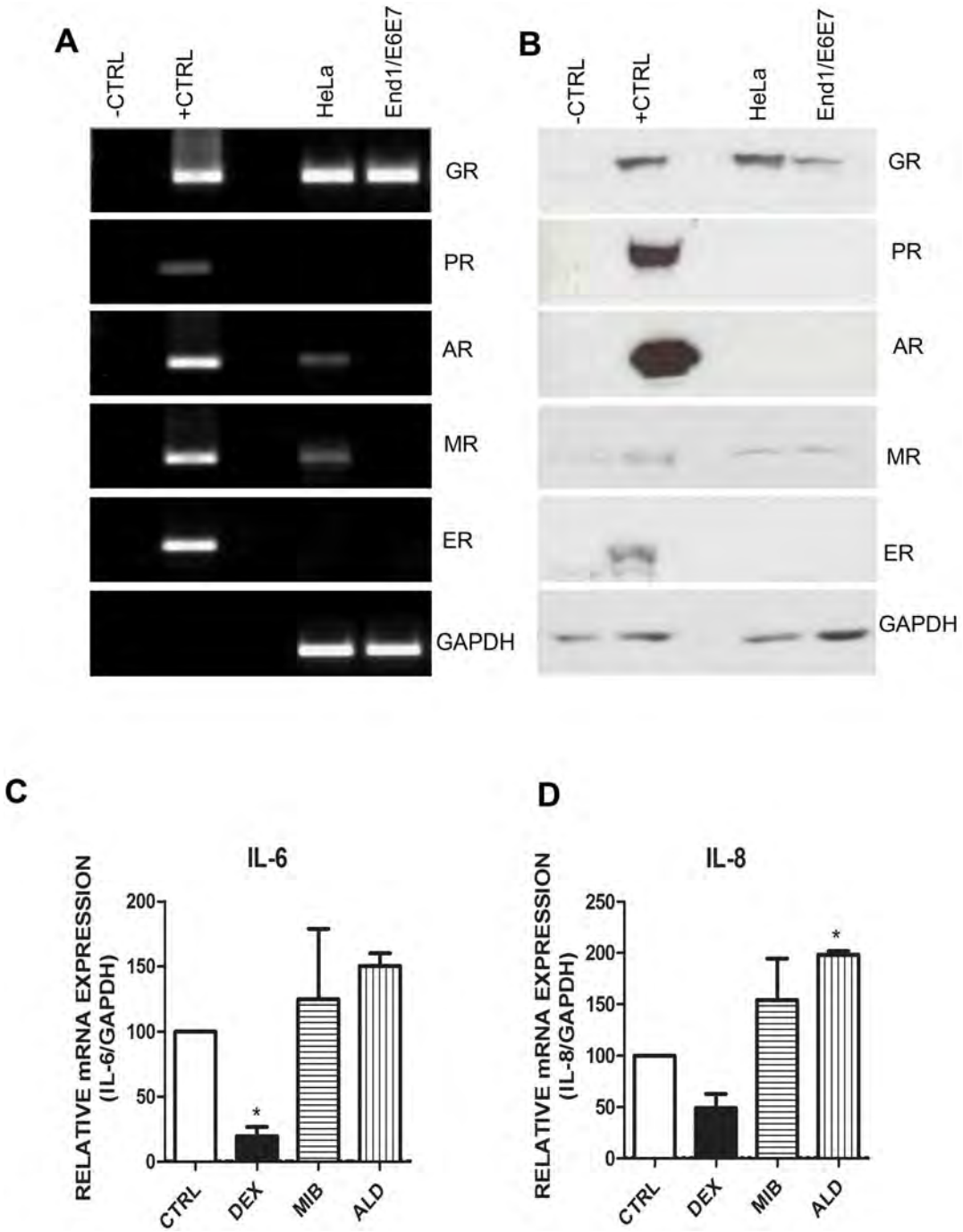

Figure 4. End1/E6E7 and HeLa cells only express detectable GR protein. (**A and B**) (**A**) HeLa and End1/E6E7 cells were harvested, total RNA was isolated and reverse-transcribed. Steroid receptor (SR) gene expression was measured by real time qRT-PCR. SR expression vectors (pcDNA3-hGR, pMT-PR-B, pSV-hAR, pRS-hMR and pSG5-hER) served as positive controls (+CTRL) for the GR, PR-B, MR and ER, respectively. COS-1 cells transiently transfected with pcDNA3 (empty vector) served as negative control (−CTRL). (**B**) Whole cell lysates were prepared from the HeLa and End1/E6E7 cell lines. Equal volumes of lysate were analysed by Western blotting with antibodies against specific SRs and GAPDH as loading control. **(C and D) SR agonist screen indicates that in the cervical cells the GR, but not the MR or AR repress IL-6 and IL-8 in the presence of receptor-specific agonist.** HeLa cells were treated with 100 nM DEX, 100 nM mibolerone (MIB), 10 nM aldosterone (ALD) or vehicle (ethanol) (CTRL) for 4 hrs. Total RNA was isolated and reverse-transcribed. Relative (**C**) IL-6 and (**D**) IL-8 gene expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (CTRL) in order to obtain fold expression. The primers and antibody used to investigate PR levels are capable of detecting both PR-A and PR-B isoforms, however the positive protein control shown is specific for PR-B isoform only. Graphs represent pooled results of at least three independent experiments and are plotted as mean ± SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with Dunnett post-test. Statistical significance is denoted by * to indicate $P<0.001$.

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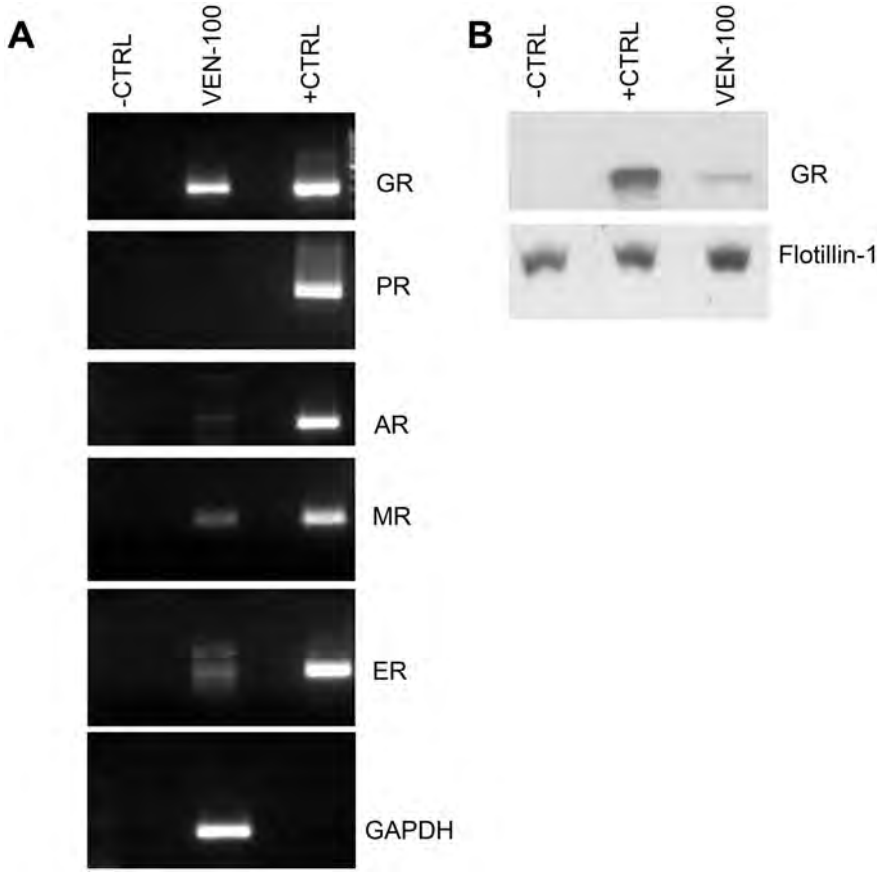

Figure 5. Only GR protein is detected in primary cervical epithelial cells (VEN-100). (**A**) Upon arrival the VEN-100 cells were rested overnight before being washed once with PBS and harvested with TRIzol®. Total RNA was isolated and 500 ng RNA was reverse-transcribed. Steroid receptor gene expression was measured by qRT-PCR with receptor-specific primers, followed by gel electrophoresis to confirm the PCR products. (**B**) VEN-100 cells were rested overnight before harvesting in 2X SDS sample buffer. COS-1 cells were transiently transfected with 1 μg/well pcDNA3 (empty vector) which served as negative control (−CTRL) or with 1 μg/well steroid receptor expression vectors (GR, PR-B, AR, MR and ERα) which served as positive controls (+CTRL). Twenty fourhrs later, the COS-1 cells were washed once and lysed with 2X SDS sample buffer. Equal volumes of cell lysate (VEN-100 and COS-1 ctrls) were analysed by Western blotting with antibodies specific for the GR and Flotillin-1 (loading control), respectively.
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matory genes investigated are at least in part regulated by direct effects of DEX without a requirement for new protein synthesis, and where this could be established, similar trends are observed for MPA.

DEX and MPA Result in Recruitment of the GR to the Promoters of the IL-6 and IL-8 Genes

In order to further investigate the mechanism of transcriptional regulation of these cytokine genes via the GR, ChIP assays were performed in HeLa cells. Attempts to perform ChIP assays in the End1/E6E7 cells were unsuccessful. This may be due to high background and low sensitivity for ChIP signals in these cells. Figure 9 A shows that stimulation with DEX, but not MPA results in the recruitment of the GR to the GILZ promoter. Furthermore, both DEX and MPA stimulation resulted in significant recruitment of the GR to the IL-6 and IL-8 promoters (Figure 9 B and C). The inability to observe GR recruitment to the GILZ promoter with MPA may be because some of the effects of MPA on GILZ are not direct, as suggested by the CHX experiments. However, since we have previously shown in A549 cells that the GR is recruited to the GRE region of the GILZ promoter by both DEX and MPA [67], it is more likely that a small amount of GR is recruited by MPA, but this is below the limits of detection of the ChIP assay in these cells. In summary these results strongly support a model whereby both DEX and MPA suppress inflammation in the cervical epithelial cells by activating and thereby recruiting the GR to promoters of these genes and consequently inducing transcription of the anti-inflammatory gene GILZ, while repressing transcription of the pro-inflammatory genes IL-6 and IL-8.

Discussion

We show for the first time that the synthetic progestins MPA and NET-A, used in contraception and hormone replacement therapy, exert differential effects on expression of mRNA levels of key pro-inflammatory and anti-inflammatory genes constitutively expressed in an endocervical epithelial cell line, as compared to P4. MPA, unlike NET-A and P4, increases mRNA expression of the anti-inflammatory genes GILZ and IκBα, in both the cervical epithelial cells lines. Interestingly, this differential regulation of IκBα mRNA is not mimicked by IκBα protein levels, suggesting that GR-mediated increase in IκBα protein levels does not play a major role in regulation of IL-6, IL-8 and RANTES genes in these cells, consistent with reports for some cells but not others [68–72].

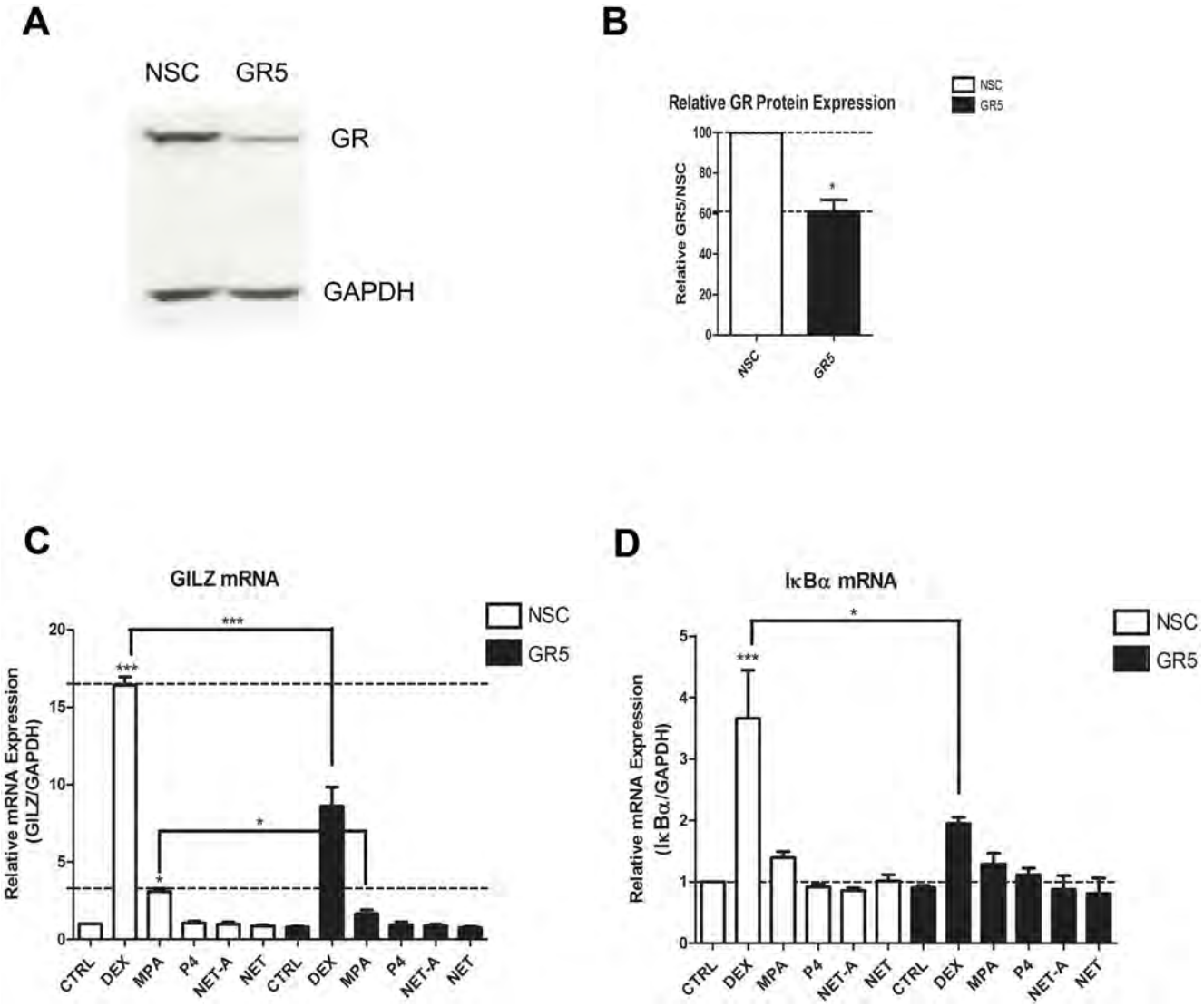

Figure 6. MPA- and DEX-mediated upregulation of anti-inflammatory mRNAs is mediated via the GR. End1/E6E7 cells were transfected with 10 nM GR or NSC siRNA (**A–D**) and then treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A, NET or vehicle (ethanol) (CTRL). For verification of GR knockdown a representative blot is shown in (**A**). (**B**) Western blots of at least three independent experiments were quantified to determine the relative GR protein expression and is plotted as mean ± SEM. Total RNA was isolated and reverse-transcribed. Relative (**C**) GILZ and (**D**) IκBα gene expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (CTRL) in order to obtain relative fold expression. Graphs in (**C**) and (**D**) represent pooled results of at least three independent experiments and are plotted as mean ± SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with either a Dunnett post-test, followed by a student's t-test to compare specific conditions to each other. Statistical significance is denoted by * or *** to indicate $P<0.05$ or $P<0.0001$, respectively.
doi:10.1371/journal.pone.0096497.g006

MPA unlike NET-A, decreases expression of the pro-inflammatory IL-6, IL-8 and RANTES genes in the endocervical epithelial cell line, as well as IL-6 and IL-8 in the HeLa cell line. These effects are mimicked at the protein levels for IL-6 and IL-8 in the epithelial cell line. Thus MPA, unlike NET-A and P4, shows an anti-inflammatory profile in both cell lines, for most genes investigated. Furthermore, we show for the first time that the predominant steroid receptor protein detected in the endocervical epithelial cell line and in primary endocervical epithelial cells is the GR, with no detectable PR mRNA or protein. Consistent with this finding, we also demonstrate by a combination of GR knockdown and ChIPs, that MPA, unlike NET-A, represses pro-inflammatory cytokine gene expression in cervical epithelial cells via a mechanism involving recruitment of the GR to cytokine gene promoters. These results are consistent with a direct effect of the GR without a requirement for new protein synthesis, as shown by cycloheximide experiments. Our findings that DEX recruits GR to the IL-6 and IL-8 promoter regions are consistent with previous reports [56,73], while we show here for the first time, that stimulation with MPA recruits GR to the IL-6 and IL-8 promoter regions, thereby repressing expression of these genes. These results are consistent with our hypothesis and our previously published data that MPA, unlike NET-A or P4, acts like a partial to full GR agonist with a relatively high affinity for the GR on endogenous genes in other cells and via synthetic reporter genes [36,41,44,74]. The findings of the present study suggest that in the context of the genital mucosa, these GR-mediated effects of MPA in cervical epithelial cells are likely to play a critical role in discriminating

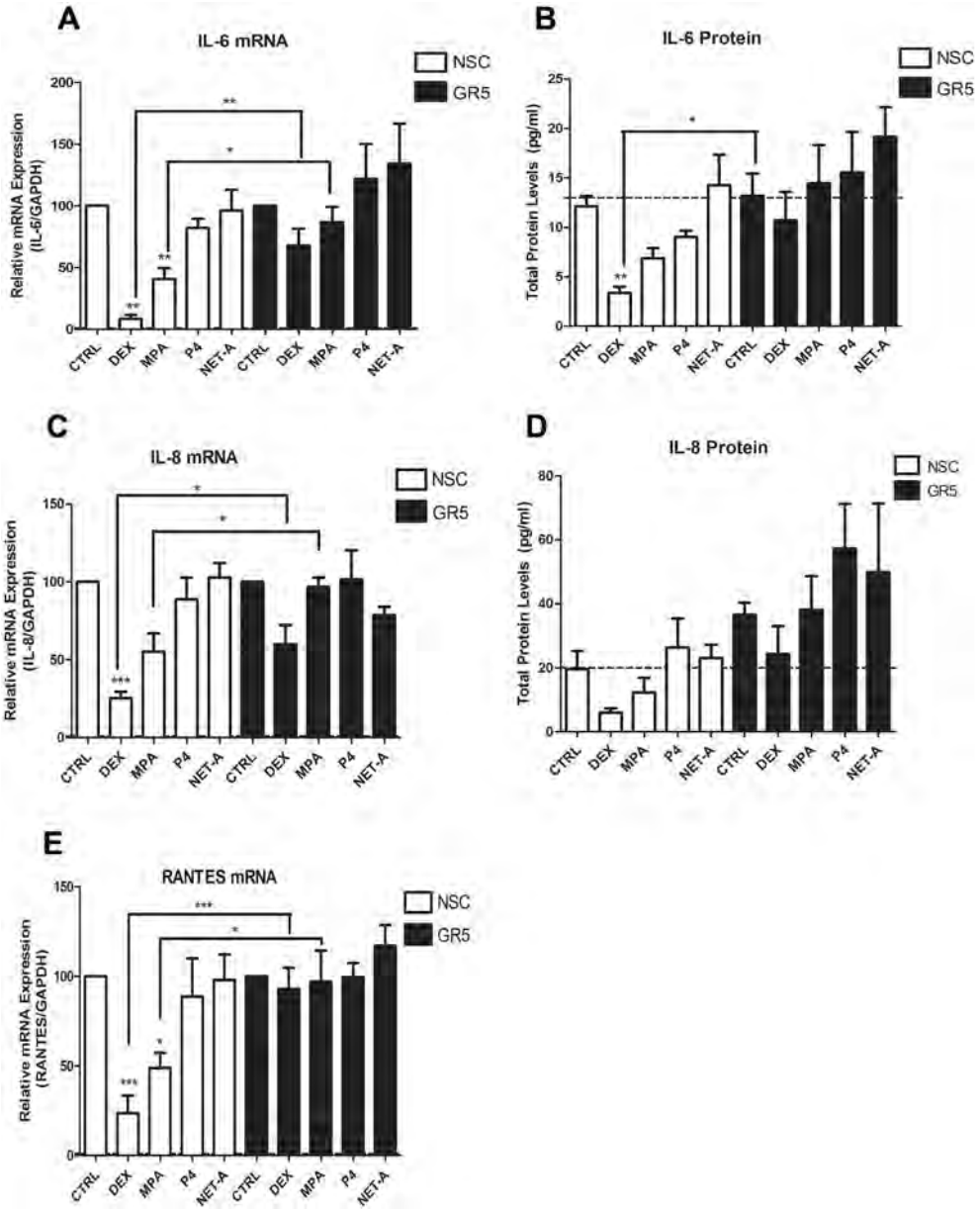

Figure 7. MPA- and DEX-mediated repression of pro-inflammatory cytokine gene mRNA is mediated via the GR. End1/E6E7 cells were transfected with 10 nM GR or NSC siRNA (**A–F**) and then treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). Total RNA was isolated and reverse-transcribed. Relative (**A**) IL-6 (**C**) IL-8 and (**E**) RANTES gene expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (CTRL) in order to obtain fold expression. The corresponding cytokine protein levels for (**B**) IL-6 and (**D**) IL-8 were determined by Luminex of supernatants collected prior to cell harvest. Graphs represent pooled results of at least three independent experiments and are plotted as mean ± SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with a Dunnett post-test followed by a student's t-test to compare specific conditions to each other. Statistical significance is denoted by * or ** to indicate $P<0.05$ or $P<0.001$, respectively.
doi:10.1371/journal.pone.0096497.g007

between the effects on inflammation caused by different progestins and progesterone and hence susceptibility to genital infections, given the predominant expression of the GR and lack or PR protein expression in these cells. The GR knockdown results furthermore suggest that changes in GR expression levels are likely to significantly modulate the inflammatory response in the endocervix, with reduced GR levels even possibly resulting in some pro-inflammatory effects by both MPA, NET-A and P4.

Our findings that MPA has anti-inflammatory gene expression effects in the endocervical cells are consistent with previous reports that show MPA suppresses pro-inflammatory immune markers in primary mouse uterine and cervical tissue and in primary human vaginal mucosal mononuclear cells [49,75]. Given the different steroid receptor selectivities of MPA, NET and P4 [36–38], it is likely, however, that the steroid receptor profile of different compartments of the female genital tract will determine the outcome of inflammatory gene expression effects of these ligands. In the current paper we show that both primary endocervical cells and the endocervical cell line express predominantly the GR. In contrast, we have previously shown that the Ect1/E6E7

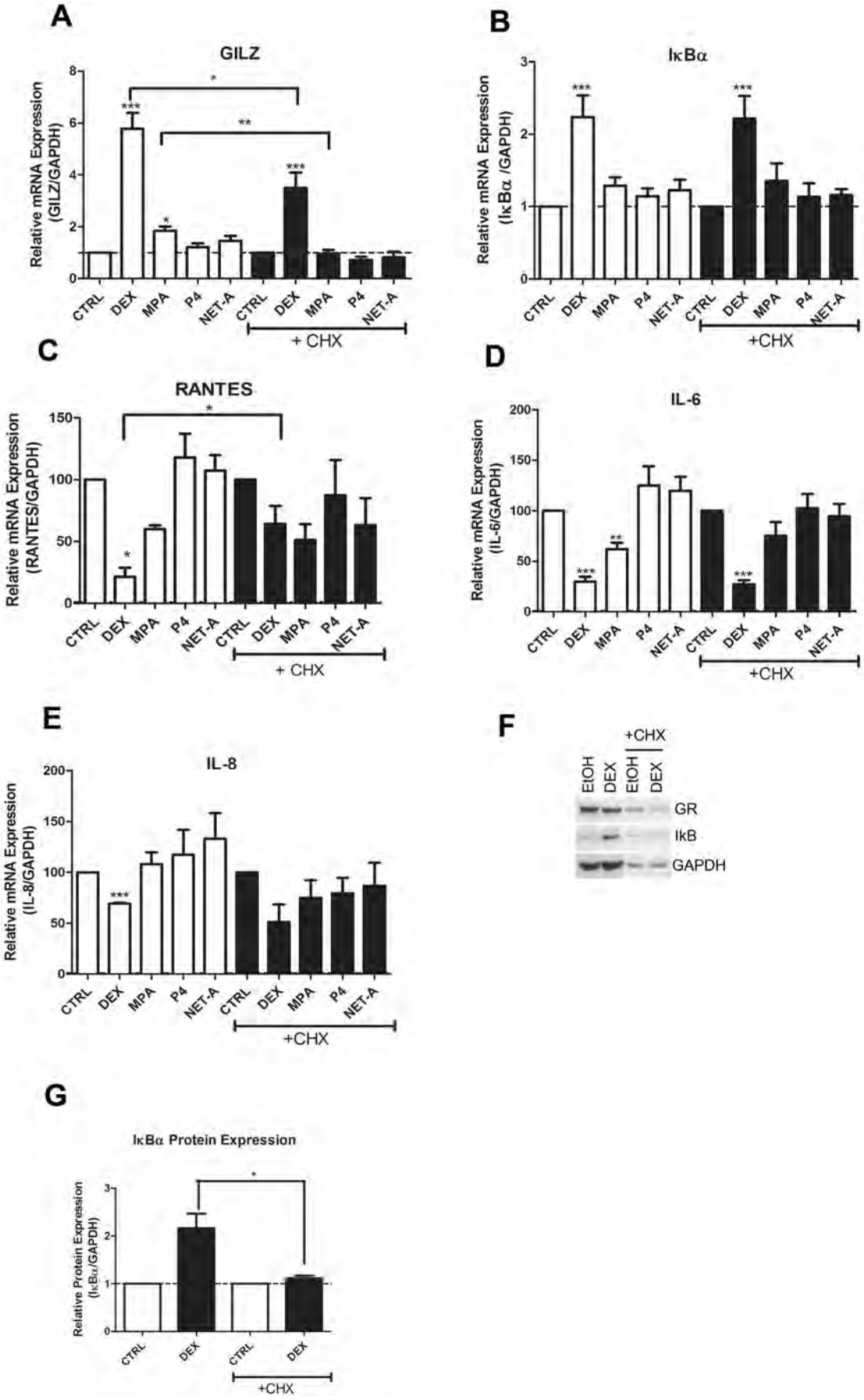
A
GILZ
Relative mRNA Expression (GILZ/GAPDH)
CTRL
DEX
MPA
P4
NET-A
+ CHX
B
IκBα
Relative mRNA Expression (IκBα /GAPDH)
+CHX
C
RANTES
Relative mRNA Expression (RANTES/GAPDH)
D
IL-6
Relative mRNA Expression (IL-6/GAPDH)
E
IL-8
Relative mRNA Expression (IL-8/GAPDH)
F
+CHX
EtOH
DEX
GR
IkB
GAPDH
G
IκBα Protein Expression
Relative Protein Expression (IκBα/GAPDH)

Figure 8. The GR at least in part directly regulates mRNA levels of the inflammatory genes. End1/E6E7 cells were pretreated with 1 μg/ml cycloheximide (CHX) then treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL), in the absence or presence of CHX. Total RNA was isolated and reverse-transcribed. Relative (**A**) GILZ (**B**) IκBα, (**C**) RANTES, (**D**) IL-6 and (**E**) IL-8 gene expressions was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (CTRL) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean ± SEM. To verify that the CHX inhibited *de novo* protein synthesis, End1/E6E7 cells were pretreated with CHX then treated with 100 nM DEX or vehicle (ethanol) (CTRL) for 24 hrs. (**F**) Cells were harvested and equal volumes of lysate were analysed by Western blotting with an antibody specific for IκBα and a GAPDH specific antibody as loading control. (**G**) Western blots of four independent experiments were quantified to determine the relative GR protein expression. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with a Dunnett post-test followed by a student's t-test to compare specific conditions to each other. Statistical significance is denoted by *, ** or *** to indicate $P<0.05$, $P<0.001$ or $P<0.0001$, respectively.
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ectocervical epithelial and Vk2/E6E7 vaginal epithelial cell lines appear to express a greater variety of steroid receptors, including the PR, GR, AR and ERα [76]. This is consistent with the report that the ectocervix is covered by a mucosal layer that is histologically similar to the vagina but different to the endocervix [77]. Our previous results show that IL-6, IL-8 and RANTES mRNA levels are regulated differently in the ectocervical and vaginal cell lines compared to the endocervical cell line, consistent with their different steroid receptor profiles. MPA and NET have no effect on IL-6 mRNA levels in both the ectocervical and vaginal cell lines, while MPA is pro-inflammatory for IL-8 in the ectocervical cell line, in contrast to the anti-inflammatory results we observed for MPA in the endocervical cell line. P4 appears to be pro-inflammatory at 1 μM concentrations for most of the pro-inflammatory genes in the ectocervical and vaginal cell lines, an effect which we also observe for some genes at 1 μM P4 for IL-6, but not IL-8 or RANTES. Interestingly MPA represses RANTES in both the ectocervical and endocervical cell lines, with no significant effect in vaginal cells. However, in the ectocervical cells, this effect is mediated predominantly via the AR, while in the endocervical cells, we show that it is mediated via the GR. Interestingly, we have recently found that MPA, unlike P4 and NET, shows a very similar pattern and potency of repression of pro-inflammatory genes in human PBMCs to that observed in the current study in the endocervical cell line, with a similar predominantly GR steroid receptor profile [78]. Collectively, our results support the hypothesis that MPA, when acting predominantly via the GR, is likely to exert anti-inflammatory effects on gene expression via classical transrepression mechanisms, unlike NET and P4, but when the steroid receptor profile is changed, the responses are likely to vary. Furthermore the results collectively suggest that different compartments of the genital tract are likely to exhibit different inflammatory responses to MPA vs NET vs P4, with their associated different effects on susceptibility to genital infections. Our lack of detection of PR expression in the endocervical primary cells or cell lines raises the question as to what is the role of the PR in mediating responses to progestins and progesterone in the endocervix. It is possible that other cells besides epithelial cells in the cervix express functional PR, as is suggested from one report [79], while others report the expression of both the GR and ERα [80].

Whether the observed effects of MPA, NET and P4 are relevant to the physiological doses of these ligands *in vivo* is a critical question, which we investigated here by dose response analysis [81] to determine potencies (EC_{50}s) and efficacies (maximal response). The MPA serum concentrations of DMPA-users are reported to be in the range 2.5 to 65 nM a few days after injection and to plateau at about 2.6 nM for about three months thereafter [38,48,82], while serum concentrations for injectable NET-EN, in the range of 1.5–59 nM have been reported [83]. The concentration of endogenous P4 in serum of premenopausal women is low during the follicular phase (0.65 nM) but rises to about 80 nM during the luteal phase, and to about 600 nM during pregnancy [37]. We show that MPA at 10 nM significantly represses both IL-6 and RANTES at 24 hrs (Figure 3 C and H). Furthermore our dose response analysis show that MPA has a potency of ~24 nM for transactivation of the anti-inflammatory GILZ gene and a potency of ~4–20 nM for repression of the pro-inflammatory IL-6, IL-8 and RANTES genes. This suggests that these immunosuppressive effects are likely to be relevant at physiological doses of MPA used in injectable contraception, particularly shortly after injection, while any possible effects of NET-EN injectable

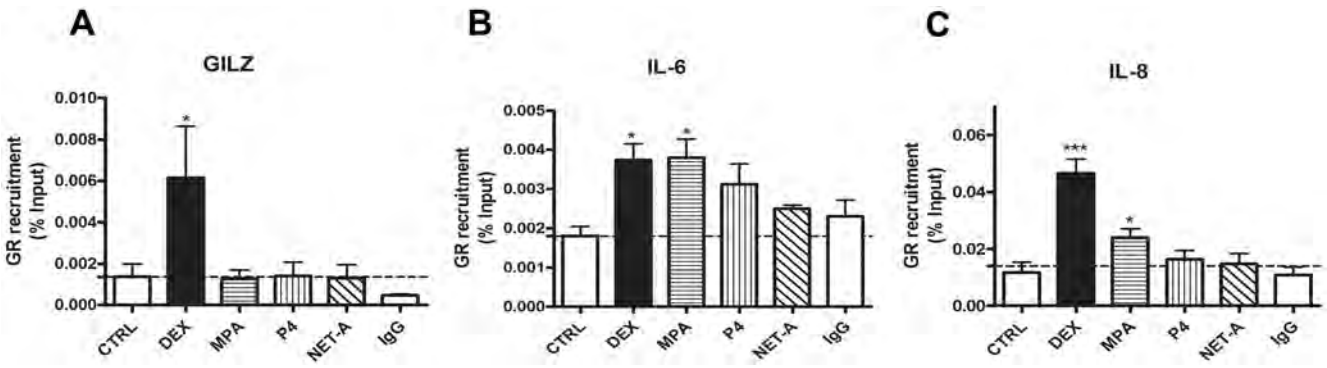

Figure 9. DEX and MPA recruit GR to the IL-6 and IL-8 cytokine gene promoters. HeLa cells were serum starved for 2 hrs and then treated for 1 hr with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). ChIP was carried out using an anti-GR antibody to immunoprecipitate endogenous GR and an anti-IgG antibody as a negative control. Real-time qRT-PCR was performed on input and immunoprecipitated DNA with primers specific for endogenous (**A**) GILZ, (**B**) IL-6 and (**C**) IL-8 promoters. GR recruitment was measured relative to input. Graphs represent pooled results of at least three independent experiments and are plotted as mean ± SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with Dunnett post-test. Statistical significance is denoted by * or *** to indicate $P<0.05$ or $P<0.0001$, respectively.
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contraceptive on inflammation via the GR are likely to be negligible, even shortly after injection. Since P4 at concentrations up to 100 nM shows very little effect on expression of the genes investigated, P4 at doses other than during pregnancy, are unlikely to exert major effects on inflammation or immune function in endocervical epithelial cells. However, about 20% repression of IL-6 is observed by P4 at 4 hrs at 10–100 nM, suggesting that in the presence of a predominant GR, P4 could exert some anti-inflammatory effects. At pregnancy concentrations, P4 may exert some pro-inflammatory effects on some genes, as suggested by our dose response analysis showing this trend for some genes at 1 μM. It should, however, be noted that the concentrations of MPA, NET and P4 in cervical tissue may not be the same as that found in the serum of contraceptive users.

The physiological significance of changes in expression of pro-inflammatory mediators like IL-6, IL-8 and RANTES in genital epithelial cells is difficult to predict. Increased pro-inflammatory mediators could increase recruitment of dendritic cells (DCs) or Langerhans cells (LCs) as well as $CD4^+$ T cells and monocytes/macrophages, thus potentially increasing HIV-1 acquisition by increasing the number of target cells. Thus progestins like MPA, unlike NET-A, that exert anti-inflammatory gene expression effects in the female genital tract may decrease HIV-1 acquisition by decreasing the number of target cells. However, decreased pro-inflammatory mediators could also inhibit immune function, such as B-cell maturation, T-cell activation and differentiation, IgA production, neutrophil/monocyte/macrophage/dendritic cell activity, reducing the host's ability to mount a defence against a pathogen [84]. Additionally, RANTES is a ligand for the CCR5 receptor, which has the ability to block HIV-1 entry [85]. Thus a decrease in RANTES expression by endocervical epithelial cells, as we show for MPA but not NET, with a potency of 5 nM, could increase HIV-1 infection of $CD4^+$ T cells*in vivo*. Interestingly, MPA shows the greatest efficacy for RANTES mRNA repression, acting like a full GR agonist. Whether or not DMPA usage increases or decreases the expression of inflammatory mediators in the female genital tract *in vivo* is unclear and requires further investigation. Several studies suggest that a pro-inflammatory environment is associated with an increase in HIV-1 acquisition [86–88]. Furthermore, DMPA-usage was recently reported to be associated with increased HIV-1 acquisition and increased levels of RANTES [88]. However, in this study it was not possible to discriminate between elevated RANTES levels being a cause of infection or a consequence of exposure to HIV-1 prior to seroconversion. Additionally, since 76% of the DMPA users in this study were positive for an STI, it may be that elevated RANTES was a consequence of STI infection, despite attempts to correct for that confounding variable. In contrast to the latter study, the study by Huijbregts et al. found that DMPA-usage is associated with immunosuppressive effects in the cervical mucosa [49]. Furthermore, we have recently shown that MPA, unlike NET or P4, increases apoptosis of T-cells, which is potentiated after HIV-1 infection [74], potentially decreasing the ability of T-cells to mount an anti-viral defence. Currently available clinical data from women on DMPA, taken together with animal data plus our and other biochemical *ex vivo* data, certainly suggest that immunosuppressive properties of long term MPA contraceptive usage may be a significant factor contributing towards increasing HIV-1 acquisition, transmission and possibly disease progression. Importantly, our results show that MPA effects on genital mucosal immune function and susceptibility to infections are likely to be very different to those of NET and P4, when mediated via the GR, and that choice and concentration of progestin in contraception are likely to be critical factors.

Supporting Information

Figure S1 Only DEX and MPA represses basal as well as TNF-induced cytokine mRNA expression. End1/E6E7 cells were treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL), in the absence or presence of 20 ng/ml TNFα. Thereafter the cells were harvested, total RNA was isolated and reverse-transcribed. Relative (A) IL-6, (B) IL-8 and (C) RANTES mRNA expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalized to basal activity (CTRL) in order to obtain relative fold expression. Graph represents pooled results of at least three independent experiments and are plotted as mean ± SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with Dunnett post-test, followed by a student's t-test to compare specific conditions to each other. Statistical significance is denoted by * or ** to indicate $P<0.05$ or $P<0.001$, respectively.
(TIF)

Figure S2 DEX, MPA and NET-A induce total IκBα protein. End1/E6E7 cells were treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). Thereafter, cells were harvested and equal volumes of lysate were analysed by **(A)** Western blotting with an antibody specific for total IκBα and a GAPDH specific antibody as loading control. **(B)** Western blots of five independent experiments were quantified to determine the relative GR protein expression. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with a Dunnett post-test followed by a student's t-test to compare specific conditions to each other. Statistical significance is denoted by *, ** or *** to indicate $P<0.05$, $P<0.001$ or $P<0.0001$, respectively.
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Figure S3 Cell Viability of VEN-100. VEN-100 cells were either incubated for 24 hrs (day 1, treatment day) or 72 hrs (day 3, end of treatment day), followed by, analysis for cell viability (MTT assay). Absorbance readings were measured at 570 nm. Cell culture media served as the control (CTRL). CTRL for each day was set to 1 to obtain relative fold cell viability. The graph represents results of at least three independent experiments, plotted mean +/− SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with a Dunnett post-test followed by a student's t-test to compare specific conditions to each other. Statistical significance is denoted by ** or *** to indicate $P<0.001$ or $P<0.0001$, respectively.
(TIF)

Figure S4 Ligand-selective GR protein turnover. End1/E6E7 cells were treated with increasing amounts (1 nM, 10 nM, 100 nM and 1 μM) of DEX, MPA, P4 or NET-A, or vehicle (ethanol) (CTRL) for 24 hrs. Thereafter, the cells were harvested and equal volumes of lysate were analysed by Western blotting with antibodies specific for GR and GAPDH as loading control.
(TIF)

Author Contributions

Conceived and designed the experiments: JPH YG CA. Performed the experiments: YG CA NG NJDV. Analyzed the data: JPH YG CA NG RR DA. Wrote the paper: JPH YG.

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Review

Potency of progestogens used in hormonal therapy: Toward understanding differential actions

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ABSTRACT

Progestogens are widely used in contraception and in hormone therapy. Biochemical and molecular biological evidence suggests that progestogens differ widely in their affinities and transcriptional effects via different steroid receptors, and hence cannot be considered as a single class of compounds. Consistent with these observations, recent clinical evidence suggests that, despite their similar progestogenic actions, these differences underlie different side-effect profiles for cardiovascular disease and susceptibility to infectious diseases. However, choice of progestogen for maximal benefit and minimal side-effects is hampered by insufficient comparative clinical and molecular studies to understand their relative mechanisms of action, as well as their relative potencies for different assays and clinical effects. This review evaluates the usage, meaning and significance of the terms affinity, potency and efficacy in different models systems, with a view to improved understanding of their physiological and pharmacological significance.

This article is part of a Special Issue entitled 'Menopause'.

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1. Introduction

Progestogens are compounds that exhibit progestational activity, and include both endogenous progesterone (Prog) and synthetic progestogens designed to mimic its actions. A wide variety of synthetic progestogens is available and their common progestogenic effects are exploited for many therapeutic applications in female reproductive medicine, including their use in contraception and for menopausal therapy. However, these synthetic progestogens also exhibit a range of biological effects that differ not only from each other, but also from that of Prog [1–3]. Choice of progestogen for maximal benefit and minimal side-effects is hampered by a limited understanding of their relative mechanisms of action due to insufficient comparative clinical and molecular studies.

Multiple factors such as route of delivery, metabolism and binding to and regulation of serum proteins affect the bioavailability of the active form of progestogens at target cells [2–6]. Progestogens mediate their intracellular effects by modulating transcription of target genes in specific cells via binding not only to the progesterone receptor (PR), but also with varying affinities to other steroid receptors (SRs) such as the glucocorticoid, mineralocorticoid and androgen receptors (GR, MR and AR, respectively) [2,3,7,8].

Abbreviations: SR, steroid receptor; PR, progesterone receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; AR, androgen receptor; ER, estrogen receptor; GREs, glucocorticoid response elements; NFκB, nuclear factor kappa B; AP-1, activator protein-1; RBA, relative binding affinity; Prog, progesterone; MPA, medroxyprogesterone acetate; NOMAC, nomegestrol acetate; R5020, promegestone; TMG, trimegestone; NET, norethisterone/norethindrone; NET, Anorethisterone/norethindrone acetate; DRSP, drospirenone; DHT, dihydrotestosterone; IC_{50}, inhibitor concentration for 50% inhibition.

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It is generally assumed that their progestational effects are mediated via the PR in female reproductive tissue while the plethora of side-effects occur via the GR, AR and MR.

SRs are ligand-activated transcription factors that function by similar genomic mechanisms, but differ in their target genes and tissues [9]. Once the inactive receptor is activated by hormone binding, the hormone receptor–complex translocates to the nucleus where it binds to specific DNA sequences in the promoter regions of target genes to activate (transactivation) gene expression. In contrast, the expression of specific target genes can also be repressed (transrepression) via protein–protein interactions between the receptor and other transcription factors such as nuclear factor-kappa B (NFκB) and activator protein-1 (AP-1) [10].

A number of assays have been developed to elucidate the intracellular mechanisms of action of progestogens via specific receptors. Binding assays are used to determine the affinity of progestogens for a specific receptor in a number of different model systems, including animal or human tissue or cell lines, as well as in vitro systems. In contrast, most of the data on the subsequent relative biological responses via different SRs following binding, including the potency, efficacy, and biocharacter of the progestogens, have been obtained from animal experiments [2,3].

The aim of this review is to evaluate 'potencies' determined by different assays and in different model systems, as well as the meaning and significance of the term 'potency', as applied to progestogens in the current literature.

2. Receptor binding and affinity

The affinity of a progestogen for binding to the PR and other SRs is a major determinant of the potency of its biological response since it affects receptor fractional occupancy and hence the percentage maximal response in a dose response curve. However it should be noted that receptor affinity may not reflect biological activity, which is also affected by the particular conformation of the receptor–ligand complex induced by ligand binding. This is well illustrated by the fact that an antagonist can have a higher affinity for a receptor than an agonist, but exhibits a very different biological response due to the induction of a different receptor conformation as compared to the agonist. Consistent with their different structures, reported affinities of different progestogens for SRs other than the PR vary widely. However, affinities reported for a particular progestogen for a specific SR also vary greatly, most likely due to different methods and sources of biological material used to determine affinity. Table 1 shows a range of relative affinities reported in the literature for progestogens binding to SRs, where the same reference agonist is used for a particular SR, while Supplementary Table 1 shows relative affinities reported using different reference agonists, as well as details of methods and model systems used.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2013.08.001.

Most reported binding data are obtained by performing heterologous competition binding experiments and expressing the results as relative binding affinity (RBA). The constructed binding curves should theoretically be sigmoidal in shape with a Hill slope value of one, for a single ligand binding to one site on a receptor molecule, without cooperative binding. The Hill slope (steep part of the curve) indicates whether cooperative binding occurs, with a slope of one indicating no cooperative binding, while of slope of less or greater than one indicates negative or positive cooperativity, respectively. The IC_{50}, the concentration of unlabeled progestogen (inhibitor or competitor) that corresponds to 50% inhibition of the total specific binding of the radiolabeled reference agonist, can then be determined. Many apparent discrepancies in RBAs reported in the literature are due to the use of different reference ligands (set as 100% RBA). These include promegestone (R5020) versus Prog for the PR, which differ in their RBAs by about five fold, and mibolerone or methyltrienolone (R118) versus testosterone or dihydrotestosterone (DHT) (Supplementary Table 1), where the synthetic agonist have about 100-fold greater RBA than the natural ligands [2,3]. Thus, these apparent RBA differences are not necessarily real differences. RBAs can be directly compared by recalculating values relative to a common ligand, if the information is available.

Another potential source of variable affinities is inherent in the method of RBA determination. While several reports simply use relative IC_{50} values as RBAs [11–15], these are only an approximate measure of relative affinity since IC_{50} varies due to experimental and biological parameters, such as the concentration of radiolabeled steroid and the concentration of the receptor being investigated. Fig. 1A illustrates how the IC_{50} changes in a competition binding assay as a function of receptor concentration. Interestingly, if the Hill slope is not fixed during plotting, it also increases as receptor concentration increases, suggesting that caution should be exerted when interpreting changes in Hill slope in competitive binding studies when receptor concentration is much greater than the true K_d. A more accurate measure of affinity of progestogens for a SR that circumvents these problems can be obtained by saturation binding to obtain an equilibrium dissociation constant (K_d). These K_d values are likely to be more accurate than RBAs determined by competitive binding, provided that the K_d is greater than the total receptor concentration in the assay and that other sources of technical and samples source error are not present. However, only a few reports use saturation binding to measure RBAs of progestogens. For example, K_d values of 10.9 nM and 4.42 nM for the PR have been determined for drospirenone (DRSP), and R5020, respectively [27], while a K_d value of 1.7 nM was determined for medroxyprogesterone acetate (MPA) for the AR [17]. Sometimes RBAs are calculated from K_d values. For example, K_d values were determined for NOMAC and Org2058 for the PR by saturation binding in rat uterus (K_d = 5 nM and 0.6 nM, respectively) and human T47D breast cancer cells (K_d = 4 nM and 3 nM, respectively). RBAs were then calculated relative to Prog set with a RBA of 100%, such that NOMAC and Org2058 displayed RBAs of 67% and 692%, respectively for the PR in rat uterus, and 192% and 212%, respectively for the PR in human T47D cells (Table 1 and Supplementary Table 1). The differences in the K_d values for Org2058 and the RBAs for both ligands relative to Prog most likely reflect different off-target and/or metabolism and/or species effects in the two systems.As an alternative to saturation binding, homologous or heterologous competition binding displacement assays can be used to determine accurate K_d or K_i values using the Cheng–Prussof equation (Supplementary Table 1), provided the concentration of radiolabeled ligand is less than the IC_{50} [19,20]. The K_i is the equilibrium dissociation constant of the unlabeled competitor or inhibitor, and is a true constant that does not vary with receptor concentration in the assay, provided certain experimental restrictions are adhered to. Using this method, similar K_i values were obtained by two groups for Prog and MPA binding to the GR (K_i: 95.2 nM and 215 nM and K_i: 3.7 nM and 10.8 nM, respectively) [21,22]. RBAs for the GR can also be calculated from K_i values (e.g. relative to dexamethasone set at 100%, RBAs for Prog: 0.84%, and 2%, RBAs for MPA: 21.6% and 39%) (Table 1 and Supplementary Table 1). However, when comparing K_i values and RBAs calculated from K_i values obtained from different groups, large discrepancies are still often found. For example, for binding to the AR, a two-fold difference in the RBA of MPA has been reported, while a five- to nine-fold difference in the K_i values has been reported (151%, K_i = 19.4 nM [23]; 75%, K_d = 1.7–3.6 nM [24], RBAs relative to DHT set at 100%), (Table 1 and Supplementary

Table 1
Relative binding affinities (RBAs) and biological activities of progesterone and synthetic progestins via steroid receptors.

Progestogen	PR		AR			GR		MR	
	RBA %	Progestogenic	RBA %	Agonistic	Anti-androgenic	RBA %	Glucocorticoid	RBA %	Anti-mineralocorticoid
Progesterone-derived									
Progesterone	100	+	n/d	±	(+)	0.84–35	±	9–1000	+
Chlormadinone acetate	91–212	+	n/d	+	–	8	+	0	–
Cyproterone acetate	46	+	13–21	+	–	6	+	8	–
Medroxyprogesterone acetate	65–298	++	36	±	–	21.6–79	+	0.1–160	–
Nestorone	186	+	0.2	–	–	38	–	n/d	n/d
Nomegestrol acetate	67–303	+	12–31	–	+	6	–	0	–
Promegestone	111–236	++	n/d	–	–	5	+	53	–
Trimegestone	588	++	2.4	–	±	9–13	–	42–120	±
Testosterone-derived									
Dienogest	N/D	+	n/d	–	+	1	–	0	–
Gestodene	864	+	71	±	–	27–38	±	11–290	±
Levonorgestrel	96–323	++	58	+	–	1–7.5	–	17–75	±
Norethindrone/Norethisterone	134	+	55	+	–	0–1.4	–	0–2.7	–
Norethindrone acetate/Norethisterone acetate	27–43	++	n/d	+	–	0.88–1.6	–	0.07	–
Spironolactone-derived									
Drospirenone	19	++	n/d	–	+	3–6	–	100–500	+

The reference radiolabelled ligands and ligands used for 100% RBA were as follows: PR, progesterone (Prog); AR, testosterone; GR, dexamethasone (Dex); MR, aldosterone (Ald). For RBAs determined relative to other reference ligands and details about the assays and how RBAs were calculated see Supplementary Table 1. Biological activities determined in animals (mostly rats and rabbits) using a range of assays are taken from Ref. [2]. Progestational activity: measured by the endometrial transformation (McPhail Index), and/or pregnancy maintenance, and/or ovulation inhibition tests. Androgenic activity: increase in weight of ventral prostate and levator ani of immature castrated male rats. Anti-androgenic activity: weight of seminal vesicles and prostate of castrated rats and/or feminization-inducing activity in male rats. Glucocorticoid activity: production of glycogen and tyrosine transaminase in rat liver. Anti-mineralocorticoid activity: sodium and potassium excretion from ovariectomized rats fed with low sodium diet. Key to hormonal activities: –, not effective; (+), weakly effective; +, effective; ++, strongly effective; ±, literature inconsistent. n/d: not determined.

Table 1). Possible reasons for these discrepancies may be failure to establish equilibrium and/or using a concentration of radiolabeled ligand greater than the IC_{50}. Very different RBAs are likely to be obtained when using K_i values obtained by the Cheng–Prussof method compared to simply using IC_{50} values. For example the values obtained by Africander et al., for MPA for the MR (aldosterone = 100%) (0.08%; K_i = 197 nM) and NET-A (0.07%; K_i = 229 nM) are 40-fold different to previously reported values of 3.1% and 2.7%, respectively (Table 1 and Supplementary Table 1) [23,25].

Another major source of variability in binding data is biological sample variability. Different samples may exhibit different degrees of off-target binding of the progestogen to non-target SRs and/or other proteins such as steroidogenic enzymes, to which progestogens bind with variable affinity. Off-target binding would effectively lower the apparent RBA, right-shift the binding curve and increase the IC_{50}, independent of the method used to determine binding, as illustrated in the simulated binding curve (Fig. 1B). Off-target binding could also be a source of non-parallel binding curves with Hill slopes greater than one with increasing concentrations of off-target receptor relative to target receptor, as illustrated in Fig. 1B. For example, remarkable differences in the RBAs of Prog (1000% vs. 100%), DRSP (500% vs. 100% or 230%) and gestodene (97% vs. 290%), all relative to aldosterone set to 100% (Table 1 and Supplementary Table 1), were observed when comparing recombinant human MR in vitro binding, relative to rat tissue models, respectively [25–27], most likely due to off-target and/or metabolism effects in the tissues.The presence of metabolizing enzymes could also potentially right-shift binding curves and lower the IC_{50} values without a change in Hill slope (assuming the metabolites do not bind the target receptor), as illustrated in the simulated binding curves (Fig. 1C). Other sources of variability are the species from which tissue is obtained, as well as the variations in age and pre-treatment of the animal or human donors. For example, when comparing rabbit versus rat uterine cytosols, different RBAs were observed for the PR with MPA (112% vs. 9.4%) (R5020 = 100%), NOMAC (135% vs. 72%) (Prog = 100%) and trimegestone (TMG) (84% vs. 152%) (R5020 = 100%), respectively, (Table 1 and Supplementary Table 1) [15,18]. Some of the above differences are likely to be due to species differences. However, species, age, or pre-treatment differences may also be confounded by differences in relative concentrations of off-target receptors and/or metabolizing enzymes.

As mentioned, a failure to reach equilibrium by the reference and competing ligand is another common cause of variability in reported binding data [20]. The few cases where this is investigated suggest that very different times are required for different progestogens and different concentrations thereof, to reach equilibrium. For example, for 0.2 nM [^{3}H]-aldosterone or [^{3}H]-mibolerone binding to the overexpressed hMR or hAR in the COS-1 cell line, this was determined as 16 h [23], while for 1.25 nM [^{3}H]-dexamethasone binding to the GR endogenously expressed in the A549 cells it was three hours [22]. Others showed that 20 nM Prog reaches a saturation plateau faster than either 20 nM R5020 or RU486 [16]. Higher RBAs have been reported for binding of several progestogens to the PR in rabbit uterine tissue after incubation for 24 h, in comparison to 2 h [18]. Similarly, differences in the RBAs of several progestogens for the cytosolic AR of the rat prostate have been reported for different incubation times [18]. When plotting binding curves, most software programs give the option to fix or not to fix the Hill slopes for binding curves. Fixing the slope could mask the presence of competing off-target effects or of high receptor concentrations (as illustrated in Fig. 1A and B) or even the presence of cooperative target SR binding effects, leading to inaccurate determination of affinities. There are indeed some reports in the literature of cooperative binding of steroids to the GR and estrogen receptor (ER) [28,29], suggesting this may be one reason for non-parallel binding curves. Other sources of error in saturation binding data could be the use of Scatchard plots rather than non-linear regression in determination of binding parameters. This method has been used extensively to determine K_d values for progestogens binding to the PR [14,16,21,27] (Supplementary Table 1 [63,64]). The Scatchard plot, which entails a linearization of binding data, suffers from the same shortcoming as linear enzyme-kinetic

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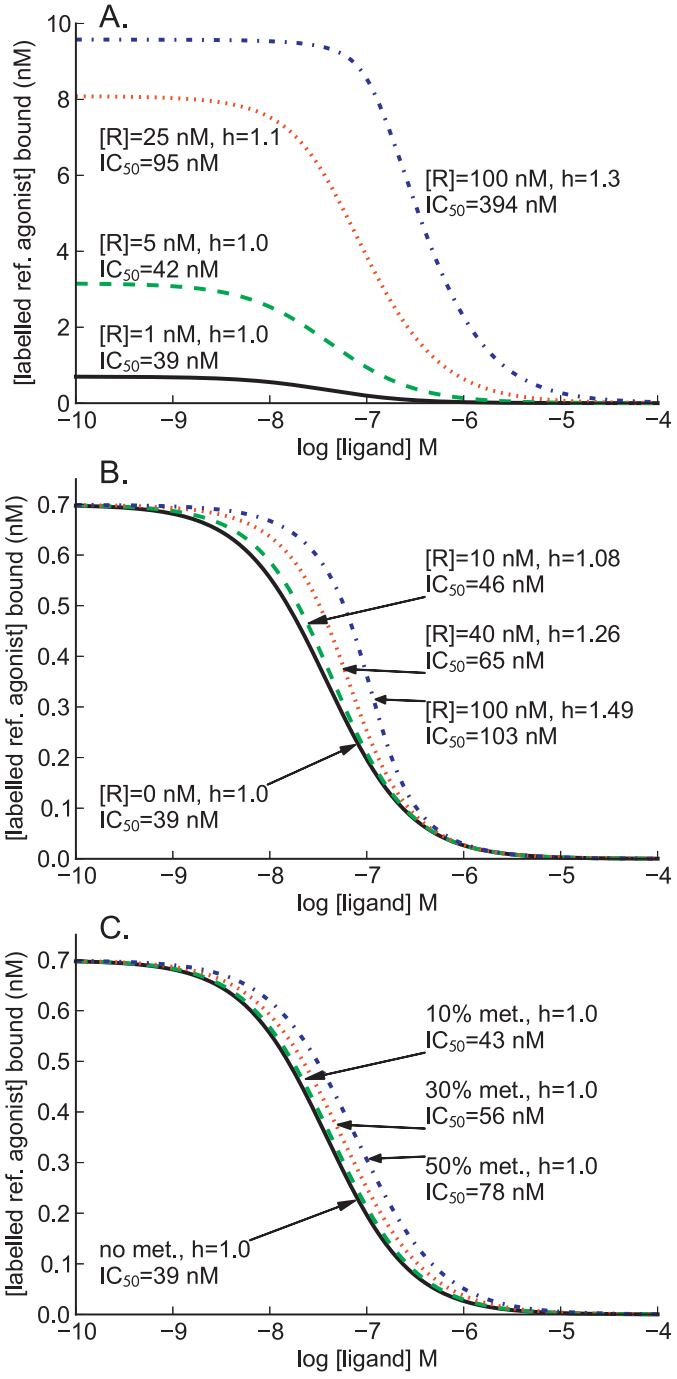

Fig. 1. Apparent affinity (IC_{50}) increases with target receptor or competing receptor concentrations and with increased metabolism of competing progestogen. Simulated competition binding curves showing IC_{50} values and Hill slopes for a progestogen binding to a target SR (A) in the presence of the indicated increasing concentrations of the target receptor, or (B) in the presence of 1 nM target receptor and the indicated increasing concentrations of competing receptor, or (C) with increasing extents of progestogen metabolism in the presence of 1 nM target receptor (met). The labeled reference agonist was assumed to bind to the target receptor but not to the competing receptor. The competing ligand was assumed to bind to both receptors; the true K_d for the competing receptor was 20 nM. For C it was assumed that the down-stream metabolites do not bind to the receptor. Simulation parameters: reference agonist concentration, 10 nM; K_d of reference agonist for target receptor, 4 nM; K_d of competing ligand for target receptor, 11 nM; K_d of competing ligand for competing receptor, 20 nM. Hill slopes (h) and IC_{50}-values are annotated on the graph and were calculated by fitting the simulated data to the competition binding equation: $y = \text{top}/(1 + 10^{(x - \log IC_{50})h})$.

treatments such as the Lineweaver–Burk plot: an uneven error distribution results in data points at low ligand concentrations being given undue weight.

Despite the sources of error discussed above, several trends have emerged and much valuable data are available regarding binding affinities of progestogens for different steroid receptors. All the progestogens bind to the PR with a high affinity, typically in the nanomolar range. For example the synthetic agonist R5020 was found to bind to the PR expressed in human or calf uterine cytosol with K_d values of 4.42 nM and 5.6 nM, respectively [16,27]. In contrast, progestogens do not bind to the ER, as expected, due to the low homology between the ER ligand binding domain and the PR, although there are reports that some progestogen metabolites do bind the ER [2]. Consistent with the structural similarities between some progestogens and testosterone, several progestogens bind with relatively high affinity to the AR (Table 1), although the reported affinities vary greatly, most likely due to many of the factors described above. For example, some of the older generation progestogens such as MPA, norethisterone (NET) and levonorgestrel bind the AR with high affinity relative to metribolone [27], DHT [11,30] and testosterone [18,25]. However other researchers report that Prog, MPA, norethisterone-actetate (NET-A), cyproterone acetate and DHT all have similar and relatively high affinities for the AR [17,23,24] (Table 1). In contrast, DRSP, dienogest and TMG exhibit low RBAs [1,6,25,31], while nestorone does not bind to the AR [12,32]. It is not surprising that several progestogens structurally similar to Prog, bind to the MR, given the high affinity of Prog for the MR (K_i for Prog 1.69 nM [23]). Indeed, TMG [25,33] and DRSP (Table 1) [26,27,31], the latter derived from the MR antagonist spironolactone, both bind the MR with high affinity. These progestogens were specifically developed for their anti-mineralocorticoid properties for contraceptive usage [34] and for their predicted beneficial effects on blood pressure and cardiovascular function [26,35,36]. In contrast, many progestogens such as chlormadinone acetate, dienogest and NOMAC exhibit undetectable binding to the MR [1,37], while MPA (K_i = 197 nM) and NET-A (K_i = 229 nM) bind weakly to the MR [2,23]. Unlike for the PR, AR and MR, few progestogens bind to the GR with affinities in the significant pharmacological range. Of note, MPA has a high affinity in the nanomolar range for the GR (Table 1) [22,25,37,38], with a K_i value of 10.8 ± 1.1 nM [22], significantly higher than the endogenous glucocorticoid cortisol in humans [38]. Interestingly gestodene also binds the GR with a relatively high affinity, while progestogens such as NET, levonorgstrel, dienogest, TMG and DRSP, like Prog, bind the GR with low relative affinity [1,6,22,25,26,38,39].

3. Potency, efficacy and biocharacter

Potency, efficacy and biocharacter define the concentrations of progestogens required to cause or inhibit particular biological or physiological effects, the upper and lower limits of those responses, as well as how these change over a range of concentrations or doses [40]. These parameters are thus important for hormonal therapy. The current literature indicates that these parameters are substantially different in cell, tissue, animal, and clinical assays, both between progestogens and for the same progestogen.Pharmacologically, potency is defined as the concentration of ligand that induces half the maximal response, or EC_{50}, while the efficacy refers to the maximal effect a ligand can elicit under defined experimental conditions. Efficacy also gives a measure of biocharacter of progestogens, which can vary from full agonist to partial agonist to different types of antagonism, depending on the extent to which a ligand can inhibit the response to a particular concentration of agonist (Fig. 2A and B) [2]. Potency, efficacy and biocharacter can only be accurately determined by performing dose response analysis and constructing a dose

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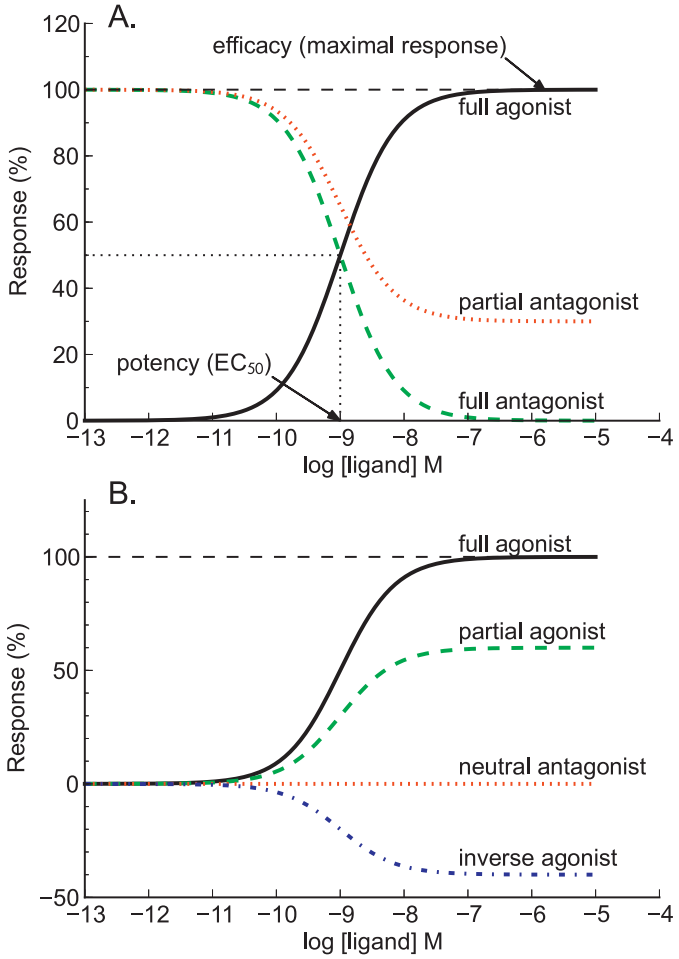

Fig. 2. Potency and efficacy are determined by dose response analysis which also reveals biocharacter of progestogens as compared to reference agonist. Schematic illustration of dose–response curves indicating (A) a typical curve for a full agonist (sigmoidal-shaped curve with Hill slope of 1) showing potency (EC_{50} value) and efficacy (maximal response), as well as for a partial antagonist and a full antagonist. (B) illustrates other possible biocharacters for progestogens, i.e. full agonist, partial agonist, inverse agonist and neutral antagonist.

response curve (Fig. 2A) [2]. It is important to note that these parameters are not constant but are particular for a specific effect or response under defined conditions. In most clinical and animal assays, potency, efficacy and biocharacter are not evaluated by these pharmacological criteria. Evidence to date from ex vivo assays suggests that all progestogens act as full agonist for transcription via the PR, while their transcriptional activities via other steroid receptors vary from no activity, to partial agonist, full agonist, and varying levels of antagonism, in a cell- and promoter-specific manner. Although best described for G-protein coupled receptors [41], inverse agonists have also been identified for the PR [42], but have to our knowledge not been reported for progestogens. Biocharacter can vary depending on context. For example, decreasing SR concentrations can convert some progestogen agonists to antagonists [4]. In addition, whether a ligand is an agonist or antagonist can depend on cell type, such as the case for asoprisnil, the selective PR modulator [43]. These modulators have been suggested as therapeutic agents for the treatment of gynecological disorders. Some progestogens may display mixed agonist-antagonist activity, as has been reported for androgenic activity by cyproterone acetate [44]. However, the selective receptor-mediated effects of most progestogens remain to be investigated. Most of the progestogen biocharacter data have been defined in animal bioassays (Table 1) and are usually not fully explored by dose response analysis. They do not necessarily reflect activity or biocharacter via a particular SR, or that obtained in other assays in either the same or different species. The large number of inconsistencies reported for biocharacter (Table 1) most likely reflects species- and/or tissue-specific differences as well as difference in methodology.

Dose response analysis (Fig. 2) can be performed on cells in culture, in tissue, in animals or even in patients. Many different responses can be measured, including mRNA levels, reporter gene activity, gene product (e.g. enzyme) activity or even physiological functions such as inhibition of ovulation. As in the case of steroid ligand binding curves, dose response curves ex vivo generally follow a sigmoidal shape with a Hill slope value of 1 (Fig. 2). Dose response curves with the same Hill slope are considered to be parallel, while those that differ are non-parallel. In the absence of dose response analysis, relative responses obtained by single doses of progestogens will vary depending on which part of the dose response curve is represented for each progestogen. For example, if the dose for all progestogens is already at the maximal response, then the progestogens could be incorrectly reported to have the same 'potency'. Furthermore, if the data from incomplete curves represents different parts of the curve, or off-target competing receptors are present, plots will be non-parallel. Taken together, this will result in misleading estimates of relative potency, efficacy and biocharacter.

Determination of relative potency (EC_{50}) by dose response assays in vitro is subject to the same sources of variability as determination of affinity, since the EC_{50} and Hill slope of the dose response curve are highly dependent on the binding of ligand to a competing receptor that may be present, and on metabolism of the ligand. This is illustrated in Fig. 3A and B (compare with Fig. 1B and C). All the determinants of potency are not fully understood, since a response involves several steps downstream of progestogen binding to receptor. However, factors such as relative concentrations of cofactors [45] and SRs [4] have been shown to shift (EC_{50}) values. For example, dose response curves for transrepression have been shown experimentally to left-shift with increasing concentrations of target receptor, with accompanying increases in Hill slope, most likely due to effects downstream of ligand binding to the receptor (Fig. 3C).Few studies have investigated relative potency and efficacy of different progestogens via particular SRs by dose response analysis in ex vivo models. For transactivation, it has been shown that Prog, MPA, dienogest, and norethisterone are all agonists for the expressed PR on a synthetic GRE promoter in COS-1 cells, but that their potencies and efficacies differ [37]. We have shown that unlike NET-A, MPA and Prog have GR agonist activity, while MPA and NET-A have AR agonist activity, and Prog has anti-AR activity via expressed receptors on synthetic GRE promoters in COS-1 cells [22,23,39]. Using a similar approach, dose response studies with expressed receptors on another GRE promoter reported that Prog and DRSP displayed no anti-AR activity, weak MR agonist and considerable anti-MR activity in CV-1 cells [2]. Several studies have shown that transactivation efficacy for progestogens appear to be highly promoter - and cell-specific. For example, MPA was found to only minimally transactivate (partial agonist) a synthetic GRE-reporter gene via overexpressed human GR in the Jurkat T-lymphocyte cell line [46], while displaying potent transactivation activity (full agonist) on another synthetic GRE-reporter gene via endogenous mouse GR in L929sA cells [47]. For transrepression, cell line studies have shown that MPA exhibits potent GR-mediated transrepression on synthetic and endogenous AP-1 or NFκB-containing promoters [47,48]. In contrast, NET-A is a weak partial agonist for transrepression [22]. Using expressed receptors in COS-1 cells, we have shown that Prog and NET-A, but not MPA, have partial MR agonist activity for transrepression on an AP-1 promoter-reporter, while MPA and NET-A, unlike

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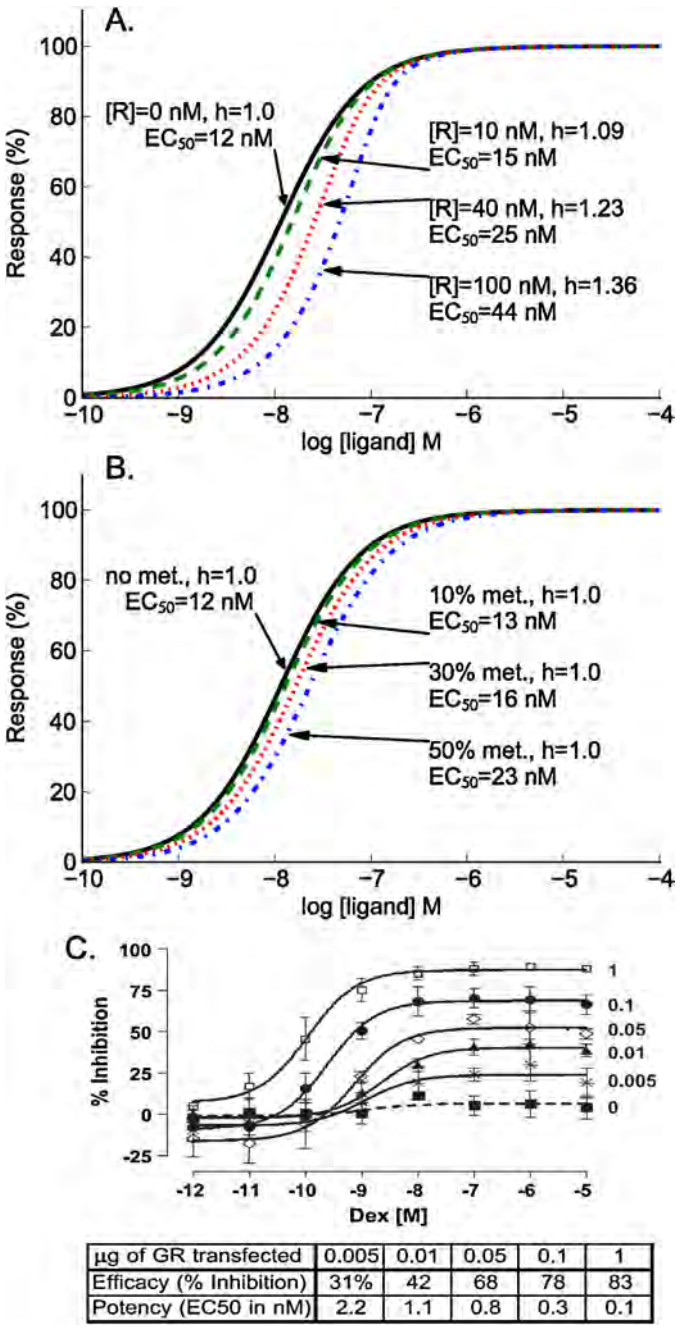

µg of GR transfected	0.005	0.01	0.05	0.1	1
Efficacy (% Inhibition)	31%	42	68	78	83
Potency (EC50 in nM)	2.2	1.1	0.8	0.3	0.1

Fig. 3. Apparent potency (EC_{50}) increases with competing receptor concentrations, with increased progestogen metabolism and with target receptor concentrations. (A and B) Simulated dose–response curves indicating potencies (EC_{50}) and Hill slopes for a progestogen via a target steroid receptor (A) in the presence of the indicated increasing concentrations of competing receptor, or (B) in the presence of increasing extents of progestogen metabolism to inactive compounds. Simulation parameters were as in Fig. 1B and C, respectively, except that labeled reference agonist was not involved. The response was assumed to be proportional to the concentration of reference agonist–receptor complex. The competing receptor was assumed not to contribute to the response. Hill slopes and EC_{50} values were obtained by fitting the simulated data to the dose–response equation: $y = \text{top}/(1 + 10^{(\log EC_{50} - x)h})$. (C) Experimental data showing dose–response curves with increasing target receptor concentrations. Agonist (Dex)-mediated repression of PMA-induced NFκB activity in COS-7 cells transfected with an NFκB-luciferase reporter gene and varying amounts of a GR expression vector was plotted. Fig. 3C was reprinted from *International Immunopharmacology*, vol. 3, Q. Zhao, J. Pang, M.F. Favata, J.M. Trzaskos, "Receptor density dictates the behavior of a subset of steroid ligands in glucocorticoid receptor-mediated transrepression", pp. 1803–1817, Fig. 2B, © (2003), with permission from Elsevier.

Prog, display similar AR agonist activity for transrepression on a NFκB promoter-reporter construct [23]. Although very little work has been done comparing relative potencies and efficacies of progestogens for transactivation or transrepression on endogenous genes, we have shown that saturating concentrations of MPA, NET-A and Prog exhibit promoter- and cell-specific regulation of endogenous cytokine genes in human cervical and vaginal epithelial cell lines [49]. Other studies have reported different 'efficacies' of some progestogens for expression of the low density lipoprotein receptor protein in a placental cell line, using a single and possibly non-saturating concentration of progestogens [50], while others reported differential effects of progestogens on expression of thrombospondin-1 mRNA in Ishikawa cells, using three concentrations of progestogens [51]. Some of these studies illustrate the difficulties in obtaining accurate potency, efficacy and biocharacter data from cell culture studies without the use of full dose response curves. We have shown by dose response analysis that MPA, as compared to other progestogens, exhibits very different potencies, efficacies and biocharacter for expression of endogenous genes in cells where the GR is the predominantly expressed SR, as assessed by receptor knock down strategies or the use of receptor-selective antagonists (unpublished data). More of such investigations are required, including investigating tissue/cell- and promoter-specific biological activities and the involvement of particular SRs and their isoforms.

In clinical and animal studies, the term 'potency' has been widely used to describe many different progestogen actions, including RBAs, relative responses to fixed constant doses in animal or clinical assays, or a dose required to obtain a particular defined response, which may not be maximal [3,52]. Some studies have included dose response analysis [53], but often only three doses are arbitrarily chosen and full curves are not generated [54,55]. Most assays describing the 'potency' of progestogens are based on the progestational effects of these ligands on uterine glandular proliferation, pregnancy maintenance, glycogen deposition in endometrial glands, delay-of menses, or inhibition of ovulation [3,5] using animal models [37,53,54] or healthy female volunteers [55–57]. An early study involving administration of three different doses of progestogens to women reported that the 'potency' of norgestrel was lower in a glycogen deposition assay than in the delay of menses test [55]. When assessing the significance of this result, it should be noted that the "potencies" determined were not obtained from full dose response curves and the plots were non-parallel. Another possible reason for differences in relative 'potencies' between different assays is the inclusion of estrogen together with the progestogen in some assays, such as in the delay of menses assay [58], but not others, since estrogen could affect the response by, for example, changing receptor levels.

A rat study by Lundeen and co-workers showed that TMG was more 'potent' than MPA when assessing complement component C3 expression in epithelial cells of the uterus, less 'potent' than MPA in the proliferation of endometrial stromal cells, and 'equipotent' in inhibiting ovulation [54]. In these assays, once again the plots generated did not reach a maximal level and were not parallel. Differences were also observed when comparing the 'potencies' of progestogens in a bioassay measuring endometrial transformation in rabbits ('potency' rank order MPA ≥ dydrogesterone > NET) to that of an ex vivo assay measuring their transactivation potential via the human PR ('potency' rank order MPA ≥ NET > dydrogesterone) [37]. Although the bioassay dose response curves did not reach a maximal level, indicating that the ED_{50} values obtained by this assay may be inaccurate, the change in rank order may also be due to difference in metabolism between the animal study compared to the in vitro cell line study. Interestingly, the rank order for 'potency' of the same progestogens in a clinical assay measuring endometrial protection, by dose

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response analysis, indicated that NET > MPA > dydrogesterone [59], possibly reflecting species-specific intracellular responses. A comparison between the rank order for potencies of progestogens in the above bioassays with their RBAs for the PR (Table 1), illustrates that generally the two do not correlate. There could be many reasons for this, including differences in the levels of competing SRs in the different target tissues/cells.

Not all of the biological activities exhibited by progestogens (Table 1) are mediated via binding to SRs. For example, the anti-estrogenic action of progestogens in the endometrium is due to the progestogen-bound PR suppressing ER gene expression, and consequently the ability of the cell to respond to estrogen. In addition, progestogen-bound PR activates the 17β-hydroxysteroid dehydrogenase type 2 enzyme which converts active estradiol to inactive estrone, as well as activating the estrone-sulfotransferase which causes the conjugation of estrone [5]. All the synthetic progestins in Table 1 display anti-estrogenic activity via this mechanism in animal models [5]. Similarly, Prog displays anti-androgenic effects, not via binding to the AR, but by inhibition of 5α-reductase activity, and hence decreasing the conversion of testosterone to the more active androgen, DHT. It is thus plausible that synthetic progestogens elicit similar effects. Indeed, the synthetic progestogen, dienogest, that has low relative binding affinity for the AR and potent anti-androgenic activity [2], has been shown to inhibit 5α-reductase activity [60]. The relative contribution of these non-receptor off-target effects will vary depending on the relative concentrations of the off-target proteins in different model systems.

4. Conclusions

Potency, efficacy and biocharacter are useful clinical parameters since they define the concentrations of progestogens required to cause or inhibit particular biological or physiological effects, the extent of that response, as well as how the response changes over a range of concentrations or doses, and are thus important to understand for hormonal therapy. Receptor affinity alone does not determine potency and appears unrelated to biocharacter. However, the affinity of a progestogen for a SR is a major contributor to potency, since together with concentrations of progestogen, competing endogenous hormone and receptor, affinity determines the fractional occupancy of the receptor, which in turn affects the percentage of maximal response. The current literature indicates that affinity, potency, efficacy and biocharacter are substantially different between progestogens and for the same progestogen in cell, tissue, animal, and clinical assays. Since potency, efficacy and biocharacter are operational definitions which always have to be defined for a particular model system and response, it is not surprising that these values will exhibit species-, tissue-, cell- and assay-specific differences for a particular progestogen. However equilibrium dissociation constants should theoretically not vary within a species.

Reported binding data in the literature are highly variable, most likely due to multiple factors, including methodological differences, and differential affinities of progestogens for binding to the MR, AR and GR, which exhibit varying relative concentrations in different assay systems. The different binding affinities of progestogens for different SRs are likely to be a major determinant of differential actions of progestogens in a tissue- and cell-specific manner and be highly relevant to side-effects of progestogens. For example, since MPA has a relatively high affinity for the ubiquitously expressed GR [2] and is widely used in hormonal therapy and contraception, this raises important questions about glucocorticoid-like side-effects of MPA on cardiovascular effects, breast cancer, immune function, bone density and susceptibility to infectious diseases [2,3,8,35,37,52,56,59,61,62]. It is thus essential that accurate affinities of progestogens are determined for different SRs, in order to better assess their potential differential side-effect profiles and aid in new drug design. When designing binding experiments, potential confounding factors such as metabolism, off-target binding to other receptors, methodology and analysis thereof, need to be carefully considered, since they can lead to shifts in the IC_{50} values, Hill slopes and hence inaccurate RBAs and K_d values.

Taken together, it is clear that the task of contextualizing the 'potencies' determined for different progestogens in different assay systems is challenging as each assay tells a different story. Clinically it would be useful to know the potencies, efficacies and biocharacters of different progestogens for specific biological and physiological responses. This would enable better prediction of the minimum dose required for a maximal response, as well as the dose range likely to result in the most varied intra and inter-individual responses (steep part of the dose response curve), along with likely side-effect profiles. Many factors would result in expected differences in relative potencies, efficacies and biocharacter for ex vivo, animal and clinical assays. These include route and time of administration, co-administration of estrogen, species-specific metabolism and physiology, affinities of progestogens for different steroid receptors, the relative concentrations of off-target receptor and non-receptor proteins, as well as promoter-specific transcriptional effects of progestogens in different target tissues/cells. For example, the fact that MPA is more 'potent' than NET in a rabbit model, does not imply that it will be more potent in an ex vivo assay via the PR, or more 'potent' as a contraceptive in humans. Indeed, the many varying factors mentioned above preclude precise comparisons of the relative 'potencies' determined by animal, clinical, and ex vivo assays. However, it is likely that many of the reported differences in 'potencies' for animal and clinical assays are confounded by the methods used to determine potency. Since the term 'potency' means different things to different people, results comparing relative activities of progestogens need to be interpreted in this context.

Clearly more clinical and molecular data is required to better quantify the relative affinities, potencies, efficacies and biocharacters of progestogens in different, animal and clinical models, toward better understanding of their mechanisms of action, assessment of their relative side-effects and improvement of choice and concentrations of progestogens in hormonal therapy. An awareness of the pharmacological background and standardization of definitions would also improve our understanding of the significance of the current and future data.

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Differential Glucocorticoid Receptor-Mediated Effects on Immunomodulatory Gene Expression by Progestin Contraceptives: Implications for HIV-1 Pathogenesis

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Whether hormonal contraceptives increase HIV-1 acquisition, transmission and disease progression are critical questions. Clinical research has been hampered by a lack of understanding that different progestins used in contraception exhibit differential off-target effects via steroid receptors other than the progesterone receptor. Of particular, relevance is the relative effects of medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN), widely used as injectable contraceptives in sub-Saharan Africa. While most high-quality clinical studies find no increased risk for HIV-1 acquisition with oral contraception or injectable NET-EN, most do find an increase with MPA, particularly in young women. Furthermore, mounting evidence from animal, *ex vivo* and biochemical studies are consistent with MPA acting to increase HIV-1 acquisition and pathogenesis, via mechanisms involving glucocorticoid-like effects on gene expression, in particular genes involved in immune function. We report that MPA, unlike NET and progesterone, represses inflammatory genes in human PBMCs in a dose-dependent manner, via the glucocorticoid receptor (GR), at concentrations within the physiologically relevant range. These and published results collectively suggest that the differential GR activity of MPA versus NET may be a mechanism whereby MPA, unlike NET or progesterone, differentially modulates HIV-1 acquisition and pathogenesis in target cells where the GR is the predominant steroid receptor expressed.

Introduction

Whether hormonal contraceptives increase HIV-1 acquisition is a critical and unresolved issue, in particular for young women in high risk areas, and is the subject of intense worldwide research and debate.[1,2] Furthermore, whether hormonal contraceptives increase HIV-1 transmission and disease progression, as well as increase susceptibility to other infectious diseases and STIs, are also critical health issues. These issues are also mostly unresolved and are topics of ongoing research. Clinical research in these areas has been hampered by a lack of understanding of the molecular mechanisms of action of the progestin components of contraceptives and by a lack of appreciation of the differences between progestins, which cannot be considered to act as a single class of compounds regarding their side effect profiles.[3–5] Although progestins are designed to act like the natural ligand progesterone (P4) via the progesterone receptor (PR), they are likely to exert very different side-effects due to their differential affinities and activities via other members of the steroid receptor family.[3–5] Currently a wide range of progestins is used in contraceptives, with several different delivery methods and varying progestin doses. Until recently, clinical studies investigating effects of contraceptives on HIV-1 have grouped together data based on methods of delivery (injectable versus oral), while ignoring both the type of progestin and

dose used within those groups. More recently, attempts have been made to investigate effects of one progestin versus another, but usually the sample size has been too small to establish significant differences, above other confounding variables. Nevertheless, a picture is emerging from clinical, animal, *ex vivo* and biochemical studies that the injectable contraceptive, medroxyprogesterone acetate (MPA), is likely to increase HIV-1 acquisition in women and transmission from women to men.

The injectable contraceptive medroxyprogesterone acetate (MPA), unlike norethisterone enanthate (NET-EN), appears to increase HIV-1 acquisition

MPA, administered for contraception as Depo-MPA (DMPA) or Depo-Provera, is a 150 mg 3-monthly intramuscular injection that is used by millions of women worldwide, particularly in sub-Saharan Africa with high HIV-1 prevalence.[1,6] Norethisterone enanthate (NET-EN) is a 200 mg 2-monthly injectable that is used less than MPA globally, although its usage is high in some regions of South Africa.[7] Injectable contraceptive usage and HIV-1 prevalence are particularly high in young women in some areas of South Africa, such as at the KwaZulu-Natal site for the CAPRISA microbicide trial. In this trial, about 82% of the women investigated were on injectable progestin-only contraceptives, as compared to 15% on oral contraceptives.[8] The majority of high-quality studies, in which the most common progestin used was levonorgestrel (LNG), showed no significant increase in HIV-1 acquisition for oral contraceptive pills.[9] Although only a few studies have investigated the risks associated with the use of injectable NET-EN on HIV-1 acquisition, none have shown a significant association with HIV-1 acquisition.[9] However, the majority of high-quality studies do show an increased risk associated with only or predominantly injectable DMPA usage.[9–11] Many clinical observational studies do not investigate risks associated with subgroups of women, such as those grouped by age or HSV-2 status, usually due to a lack of sufficient study size for a particular subgroup. However, of particular concern is the finding of an adjusted hazard ratio (HR) for DMPA of 9.29 in women aged 18–20.[12] Although others have not reported such age-related findings, they may not have had sufficient young women with high HIV-1 exposure in their studies.[13–16] Also of concern is the finding of an HR of 4.5 for DMPA for HSV-2 negative women.[12] However, establishing indisputable evidence from such studies is extremely difficult due to methodological challenges and multiple confounding factors, such as the degree of exposure to HIV-1, condom usage, HSV-2 exposure as well as varying ages of women that have been enrolled in these studies.[9] Furthermore increases in both HIV-1 and HSV shedding have been reported in women using contraception,[17–19] as well as the presence of more viral variants and higher viral loads in HIV-1-infected DMPA users than non-users.[20] These findings are consistent with an increase in HIV-1 transmission found for injectable DMPA users.[16] DMPA usage has also been associated with increased acquisition of cervical chlamydial and gonococcal infections.[21] These STIs have also been linked to increased HIV-1 acquisition. Consistent with the clinical data, MPA increases susceptibility to vaginal simian–human immunodeficiency virus (SHIV) transmission and suppresses the antiviral cellular immune response in SHIV-infected rhesus macaques.[22] No information is available regarding other progestins and HIV-1 acquisition. Contraceptives may also affect diseases progression. Most of the clinical studies to date indicate no effect of hormonal contraception on disease progression. However, this may be due to several study limitations such as insufficient power to discriminate between progestin groups or methods of contraception delivery, analysis of only chronically infected patients, and confounding variables such as antiretroviral (ARV) usage and pregnancy. This suggests that effects of specific progestins like MPA on disease progression from the time of HIV-1 acquisition, cannot as yet be excluded.[23,24] Collectively, these findings raise questions as to the choice of progestin, dose and method of administration in determination of susceptibility to and transmission of pathogens, such as HIV-1, particularly in young women. Since the current data from observational studies are not considered persuasive enough to recommend using methods of contraception other than DMPA,[1,2] more research is urgently required. The key question is what information is needed and how feasible it is to obtain. Some have argued that more large-scale randomized trials are needed,[25] but these may not be ethical or conclusive. Another approach is to investigate direct biological effects and mechanisms *ex vivo* of different progestins at specific doses for different target cells relevant to HIV-1 pathogenesis.

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The mechanism of action is likely to involve differential effects on gene expression on immune function genes by MPA versus NET, mediated via the glucocorticoid receptor

Effects of progestins on HIV-1 acquisition and transmission are proposed to occur via multiple mechanisms, including changes in vaginal structure, endometrial thinning, effects on genital flora, effects of other sexually transmitted infections, as well as changes in immune function.[26] At the cellular level, progestins and sex steroid hormones mediate their effects via alterations in transcription of specific genes via binding to and regulating the activity of steroid receptors.[4] Progestins differ widely in their steroid receptor selectivity profiles.[3–5] MPA, NET and LNG all exhibit some binding to the androgen receptor with undetectable anti-androgenic activity and some androgenic activity. MPA and NET exhibit very little antimineralocorticoid activity at contraceptive doses,[27] unlike some reports for LNG.[4,27] However, MPA binds to the GR with an affinity (Kd of 4–11 nM) similar to that of the endogenous glucocorticoid cortisol and acts as a full to partial agonist for the GR, whereas P4 and NET bind to the GR with about a 50- to 100-fold lower affinity and are very weak partial GR agonists with much lower potency and efficacy.[4,5] In contrast, the fourth-generation progestins are much more selective for the PR. The GR is a well-established regulator of immune function via regulation of transcription of a wide variety of immune function genes. Glucocorticoids are potent immunosuppressive agents which exert multiple complex actions on dendritic cells, myeloid cells and B- and T-lymphocytes, including effects on apoptosis and differentiation fate and inhibition of cytokine release and cell migration.[28–30] Consequently, glucocorticoids are widely used as anti-inflammatory and immunosuppressive drugs. The immunosuppressive actions of glucocorticoids are largely due to their repression of transcription of target genes such as cytokine and chemokine genes, via inhibition of the transcription factors nuclear factor kappa B (NFκB) and activator protein 1 (AP-1).[30] Glucocorticoids, like cortisol, bind to inactive GR in the cytoplasm. Ligand-bound GR is subsequently hyperphosphorylated and translocates into the nucleus where it is able to modulate transcription. Once in the nucleus, ligand-bound GR binds as a dimer to glucocorticoid response elements (GREs) resulting in positive transcriptional regulation, called transactivation.[28–30] Additionally, ligand-bound monomeric GR is also known to negatively regulate transcription by interfering either directly or indirectly with other DNA-bound transcription factors, like NFκB or AP-1.[30] This mechanism, known as transrepression, prevents NFκB- and AP-1-mediated transcription.[28–30] Given that MPA, unlike other progestins, acts as a relatively potent full to partial agonist for the GR, MPA is likely to exert much more potent and efficacious effects on gene promoters involved in immune function than P4 or NET via the GR, as we have demonstrated in cell lines.[31–33] We proposed in 2004 that due to the differential activity of MPA versus NET via the GR, MPA is likely to exert different effects on immune function and hence modulate susceptibility to infections like HIV-1.[3] MPA has been reported to modulate transcription of a number of genes via the GR, including IL2 in normal human lymphocytes,[34] and IL6 and IL8 in a mouse fibroblast cell line.[31] We have also recently shown that MPA, unlike NET and P4, exhibits differential regulation of apoptotic genes and dose-dependently enhances HIV-1-mediated apoptosis in primary human $CD4^+$ T cells via a GR-dependent mechanism,[35] consistent with the steroid receptor selectivity of these ligands. Furthermore, MPA, to a greater extent than P4, suppresses *ex vivo* the production of key regulators of cellular and humoral immunity involved in orchestrating the immune response to invading pathogens such as HIV-1, most likely via the GR.[36] It remains to be determined how different progestins affect expression of key genes involved in immune function in primary cells relevant to HIV-1 pathogenesis.

We have addressed this question in peripheral blood mononuclear cells (PBMCs), representing key targets for HIV-1 infection and replication. We determined the effects of varying doses of MPA, NET and P4, in comparison with dexamethasone (DEX), a synthetic GR agonist, on expression of four-key immunomodulatory genes namely, glucocorticoid-interacting leucine zipper (GILZ), interleukin (IL) 6, IL8 and regulation on activation normal T cell expressed and secreted (RANTES). The GILZ gene is GRE-regulated and encodes an anti-inflammatory protein that inhibits the activation of pro-inflammatory transcription factors.[37] IL6 and IL8 genes, which are upregulated by NFκB and AP-1, are pro-inflammatory cytokines expressed by a number of innate

and adaptive immune function cells.[38] RANTES (CCL5), also upregulated by NFκB, is a chemokine that is involved in the recruitment of mononuclear cells to sites of chronic inflammation[39] and interferes with HIV-1 entry into target cells by competitive binding to the CCR5 co-receptor.[40] A decline in RANTES protein expression is also associated with an increase in HIV-1 disease progression.[39] Freshly isolated PBMCs were stimulated with varying concentrations of the different ligands for 48 hrs, followed by RNA isolation, reverse transcription and real-time quantitative PCR. The results show that DEX and MPA, unlike NET and P4, repress IL6 and IL8 pro-inflammatory gene mRNA levels while increasing GILZ anti-inflammatory mRNA levels (Fig. 1), while RANTES mRNA repression was only detectable with DEX under these conditions (Fig. 1). Concurrent with mRNA expression analysis, protein expression analysis by flow cytometry indicated that the progestins mediated differential regulation on IL6 and GILZ protein levels (Fig. 1 E and F). MPA and DEX significantly repressed IL6 protein expression after 48 hrs, while P4, NET-A, NET and LNG did not affect basal expression in $CD14^+$ monocytes (Fig. 1e). DEX significantly increased GILZ protein expression levels in $CD4^+$ T cells, while MPA indicated a moderate, but not significant, increase in basal expression of GILZ after 48 hrs. P4, NET, NET-A and LNG did not affect GILZ protein expression levels (Fig. 1 e and f). Both NET and NET-A were included in these experiments to show that the lack of an effect with NET-A is not due to an inability to be metabolized to NET in PBMCs. We did not detect substantial GILZ expression in monocytes or IL6 expression in $CD4^+$ T cells. These results showing immunosuppressive effects of MPA on gene expression are consistent with our previous data,[31–33] as well as with results reported by others in Bacillus Calmette–Guerin-stimulated PBMCs and murine *M. tuberculosis* models,[41,42] and those from the Hel laboratory in activated T cells and in primary vaginal mucosal mononuclear cells (VMMCs)[36] and cytobrushes from patients on DMPA.[36]

To investigate the role of the GR in mediating the responses, PBMCs were treated with DEX and MPA in the absence and presence of RU486, a known GR antagonist. In Fig. 2, IL6, IL8 and RANTES mRNA repression by DEX and MPA was lifted in the presence of RU486, while DEX and MPA induction of GILZ mRNA was attenuated by RU486 (Fig. 2 a-d). Similarly Huijbregts et al. found that RU486 treatment together with MPA lifted the MPA-mediated repression of IFN-γ protein expression in VMMCs and activated PBMCs, $CD3^+$ and $CD14^+$ cells.[36] Although RU486 can also antagonize the PR, we have recently shown that PBMCs under our conditions express no detectable PR mRNA or protein.[35] Thus, our RU486 results support a role for the GR in mediating these anti-inflammatory effects in PBMCs. Importantly we show for the first time that the GR is likely to play a key role in discriminating between immunosuppressive responses by MPA versus NET and P4 in primary human cells representing key targets for HIV-1 infection and replication. We have recently shown that this discriminatory role of the GR is also observed in the End1/E6E7 endocervical epithelial cell line, a model for the endocervical mucosa and site of heterosexual HIV-1 transmission (data not shown). Interestingly in these cells, as well as in primary endocervical epithelial cells, the GR is the predominant steroid receptor expressed (data not shown).

The concentrations of progestins used are critical and determine dose–response

Previous research on the biological effects of progestins and P4 has been hampered by a lack of understanding of the significance that doses have in determination of physiologically relevant responses.[43] Unfortunately there is also a paucity of information about serum concentrations of progestins in contraception users, which show high interindividual variability, as well as concentrations in tissues at target sites, which may not mimic serum concentrations. We investigated whether the observed effects of MPA, NET and P4 are relevant to the physiological doses of these ligands[4,5] by dose–response analysis to determine potencies and efficacies.[43] Endogenous P4 serum concentrations vary substantially during the menstrual cycle in pre-menopausal women and are low during the follicular phase (0.65 nM), rising to about 80 nM during the luteal phase, with higher concentrations of about 600 nM during pregnancy.[4] MPA serum concentrations are reported to be in the range 2.5 to 65 nM a few days after injection in injectable users and to plateau at about 2.6 nM for about 3 months, while serum concentrations of NET-EN are reported to be in the range 1.5–59 nM.[4,5] Our dose–response analysis in these experiments suggests that MPA is likely to exert anti-inflammatory immunosuppressive effects via GILZ gene expression within the range of concentrations found in the serum of

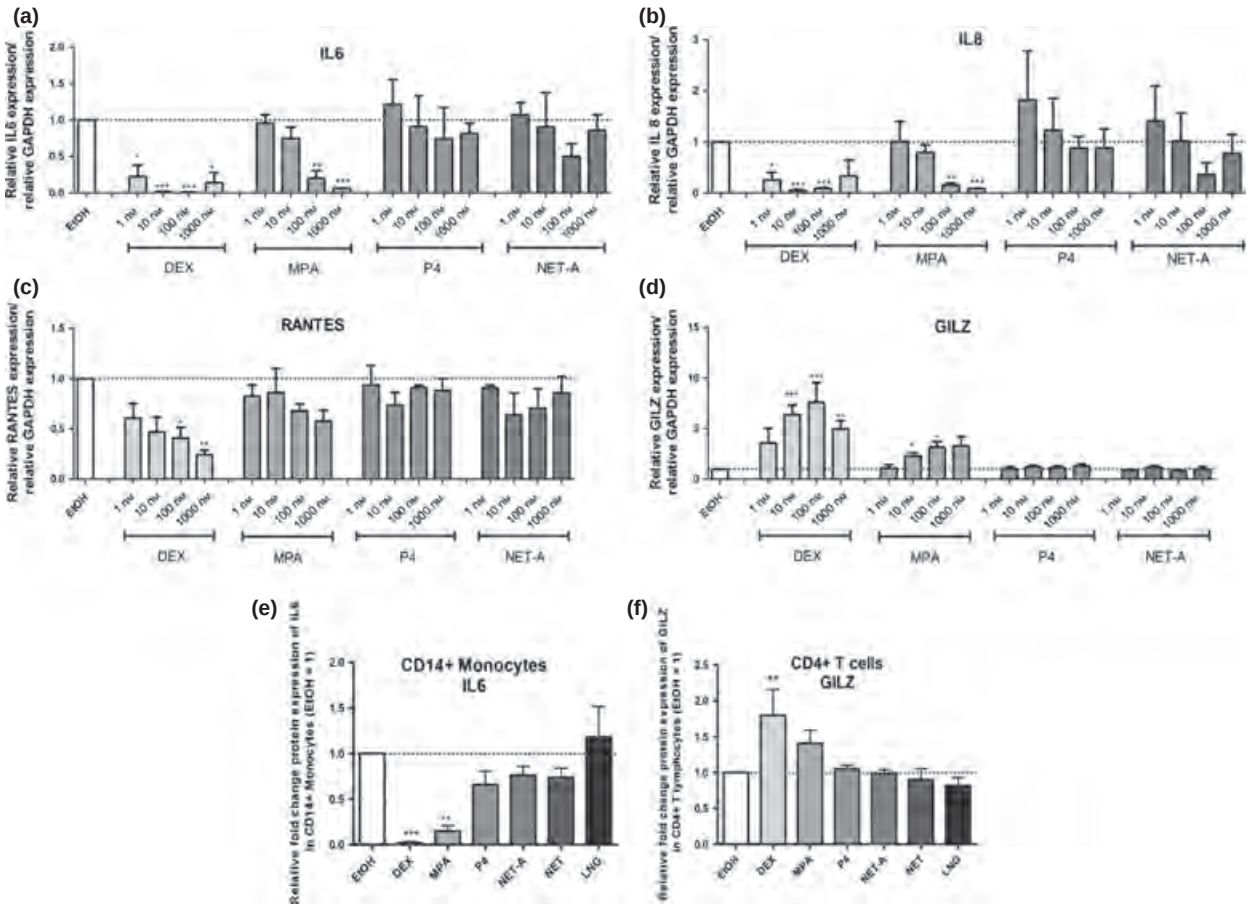

Fig. 1 DEX and MPA, dose-dependently regulate IL6, IL8, RANTES and GILZ mRNA levels in primary human PBMCs and decrease IL6 protein expression in $CD14^+$ monocytes and increase GILZ expression in $CD4^+$ T cells. (a-d) PBMCs were stimulated with increasing amounts of DEX, MPA, P4, NET-A or vehicle (EtOH) for 48 hrs. Relative levels of IL6 (a), IL8 (b), RANTES (c) and GILZ (d) mRNA were normalized to GAPDH mRNA levels. (e-f) PBMCs were treated with 100 nM DEX and MPA, 1 μM P4, 10 μM NET-A, NET, and LNG (GR saturation concentrations) or vehicle (ETOH) for 48 hrs. Thereafter, cells were stained with surface antibody markers ($CD4^+$ and $CD14^+$) followed by IL6 (e) or GILZ (f) intracellular antibody staining for protein expression. Protein expression was measured using a Becton–Dickinson FACS Calibur flow cytometer and analysed using FlowJo software (Version X, Treestar Inc., Ashland, Ore). Fold changes in protein expression are indicated, with vehicle (EtOH) set to 1. Histograms show pooled results from three to five independent experiments with samples from female donors. Statistical significance was determined by one-way ANOVA with Student's *t*-test to control where, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

injectable contraceptive users (1–100 nM), as well as possibly on IL6 and IL8, particularly shortly after injection. However, the immunosuppressive effects of NET-EN on PBMCs via the GR are likely to be negligible, even shortly after injection, while P4 is unlikely to exert immunosuppressive effects in PBMCs even during pregnancy via these genes. Interestingly, we have recently shown that the GR may also play a key role in PBMCs in discriminating between apoptotic effects by MPA, unlike NET and P4, at doses within the contraceptive range, in the absence and presence of HIV-1 infection.[35] The physiological consequences of immunosuppressive and apoptotic effects of MPA on PBMCs could include a wide range of effects beneficial to the virus. These could include compromising the ability of the host to mount effective innate and adaptive immune responses, as well as increasing binding of HIV-1 to CCR5 co-receptors. Effects seen with MPA in PBMCs may also occur in T cells and monocytes in the genital mucosa.

Conclusions

While controversy continues, a trend is emerging from multiple clinical studies that the injectable contraceptive DMPA appears to increase HIV-1

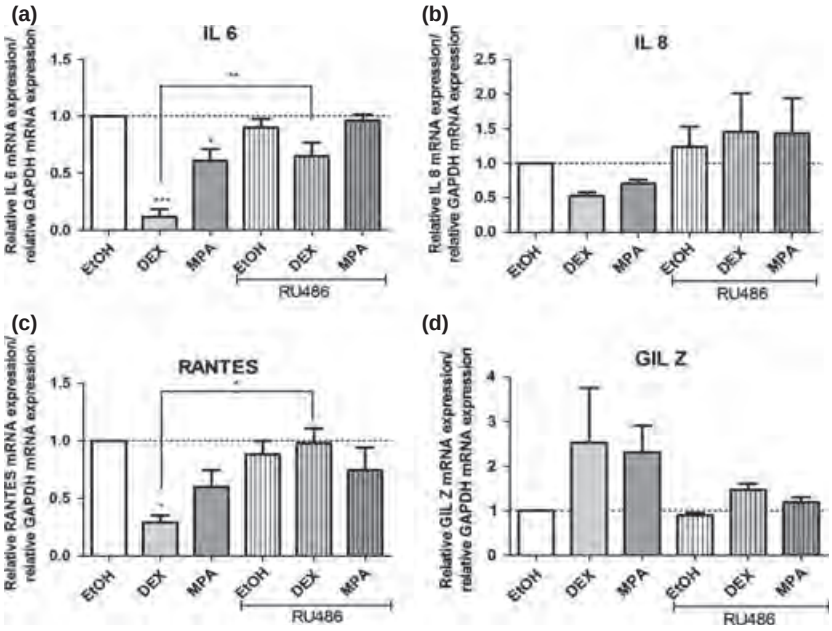

Fig. 2 DEX- and MPA-mediated regulation of GILZ, IL6, IL8 and RANTES mRNA requires the presence of the GR. (a-d) PBMCs were stimulated with 100 nM DEX and MPA in the presence and absence of 1 μM RU486 for 48 hrs. Relative levels of IL6 (a), IL8 (b), RANTES (c) and (d) GILZ transcripts were determined by qPCR and normalized to GAPDH. Histograms show results from three independent experiments. Statistical significance was determined by one-way ANOVA with Dunnet's post-test or paired student *t*-tests. $P < 0.05$ denoted as *, $P < 0.01$ denoted as ** and $P < 0.001$ denoted as ***.

acquisition and transmission, in particular in young women with high exposure to HIV-1, unlike other progestin contraceptives, such as the injectable contraceptive NET-EN. Several modelling studies have attempted to weigh up the competing risks between contraception and HIV-1 acquisition versus withdrawal of contraception and associated maternal mortality and other factors associated with unwanted pregnancies.[44–46] Their results suggest that with HRs <2, the risks of withdrawal from DMPA outweigh potential benefits. However, it should be noted that risk assessments have not, to our knowledge, been published for HRs associated with only younger women in high risk areas for HIV-1 infection, or for changing to another method of contraception such as NET-EN, which has similar pregnancy risks and compliance factors to DMPA. This would seem highly relevant, given that risks for young women in sub-Saharan Africa carry a disproportionate percentage of the worldwide HIV-1 burden and are the highest users of DMPA. Although the findings showing increased HIV-1 acquisition and transmission with DMPA could be due to confounding behavioural factors, the finding that NET-EN does not increase HIV-1 acquisition argues against this, since DMPA and NET-EN users would be expected to display similar behavioural factors such as condom usage. Furthermore, both animal and *ex vivo* biochemical studies, using contraceptive concentrations of MPA, support the possibility that MPA increases HIV-1 infection and transmission.[47] We show directly for the first time that the GR is likely to play a key role in discriminating between immunosuppressive responses by MPA versus NET and P4 in primary human cells representing key targets for HIV-1 infection and replication. Biochemical studies such as we report here provide strong support for a mechanism whereby MPA, unlike other progestins like NET-EN, is likely to modulate gene expression and immune function, at concentrations within the range of peak serum MPA concentration in injectable users, acting via the GR. The GR is ubiquitously expressed, unlike the PR. More research is needed to investigate the concentrations of different progestins in target tissues and to assess their likely dose–response effects. Given its central role in transcription regulation in all mammalian cells, effects of MPA on gene expression via the GR are likely to be the molecular basis for several differential effects of MPA versus other progestins that do not act via the GR. These could include effects on HIV-1 replication, host cell apoptosis, expression levels of several host cell receptors, cell cycle and proliferation effects and epithelial cell barrier function. Future research on the effects of different progestins on modulation of HIV-1 pathogenesis, at doses within the contraceptive range, in primary cell and tissue models will undoubtedly provide necessary insight into the question of choice of progestin and dose effects. However, it remains to be seen whether confirmatory information from such studies will be

enough to change health policy, in the absence of clinical data, which may not be possible or feasible to obtain.

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The Progestin-Only Contraceptive Medroxyprogesterone Acetate, but Not Norethisterone Acetate, Enhances HIV-1 Vpr-Mediated Apoptosis in Human CD4$^+$ T Cells through the Glucocorticoid Receptor

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Abstract

The glucocorticoid receptor (GR) regulates several physiological functions, including immune function and apoptosis. The HIV-1 virus accessory protein, viral protein R (Vpr), can modulate the transcriptional response of the GR. Glucocorticoids (GCs) and Vpr have been reported to induce apoptosis in various cells, including T-cells. We have previously shown that the injectable contraceptive, medroxyprogesterone acetate (MPA) is a partial to full agonist for the GR, unlike norethisterone acetate (NET-A). We investigated the functional cross talk between the GR and Vpr in inducing apoptosis in CD4$^+$ T-cells, in the absence and presence of GCs and these progestins, as well as progesterone. By using flow cytometry, we show that, in contrast to NET-A and progesterone, the synthetic GR ligand dexamethasone (Dex), cortisol and MPA induce apoptosis in primary CD4$^+$ T-cells. Furthermore, the C-terminal part of the Vpr peptide, or HIV-1 pseudovirus, together with Dex or MPA further increased the apoptotic phenotype, unlike NET-A and progesterone. By a combination of Western blotting, PCR and the use of receptor- selective agonists, we provide evidence that the GR and the estrogen receptor are the only steroid receptors expressed in peripheral blood mononuclear cells. These results, together with the findings that RU486, a GR antagonist, prevents Dex-, MPA- and Vpr-mediated apoptosis, provide evidence for the first time that GR agonists or partial agonists increase apoptosis in primary CD4$^+$ T-cells via the GR. We show that apoptotic induction involves differential expression of key apoptotic genes by both Vpr and GCs/MPA. This work suggests that contraceptive doses of MPA but not NET-A or physiological doses of progesterone could potentially accelerate depletion of CD4$^+$ T-cells in a GR-dependent fashion in HIV-1 positive women, thereby contributing to immunodeficiency. The results imply that choice of progestin used in contraception may be critical to susceptibility and progression of diseases such as HIV-1.

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Introduction

Globally women account for ~49% of HIV infections [1], with greater prevalence among young women vulnerable to both pregnancy and HIV-infection than in men [2]. In Sub-Saharan Africa, 59% of all those infected are women [3]. There is substantial evidence from clinical studies that hormonal contraception increases HIV-1 acquisition and transmission in young women and disease progression, although some of the findings are controversial, and some studies report no significant effects [4–15]. Of particular interest are the relative effects of the two most commonly used injectable contraceptives, MPA and norethisterone enanthate (NET-EN), in HIV-1 infection and AIDS progression. MPA, administered for contraception as Depo-MPA (DMPA) or Depo-Provera, is a 150 mg three-monthly intramuscular injection that is used by millions of women worldwide and is widely used in Sub-Saharan Africa and other areas with high HIV-1 prevalence [6,14–17]. NET-EN is a 200 mg two-monthly injectable that is used less than MPA, although in countries like South Africa, its usage varies and is high in some regions [8,17–19]. Both contraceptives have been shown to be highly effective and relatively safe regarding most risk factors investigated [20]. However, there is evidence that DMPA but not NET-EN increases HIV infectivity [5,6,8,10,15,21,22]. Increases in both HIV-1 and HSV shedding have been reported in women using contraception [23–25], as well as the presence of more viral variants and higher viral loads in HIV-1 infected DMPA users than non-users [7], consistent with an increase in HIV-1 transmission found for DMPA users [5]. DMPA usage has also been associated with increased acquisition of cervical chlamydial and gonococcal infections [26]. In addition, while there is evidence both for and against an increase in disease progression in the absence of antiretroviral drugs (ARVs) for HIV-1 positive DMPA

users [7,9,11–13], to our knowledge no information is available for effects of NET-EN. Whether DMPA or NET-EN affect disease progression in HIV-1 positive antiretroviral users remains to be established, although one study suggests no significant change in $CD4^+$ counts for DMPA users with and without antiretroviral drug usage [27]. Adjusted hazard ratios (HRs), (reflecting the fold increased risk relative to no contraception), of between 1.5 and 4.5 fold have been recently reported for DMPA (author's response in Gray [4,5,8,10]), while one study reported an HR as high as 10.4 for DMPA [28]. However, establishing indisputable evidence from such clinical observational studies is extremely difficult due to multiple confounding factors such as the degree of exposure to HIV-1, condom usage, HSV-2 exposure as well as varying ages of women that have been enrolled in these studies [15]. Therefore, a central question remains as to what extent and via which mechanisms different synthetic progestins affect HIV-1 pathogenesis at contraceptive doses and at various target sites, a question perhaps best answered by *ex vivo* studies.

At the cellular level, progestins mediate their effects via alterations in transcription of specific genes in target cells by binding to and regulating the activity of steroid receptors, which are ligand-activated transcription factors [29,30]. Although progestins are designed to act like the natural ligand progesterone (P4) via the progesterone receptor (PR), they are likely to exert very different off target side-effects due to their differential affinities and activities via other members of the steroid receptor family of receptors [29–31]. NET-EN is converted to norethisterone (NET) in the body, while water soluble derivatives of NET-EN such as NET or norethisterone acetate (NET-A are used orally for hormone replacement therapy) [27,28]. We have shown that MPA and NET-A have different affinities for and activities via the glucocorticoid receptor (GR) [32–34]. MPA binds to the GR with a relatively high affinity and acts as a full to partial agonist for the GR, whereas NET-A and P4 bind to the GR with about 100-fold lower affinity and have little or no activity via the GR. This differential activity via the GR suggest that MPA and NET may exert different effects on HIV-1 pathogenesis via the GR, in particular different effects on immune function, since the GR regulates transcription of a wide variety of genes involved in inflammation, immunity, and apoptosis [35,36]. Several different mechanisms could contribute to the observed effects of DMPA usage on HIV-1 pathogenesis, including alterations in the composition of mucosal microflora and thinning of the cervical/vaginal epithelium. However, the high affinity of MPA for the GR and the known effects of the GR on immune function suggest that effects of DMPA on both systemic and local immunity via the GR may be highly significant.

Although very few studies have investigated this hypothesis, some do show that MPA affects immune function *in vivo* in animals and humans. Two studies in mice provide evidence that MPA suppresses immune function to increase susceptibility to infections or reduce defense against disease in mice, at similar doses to those of women using DMPA [37,38]. Studies in primates have shown that DMPA reduces systemic immune responses in SIV-infected macaques [39,40]. MPA used at high doses in cancer therapy is known to cause significant systemic immunosuppression in patients [41–44] and a decrease in T-cell numbers and proliferation in breast cancer patients [41,44]. Furthermore, DMPA as a contraceptive has been shown to compromise cell-mediated immune status [45] and causes increased recruitment of inflammatory cells in cervical vaginal lavages in women [46]. A recent *ex vivo* study in primary immune function and cervical cells from patients showed that MPA, unlike P4, suppresses both innate and adaptive immune mechanisms at concentrations within the range of peak serum concentrations found in DMPA users [47,48]. Importantly, the findings from the group of Hel [47] showing significant repression of IFNλ in peripheral blood mononuclear cells (PBMCs) and lavages from DMPA users but not non-contraceptive users strongly supports the idea that DMPA concentrations *in vivo* are sufficient for immunosuppression. Interestingly, P4 is also known to regulate both the innate and adaptive immune response in the female reproductive tract, other mucosal tissues as well as systemic immune function in humans. However, the precise mechanisms and receptors involved in this regulation are not well understood, but appear to be specific for different target sites and cell types [6,49,50]. In contrast, very little is known about the effects of NET-EN/NET/NET-A on immune function in women.

HIV-1 infection is characterized by rapid and extensive $CD4^+$ T-cell depletion and eventual immunodeficiency. HIV-1-induced apoptosis appears to play an important role in depletion of $CD4^+$ T-cells, decreasing immune responses to infection and facilitating viral persistence and increased viral loads and transmission rates [51]. Furthermore, the loss of $CD4^+$ T-cells correlates with disease progression and increases in opportunistic infections [52]. Although the exact mechanisms and role of apoptosis during disease progression remain to be resolved, several HIV-1 proteins have been implicated in inducing apoptosis in T-cells, including the 96 amino acid HIV-1 accessory protein viral protein R (Vpr) [53]. Besides apoptosis, Vpr has been implicated to play a part in other cellular functions such as cell cycle arrest at G2/M phase and transport of the pre-initiation complex [53]. Vpr is packaged within the virus particle where it is thought to be involved in the early stages of viral replication through transactivation of the HIV-1 long terminal repeat (LTR) [53]. The clinical observations that mutations in key Vpr residues are associated with normal capacity to replicate but loss of cytotoxicity [54] and long-term non-progressive HIV-1 infections [55], support an important cytotoxic role for Vpr in HIV-1 infection. Such a cytotoxic role may be exerted by both virus-associated as well as virus-free Vpr, since functional Vpr protein has been purified from serum and cerebrospinal fluid of infected patients [56–58]. Vpr in the plasma of HIV-1 infected individuals is present at similar concentrations as the p24 antigen and has the ability to self-penetrate cells (transduction properties) and to elicit its effects, including apoptosis, in non-infected bystander cells [56,59]. Mapping experiments of the Vpr protein indicated that amino acids 1–52 are important for the transduction properties of Vpr, but not for induction of apoptosis [60]. The C-terminus (amino acids 52–96) of Vpr, in particular amino acids 71–82 (71-HFRIGCRHSRI-82), have been shown to be indispensable for apoptotic function [61–64]. Although Vpr can induce apoptosis via the extrinsic pathway in neuronal and epithelial cells [65–68], it has been implicated to act predominately through the intrinsic pathway in a number of other cell lines and primary cells, including T-cells [69].

Reminiscent of the effects of Vpr on apoptosis, GCs, like cortisol, (F) acting via the GR, are also potent inducers of apoptosis in a number of different cells, including T-cells [35]. Several lines of evidence suggest that Vpr regulates transcription of host and viral genes via the GR [70]. The mechanism may involve an interaction of Vpr with the GR to modulate GR transcriptional activity, as Vpr has been reported to associate with the GR *in vitro* and modulate transcription of both host and viral genes [71,72]. This interaction was reported to occur via a signature LXXLL steroid receptor co-activator motif [73]. Consistent with a role for the GR in mediating Vpr effects on apoptosis at the transcriptional level, it has been shown that RU486, a GR antagonist, prevents Vpr-mediated apoptosis in the Jurkat T-cell line [74].

Cross talk between Vpr and the GR raises the question as to how different GR ligands such as MPA would affect GR- and Vpr-mediated T-cell apoptosis. As both the GR and Vpr have been implicated to play a role in apoptosis in a number of cell lines and primary cells, we sought to investigate the possible cross talk between the GR and Vpr in modulating apoptosis in the presence of the progestins, MPA and NET-A, and P4 in PMBCs.

Materials and Methods

Ethics Statement

Anonymous buffy packs, otherwise normally discarded, were obtained from the Western Province Blood Transfusion (WPBT) services in Pinelands, Cape Town. Written informed consent was obtained from donors by WPBT and records kept by WPBT. The Ethics Committee of the University of Cape Town (N05/11/187) approved the procedure (SFREC_ 04_2010).

Plasmids, Western Blotting and Antibodies

The plasmids used in this study were as follows: pcDNA3-hGR (GR) plasmid was a gift from Prof. D.W. Ray (Centre for Molecular Medicine, School of Clinical and Laboratory Sciences, Faculty of Medical and Human Sciences, University of Manchester, UK). pMT-PR-B (PR) was obtained from Prof. S. Okret (Karolinka Institute, Sweden). pRS-hMR (MR) expression plasmid was obtained from Prof. R.M. Evans (University of California, USA). pSV-hAR (AR) was a kind gift from Frank Claessens (Catholic University of Leuven, Belguim). pSG5-hER (ER) was obtained from F. Gannon (EMBL, Germany). The positive controls for each steroid receptor were generated in COS-1 cells (ATCC). Briefly, COS-1 cells were seeded at a density of 1×10^5 cells in a 12-well plate. After 24 hrs the cells were transfected with 1 μg of the steroid receptor expression vector using X-treme-GENE 9 DNA Transfection Reagent (Roche Applied Sciences) according to the manufacturer's specifications. The next day whole cell lysates were prepared using a N-[Tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid (TAPS) buffer (0.1 M TAPS, pH 9.5) on ice as described by Ronacher *et al* [34]. PBMC lysates were also prepared in TAPS buffer from approximately 4×10^6 cells.

Western blotting was performed essentially as previously described [75]. All antibodies were purchased from Santa Cruz biotechnology (USA, California). Antibodies included anti-androgen receptor (AR, C-441, sc-7305) anti-estrogen receptor (ER, MC-20, sc-542), anti-GR (H-300, sc-8992), anti-mineralocorticoid receptor (MR, C-19, sc-6861), anti-PR (C-20, sc-539) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-47724).

Conventional PCR

Conventional PCR was performed using GoTaq DNA polymerase (Promega, USA, M3001) with the steroid receptor specific primers (Table 1) according to the manufacturer's specifications. Initial denaturation was for 90 sec at 95°C, while final extension was for 5 mins at 72°C. The cycling parameters for 35 cycles are shown in Table 1.

PBMC Isolation, Cell Culture and Test Compounds

Buffy packs were obtained from healthy donors who were negative for HIV, syphilis and hepatitis B and C. PBMCs were isolated using Histopaque (H1077 Hybri-Max^{TM}; Sigma-Aldrich, South Africa) density centrifugation with Leucosep tubes (Greiner Bio-One, Germany) according to the manufacturer's instructions [76]. PBMCs were cultured in high glucose (4.5 g/ml) Roswell Park Memorial Institute medium (RPMI) (Gibco, South Africa) supplemented with 10% (*v/v*) charcoal-stripped fetal calf serum (c-s FCS) (Highveld Biological, South Africa), 2 mM L-glutamine (Sigma-Aldrich, South Africa), 0.1 mg/mL sodium pyruvate (Sigma-Aldrich, South Africa), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, South Africa) at 37°C in a water jacket incubator (90% humidity and 5% CO_2). Note that each figure shows the results of experiments using PBMCs isolated from different donors. Dex ((11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione), MPA (Medroxyprogesterone 17-acetate), NET-A (19-Norethindrone acetate), NET (19-Norethindrone), P4 (progesterone), R5020 (17,21-dimethyl-19-norpregna-4,9-dien-3,20-dione), Mib (mibolerone), E2 (estradiol), Ald (Aldosterone) and RU486 (Mifepristone) were purchased from Sigma-Aldrich, South Africa.

Cell Treatment without Vpr Peptide and Flow Cytrometric Detection of Apoptosis

Approximately 1×10^6 PBMCs/ml RPMI were seeded into a 5 mL Becton-Dickinson Falcon tube (352063). Cells were then treated with either Dex, F, MPA, NET-A, P4 or vehicle control (EtOH) at the concentrations indicated in the figure legends for 24 hrs at 37°C. After treatment, cells were surface stained and the apoptotic phenotype detected using the annexin V PE apoptosis detection kit I according to the manufacturer's specifications (Becton-Dickinson-Biosciences; 559763). The following antibodies

Table 1. Primers used for conventional PCR.

Target Gene	Primer Sequence	Cycling Parameters	Product size (bp)
AR	F: 5′-CAGGAAAGCGACTTCACCGCACC-3′ R: 5′-ATCAGGCAGGTCTTCTGGGGTGG-3′	95°C (45 sec), 60°C (45 sec), 72°C (45 sec)	209
ER alpha	F: 5′- TCGACGCCAGGGTGGCAGAG R: 5′-TGGTGCACTGGTTGGTGGCTGG-3′	95°C (45 sec), 60°C (45 sec), 72°C (45 sec)	218
GR	F: 5′-TGCTGTGTTTTGCTCCTGATCTG-3′ R: 5′-TGTCAGTTGATAAAACCGCTGCC-3′	95°C (45 sec), 53°C (45 sec), 72°C (45 sec)	299
MR	F: 5′-GAGCAGTGGAAGGGCAACAC-3′ R: 5′-TGGCTGCTCCTCGTGAATCC-3′	95°C (45 sec), 60°C (45 sec), 72°C (45 sec)	182
PR A and PR B	F: 5′-GTGCTCAAGGAGGGCCTGCCG-3′ R: 5′-TGTGCTGCCCTTCCATTGCCC-3′	95°C (45 sec), 60°C (45 sec), 72°C (45 sec)	214

F = Forward; R = Reverse.

doi:10.1371/journal.pone.0062895.t001

Table 2. Primers used for real time PCR.

Target Gene	Primer Sequence	Cycling Parameters	Product size (bp)
GAPDH	F: 5′-TGAACGGGAAGCTCACTGG-3′ R: 5′-TCCACCACCCTGTTGCTGTA-3′	95°C (10 sec), 55°C (10 sec), 72°C (10 sec)	307
Bcl-2	F: 5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′ R: 5′-GTACAGTTCCACAAAGGCATCCCA-3′	95°C (10 sec), 60°C (10 sec), 72°C (10 sec)	167
Bim	F:5′-GAGTGTGACCGAGAAGGTAGACAATTGC-3′ R: 5′-CCTTCACCTCCGTGATTGCCTTC-3′	95°C (10 sec), 55°C (10 sec), 72°C (10 sec)	125

F = Forward; R = Reverse.
doi:10.1371/journal.pone.0062895.t002

were used to discriminate between cellular populations in PBMCs: 1 μL anti-CD4 fluorescein isothiocyanate (FITC) (Becton-Dickinson, 555346), 2 μL anti-CD14 allophycocyanin (APC) (Becton-Dickinson, 555399) and 1 μL anti-CD3 APC (Becton-Dickinson, 555342) in 50 μL PBS. Samples were acquired using a Becton-Dickinson FACS Calibur flow cytometer and analysed using Flo Jo software (Treestar, Inc, Ashland, Ore).

Cell Treatment with Vpr Peptide

The C-terminal Vpr peptide (including amino acids 52–96; GNTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRGR-RARNGASRS) was a kind gift from Dr Jeffrey Kopp (NIDDK, National Institutes of Health, Bethesda, USA). Freshly isolated PBMCs were treated with 5 μM Vpr peptide as previously described [61]. Briefly, approximately ten million PBMCs were re-suspended in 1 mL of balanced isotonic glucose-HEPES buffer (2.4% glucose, 13 mM HEPES, 68 mM NaCl, 1.3 mM KCl, 4 mM Na_2HP0_4 and 0.7 mM KH_2PO_4, pH 7.2). One million

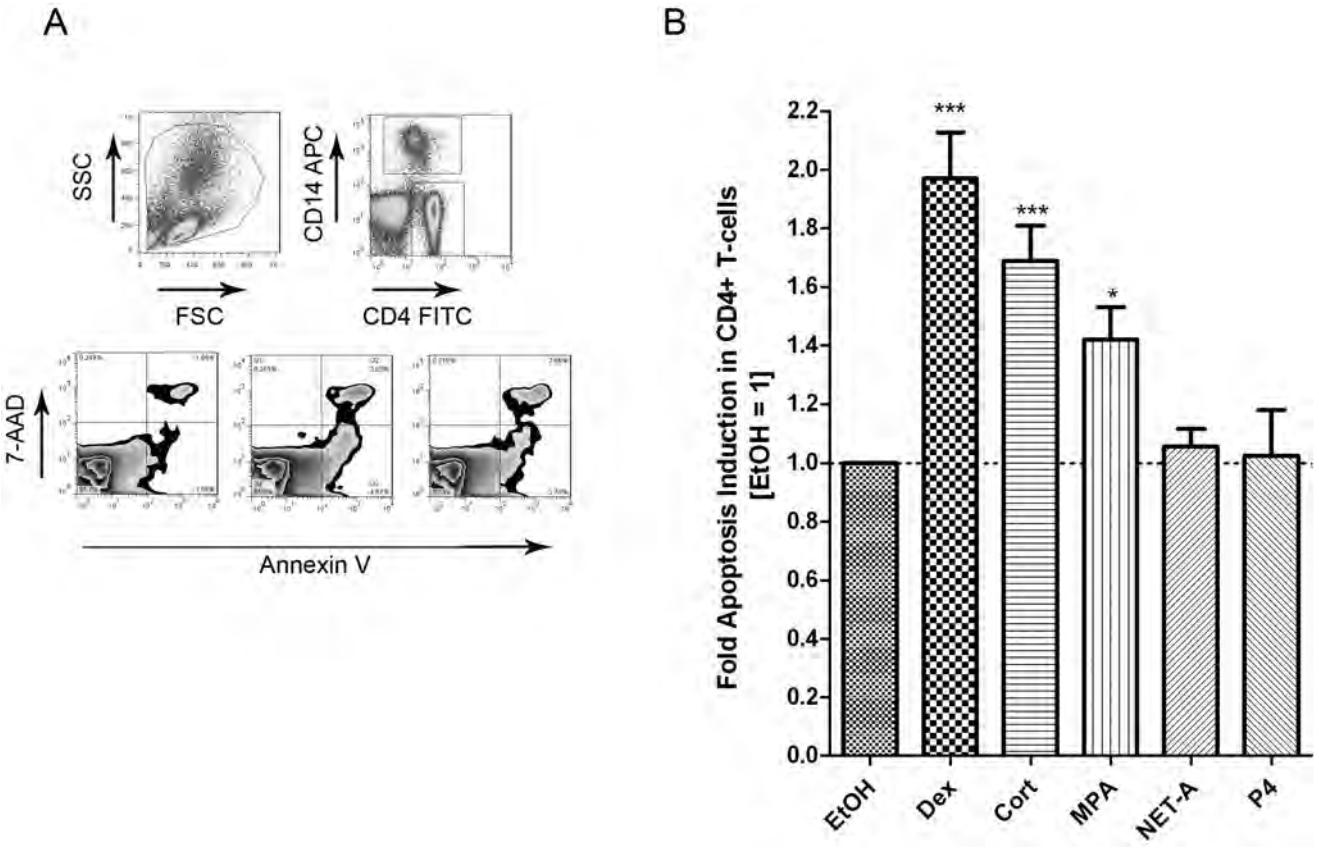

Figure 1. The progestin MPA, but not NET-A or P4, induces apoptosis in CD4$^+$ T-cells. Cells were treated with or without 100 nM Dex, 100 nM F, 1 μM MPA, 10 μM NET-A, 1 μM P4 or vehicle control (EtOH) for 24 hrs. Cells were stained with anti-CD4, anti-CD14, annexin V and 7-AAD using the Apoptosis Detection kit I (BD biosciences). (A) Gating strategy and representative zebra plots of untreated (EtOH), MPA or Dex treated PBMCs. (B) The histogram shows pooled results from two independent experiments with samples from three donors. Data were acquired on a FACS calibur system (BD Biosciences) and analyzed using Flo-Jo software (Tree Star Inc., San Carlos, CA, USA). Statistical significance was determined by one-way ANOVA with Dunnett's post-test, where *, **, and *** indicate $p<0.05$, 0.01 and 0.005 respectively. Error bars represent standard deviation.
doi:10.1371/journal.pone.0062895.g001

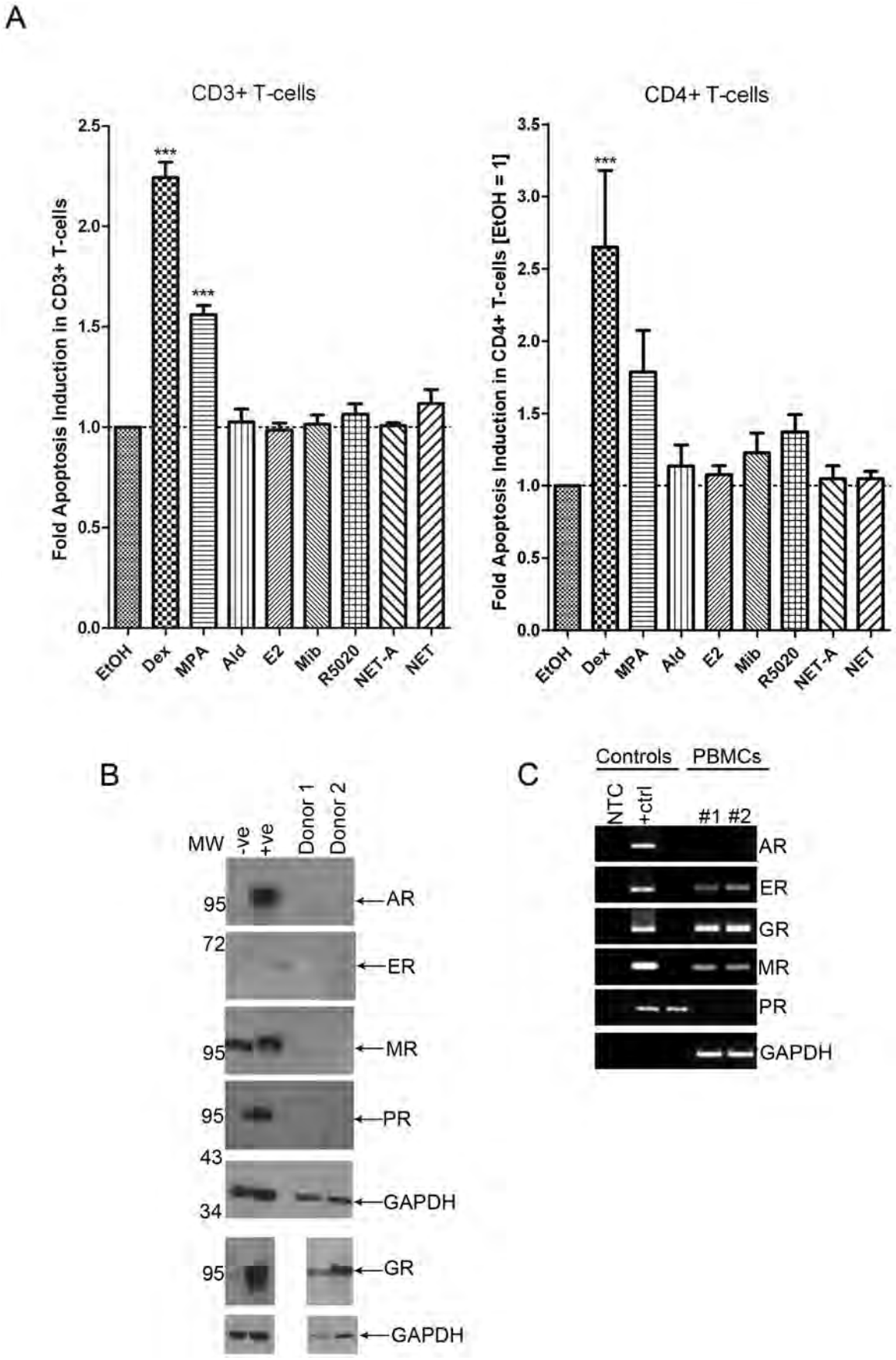
A
CD3+ T-cells
Fold Apoptosis Induction in CD3+ T-cells
2.5
2.0
1.5
1.0
0.5
0.0
***
***
EtOH
Dex
MPA
Ald
E2
Mib
R5020
NET-A
NET
CD4+ T-cells
Fold Apoptosis Induction in CD4+ T-cells [EtOH = 1]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
EtOH
Dex
MPA
Ald
E2
Mib
R5020
NET-A
NET
B
MW
-ve
+ve
Donor 1
Donor 2
95
AR
72
ER
95
MR
95
PR
43
34
GAPDH
95
GR
GAPDH
C
Controls
PBMCs
NTC
+ctrl
#1
#2
AR
ER
GR
MR
PR
GAPDH

Figure 2. Apoptosis induction by Dex and MPA is most likely mediated primarily through the GR. (A) PBMCs were treated with vehicle (EtOH), 100 nM MPA, 10 nM Ald, 100 nM E2, 100 nM Mib, 100 nM R5020, 10 μM NET-A or 10 μM NET for 24 hrs at 37°C. Cells were surface stained with ant-CD3 and anti-CD4 antibodies, and apoptosis was detected using flow cytometry as described in Figure 1. The histogram shows pooled results from two independent experiments with samples from three donors. Statistical significance was determined by one-way ANOVA with Dunnett's post-test, where *** indicates $p<0.001$. (B) Western analysis of lysates prepared from approximately 4×10^6 PBMCs. Whole cell lysates of COS-1 cells overexpressing the relevant steroid receptor (+ve) or empty vector (−ve) served as the controls. GAPDH was used as a loading control. Note that the upper strong band on the MR blot is a COS-1 cell-derived non-specific signal which is absent for PBMCs, while the MR signal is the faint band just below the non-specific band which is only seen in the positive control. We were unable to obtain a more-specific anti-MR antibody. (C) Conventional PCR of cDNA prepared from human PBMCs using primers specific for the relevant steroid receptor. The controls were prepared by PCR amplification of the relative steroid receptor cDNA from plasmid DNA. GAPDH served as a control for mRNA levels. MW: molecular weight; NTC: no template control. Error bars represent standard deviation.
doi:10.1371/journal.pone.0062895.g002

cells (100 μL) were treated with 2.5 μg (5 μM) Vpr peptide for 30 min at 37°C. The cells were washed and then cultured in RPMI. A bovine serum albumin (BSA) trypic digest was prepared according to a protocol obtained from the Sanford-Burnham Medical Research Institute (Lo Jolla California, USA [77]) and served as a control peptide wherever Vpr peptide was not added. Control peptide was added at a final equivalent concentration as Vpr, in mass/volume i.e. 25 mg/mL. Cells were treated with the test compounds as indicated in the figure legends for 24 hrs at 37°C and apoptosis was detected as indicated above. Note that we found that incubation with this buffer resulted in an increase in basal apoptosis of the $CD4^+$ T-cell population from about 2.5% to about 5% (data not shown), which masked apoptotic effects with MPA alone.

HIV-1 Pseudovirus Generation and Infection

HIV-1 pseudovirus was generated as described by Jochmann *et al* [78]. HEK293T cells (obtained from ATCC) were seeded at a density of 8×10^5 cells/well in a 6-well plate in high glucose (1 g/ml) phenol red-containing Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich) supplemented with 10% (*v/v*) fetal calf serum (FCS) (Highveld Biological, South Africa), 0.1 mg/mL sodium pyruvate (Sigma, South Africa), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen) at 37°C in a water jacket incubator (90% humidity and 5% CO_2). The next day the cells were washed with PBS and phenol red free DMEM (supplemented as described above) was added to each well. Cells were then transfected with 5 μg pSG3.1 (containing the HIV-1 genomic sequences with a mutated envelope (env) gene. (AIDS Research and Reference Reagent Program; [79]) and pDU15A (encoding the HIV-1 envelope) [80] using X-tremeGENE 9 DNA

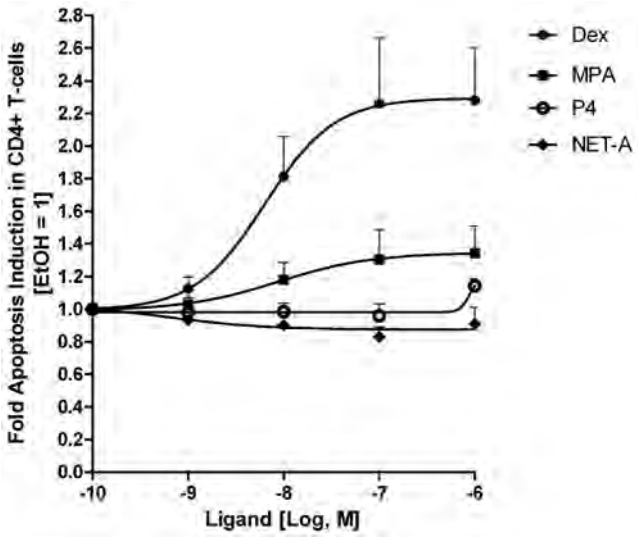

Figure 3. Dose-dependent apoptosis induction with Dex, MPA, NET-A and P4 in $CD4^+$ T-cells. PBMCs were treated with vehicle (EtOH), Dex, MPA, NET-A or P4 at the concentrations indicated for 24 hrs at 37°C. Cells were stained and analysed as described for Figure 1. The figure shows pooled results from two independent experiments with samples from three donors. Error bars represent standard deviation. Statistical trend analysis for each dose response was performed by the Wilcox rank-sum test, as further extended by Cuzick [85], and showed a significant trend only for Dex ($p<0.001$) and MPA ($p=0.047$).
doi:10.1371/journal.pone.0062895.g003

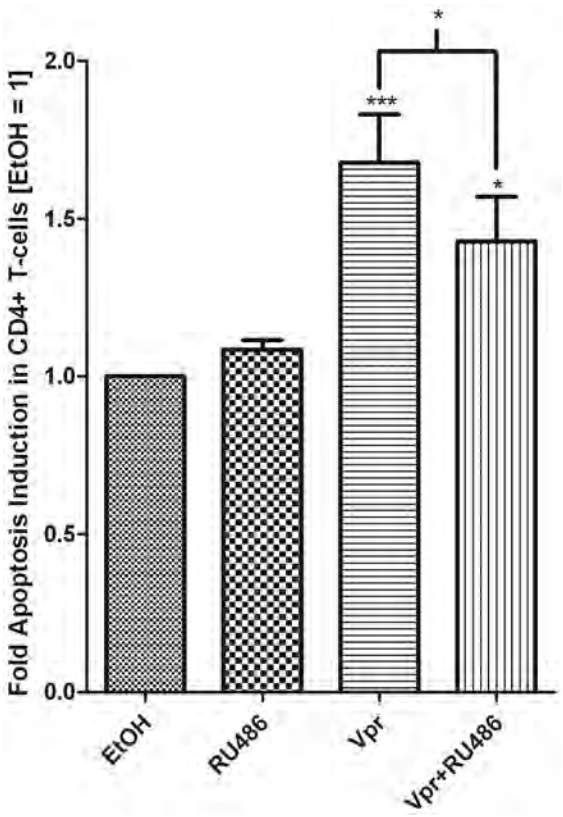

Figure 4. The GR is involved in Vpr-mediated apoptosis in $CD4^+$ T-cells. PBMCs were treated with 1 μM RU486 in the absence or presence of 5 μM Vpr peptide (amino acids 52–96) for 24 hrs. A tryptic BSA digest served as a control (bars 1 and 2) wherever Vpr peptide was not added and was added at an equivalent mass/volume of peptide. Cells were obtained and stained as described in the methods. The histogram shows pooled results from two independent experiments with samples from three donors. Statistical significance was determined by one-way ANOVA with Dunnett's post-test or a paired t-test, where *, **, and *** indicate $p<0.05$, 0.01 and 0.005 respectively. Error bars represent standard deviation.
doi:10.1371/journal.pone.0062895.g004

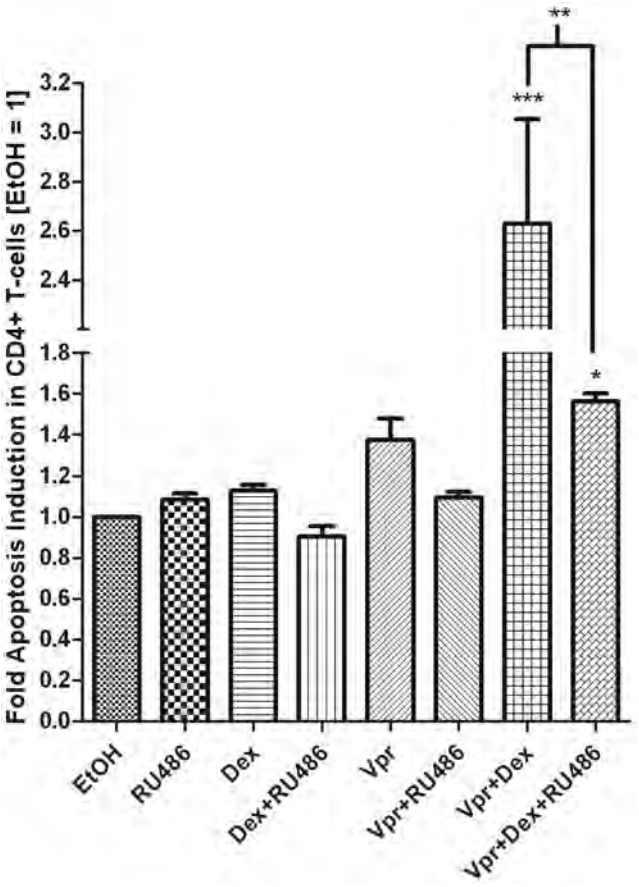

Figure 5. The GR is involved in GC- and Vpr-mediated apoptosis in CD4$^+$ T-cells. PBMCs were treated with 100 nM Dex or 1 μM RU486 in the absence or presence of 5 μM Vpr peptide (amino acids 52–96) for 24 hrs. A tryptic BSA digest added at an equivalent mass/volume ratio of peptide, served as a control wherever Vpr peptide was present. Cells were obtained and stained as described in the methods. The histogram shows pooled results from two independent experiments with samples from three donors. Statistical significance was determined by one-way ANOVA with Dunnett's post-test or a paired t-test, where *, **, and *** indicate $p<0.05$, 0.01 and 0.005 respectively.
doi:10.1371/journal.pone.0062895.g005

transfection reagent (Roche, South Africa) according to the manufacturer's specifications. Cells were incubated for 3 days at 37°C, the medium passed through a 0.22 μM filter and charcoal-striped (cs) FCS (Highveld Biological, South Africa) was added to a final concentration of 40%. The viral stocks were aliquotted and stored at −80°C until use. The titre of the pseudotyped viruses was determined using the Reed Muench method and expressed as log $TCID_{50}$/ml [81]. Prior to infection, PBMCs were activated with 5 μg/ml phytohemagglutinin (PHA) (Sigma Aldrich, South Africa) and 20 U/ml recombinant human interleukin-2 (rhIL-2) (Roche,-South Africa) for 3 days as previously described [82]. For pseudovirus infection, pseudovirus was added to obtain a multiplicity of infection (MOI) of 0.00005 and incubated for 3 days before stimulation and flow cytometric detection of apoptosis. A standard p24 assay (Aalto Bio Reagents Ltd, Dublin Ireland) was used to confirm that the cells were infected.

RNA Isolation, cDNA Synthesis and Real Time PCR

Approximately twenty million PBMCs were treated with 5 μM Vpr or control peptide in 1 mL of balanced isotonic glucose-HEPES buffer as described previously. Cells were then treated in the presence or absence of 100 nM Dex, MPA, NET-A or P4 for 24 hrs. The cells were harvested by centrifugation at 350×*g* and RNA was extracted using Tri Reagent (Sigma-Aldrich, South Africa) according to manufacturer's instructions. RNA was reverse transcribed with oligo-dT priming, using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, South Africa), and an equal volume of each cDNA synthesis reaction was used as template for real time PCR, using the Sensimix dT Kit (Quantace, London). Quantitative PCR was carried out using primers for Bim and Bcl-2 (Table 2). GAPDH was used as a housekeeping gene for normalization (Table 2; [83]). Initial denaturation and final extension was as for conventional PCR while the cycling parameters for 40 cycles are shown in Table 2. Standard curves were used to determine the efficiency of each primer set, and the relative expression of the genes of interest in each sample was calculated according to the Pfaffl mathematical model [84].

Statistical Analysis

All experiments were performed with PBMCs isolated from at least 3 different donors and at least two independent experiments were performed. All data was normalized to appropriate controls. Data were analysed for statistical significance by One-way ANOVA and appropriate post-tests as indicated in the figure legends using GraphPad Prism software. *, **, and *** indicate $p<0.05$, $p<0.01$ and $p<0.005$, respectively. For dose response analysis, a non-parametric statistical trend test was performed across the concentration range for each compound, using the Wilcox rank-sum test, as further extended by Cuzick [85].

Results

An important question is whether doses of DMPA and NET-EN used for injectable contraception, and physiological concentrations of endogenous P4, are sufficient to cause significant effects on immune function via the GR *in vivo*. MPA concentrations in the serum of DMPA users are reported to be in the range of 4.5 to 65 nM a few days after injection of 150 mg and then to average at about 2.6 nM for about three months [48,86–89]. NET has been shown to reach a peak plasma concentration of 50 nM a few days after intramuscular injection of NET-EN, followed by an average concentration of about 13 nM for about four months [90–92]. The concentration of endogenous P4 in the serum of premenopausal women varies from 0.65 nM to about 80 nM between the follicular and luteal phases, respectively, while reaching about 600 nM during pregnancy [29]. MPA has a high relative binding affinity for the GR (K_i of 10.8 nM), similar to that of F (10–20 nM) [93], whereas NET-A and P4 have lower affinity for the GR (K_i of 270 and 215 nM respectively) [34,94,95]. In order to investigate effects of P4 and progestins via the GR on apoptosis, experiments were thus performed at single concentrations required for near or full saturation of the GR (10–50×K_d or K_i), as well as by dose response analysis using doses spanning the range of concentrations found in the serum of DMPA and NET-EN users, or doses spanning physiologically relevant concentrations for P4. Since NET-EN is not soluble in aqueous solution, we used the water soluble derivatives NET-A or NET [29].

GCs and the Progestin MPA, but not NET-A or P4, Induce Apoptosis in CD4$^+$ T-cells

GCs have been shown to induce apoptosis in several different cell lines, including CD4$^+$ T-cells [35]. The progestin MPA is

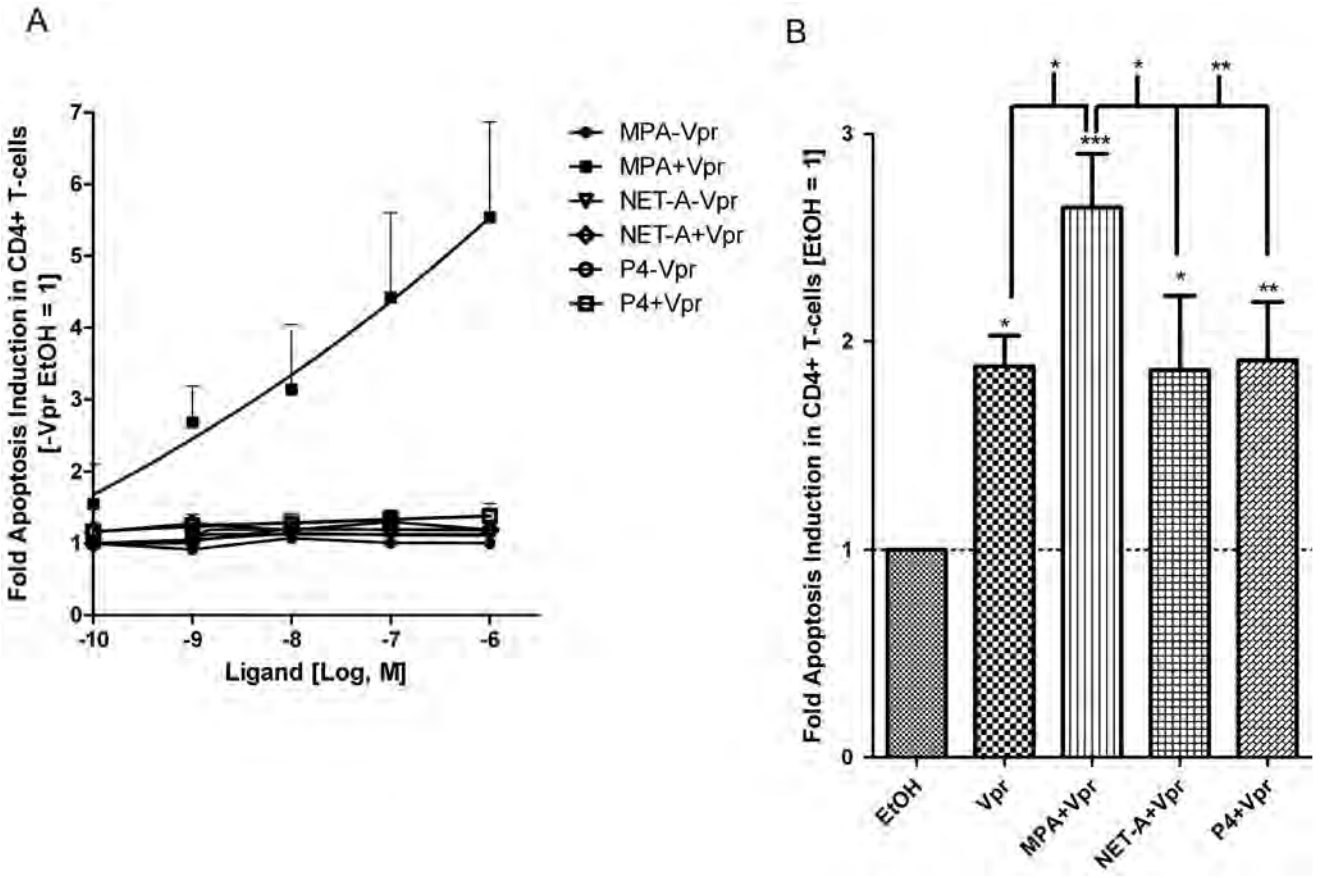

Figure 6. MPA but not NET-A or P4 increases Vpr-mediated apoptosis in a dose-dependent manner. (A) PBMCs were treated with or without 5 μM Vpr peptide (amino acids 52–96) and increasing concentrations of MPA, NET-A or P4 for 24 hrs. The graph shows results pooled from two independent experiments with samples from three donors. (B) PBMCs were treated with 100 nM MPA, 10 μM NET-A, 1 μM P4 or in combination with 5 μM Vpr peptide (amino acids 52–96) for 24 hrs. Cells were stained and acquired by flow cytometry as described in the materials and methods. A tryptic BSA digest served as a control wherever Vpr peptide was not added, as for results in figure 4. The histogram shows results pooled from two independent experiments with samples from three different donors compared to those in figure A. donors. Statistical trend analysis for panel A was performed by the Wilcox rank-sum test, as further extended by Cuzick [85], and showed a significant trend only for MPA plus Vpr ($p = 0.012$) and P4 minus Vpr ($p = 0.012$). Statistical significance in panel B was determined by one-way ANOVA with Dunnett's post-test or a paired t-test, where *, **, and *** indicate $p<0.05$, 0.01 and 0.005 respectively. Error bars represent standard deviation.
doi:10.1371/journal.pone.0062895.g006

a partial to full GR agonist, unlike NET-A and P4 which have weak to no GR activity [30–33]. We investigated the relative capability of MPA and NET-A to induce apoptosis in $CD4^+$ T-cells and $CD14^+$ monocytes, as compared to the endogenous GC agonist F, the synthetic GR agonist Dex and P4. Briefly, PBMCs were isolated and treated with 100 nM Dex, 100 nM MPA, 10 μM NET-A, 1 μM P4 or vehicle control (EtOH) for 24 hrs. Cells were stained with anti-CD4 (T-cells), anti-CD14 (monocytes), 7-aminoactinomycin D (7-AAD), annexin V and the data were acquired using the Becton Dickinson FACS Calibur. 7-AAD was included to discriminate between live and dead cells. $CD4^+$ T-cells and $CD14^+$ monocytes were gated from the total PBMC population as indicated and the apoptotic cells were detected with the apoptosis marker annexin V (Figure 1A). As expected Dex and F induced apoptosis in a statistically significant manner in $CD4^+$ T-cells by about 2-fold and 1.6-fold, respectively, compared to untreated cells (Figure 1B). Importantly, MPA also statistically significantly induced apoptosis in these cells (1.5-fold), yet to a lesser extent than Dex. By contrast, when cells were treated with NET-A or P4 no increase in apoptosis compared to control, was detected in $CD4^+$ T-cells (Figure 1B). The apoptotic effect of Dex, F and MPA was however not observed in $CD14^+$ monocytes (data not shown) and therefore the following experiments were carried out in $CD4^+$ T-cells.

MPA Acts Primarily via the GR to Induce Apoptosis in $CD4^+$ T-cells

Next we sought to provide evidence that the increase in apoptosis observed with Dex and MPA was mediated via the GR and did not involve other steroid receptors. Since MPA is a PR agonist, and a partial agonist for the androgen receptor (AR) and a partial to full agonist for the GR [29], the possibility that MPA exerts its apoptotic effects via the PR or AR was investigated indirectly by using receptor-selective agonists. In order to determine whether other steroid receptors (apart from the GR) could induce apoptosis, PBMCs were treated with agonists that are selective for the AR (100 nM Mib), estrogen receptor (ER) (100 nM E2), mineralocorticoid receptor (MR) (10 nM Ald) and PR (100 nM R5020), as well as 100 nM Dex, 100 nM MPA, 10 μM NET-A or 10 μM NET for 24 hrs, and apoptosis was detected using flow cytometry as described previously. The ligands were used at saturating concentrations for each steroid receptor to control for the differences in relative binding affinities of each

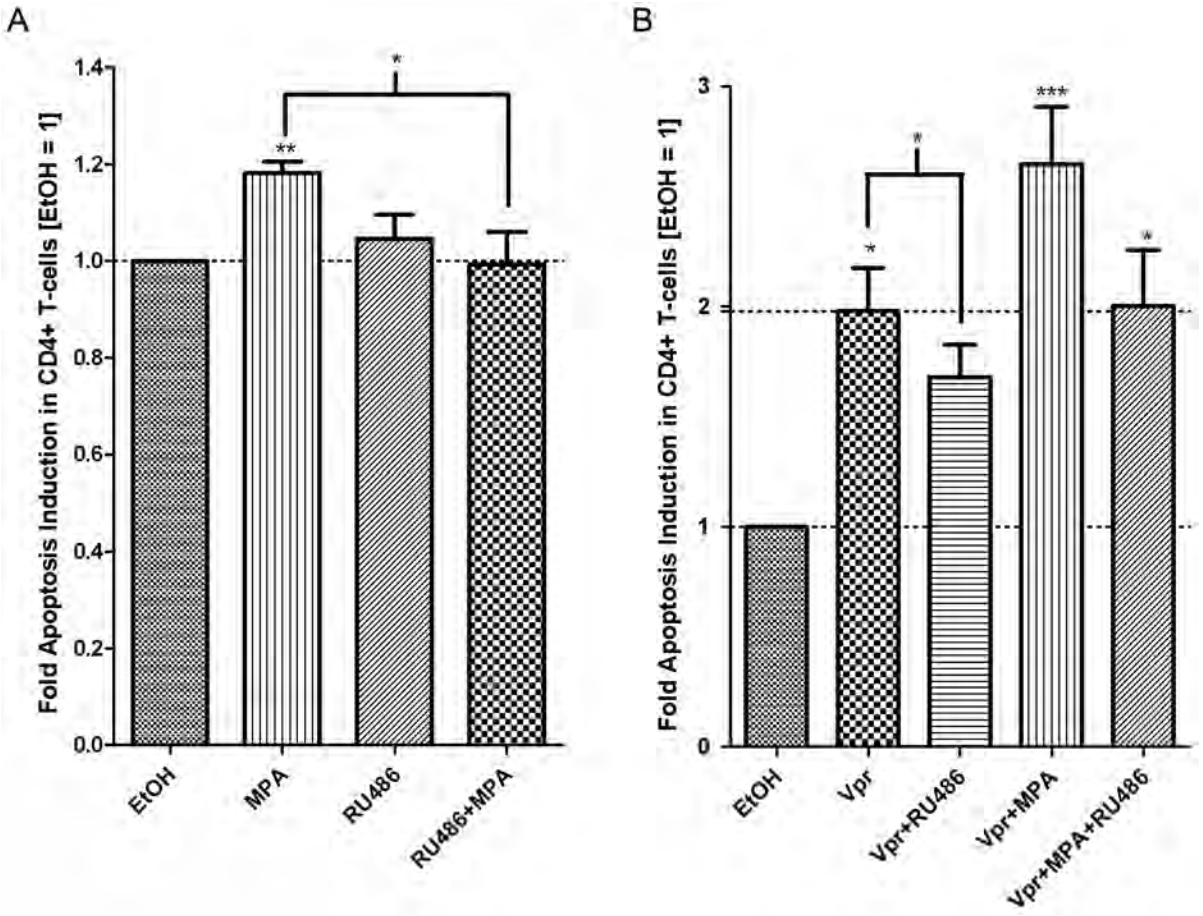

Figure 7. The GR is involved in MPA- and Vpr-mediated apoptosis in CD4$^+$ T-cells. PBMCs were treated with vehicle (EtOH), 100 nM MPA, 1 μM RU486 or 100 nM MPA plus 1 μM RU486, in the absence (A) or presence (B) of 5 μM Vpr peptide for 24 hrs. Cells were stained and acquired by flow cytometry as indicated in the methods. In A cells were not incubated with balanced isotonic glucose-HEPES buffer while in B, this buffer was used and a tryptic BSA digest served as a control wherever Vpr peptide was not added, as described in Methods. The histograms show pooled results from two independent experiments with samples from three donors. Statistical significance was determined by one-way ANOVA with Dunnett's post-test or a paired t-test, where *, **, and *** indicate $p<0.05$, 0.01 and 0.005 respectively. Error bars represent standard deviation.
doi:10.1371/journal.pone.0062895.g007

ligand for their respective receptors [33,34,94]. As found earlier, Dex significantly induced apoptosis by about 2-fold and about 3-fold in CD3$^+$ and CD4$^+$ T-cells, respectively (Figure 2A). MPA significantly induced apoptosis by about 1.5-fold compared to untreated control cells in the CD3$^+$ T-cells and appeared to increase apoptosis in CD4$^+$ T-cells to a similar extent as observed before (Figure 1B). In both CD3$^+$ and CD4$^+$ T-cells, the other steroid receptor-selective agonists did not induce apoptosis in a statistically significant manner (Figure 2A). Therefore, it is likely that the effects of MPA on apoptosis are mediated via the GR in T-cells. In support of these findings, PBMCs expressed GR protein whereas AR, PR, MR or ER protein expression was not detected by Western blot analysis (Figure 2B). The ER and MR mRNAs, but not AR or PR mRNAs, were however detected by PCR, indicating that ER and MR proteins may be expressed, but at a level undetectable by Western blot analysis (Figure 2C). Together with the results presented in Figure 2A, these data show that if low levels of ER and MR protein are expressed, they have no effect on apoptosis in CD3$^+$ or CD4$^+$ T-cells (Figure 2A). Taken together, these results strongly support the finding that the PR, AR, MR and ER do not induce apoptosis in these cells, and that MPA acts primarily via the GR to induce apoptosis in PBMCs. It is noteworthy that NET was included in this experiment as a control to exclude the possibility that the acetate form (NET-A) may be less active. However, similarly to NET-A, NET does not result in apoptosis.

Dex and MPA but not NET-A or P4 Increase Apoptosis in a Dose-dependent Manner

Having established that both MPA and Dex induce apoptosis in CD4$^+$ T-cells, we next sought to determine whether this pro-apoptotic effect was dose-dependent. Statistically significant trends [85] were observed for Dex ($p<0.001$) and MPA ($p = 0.047$), but not for P4 or NET-A, showing increased apoptosis with increasing concentrations of ligand, in the absence of Vpr, under these experimental conditions (Figure 3). Furthermore, apoptotic induction in response to both Dex and MPA was observed, starting at concentrations as low as 10 nM (Figure 3). The maximal apoptotic response observed for both Dex and MPA was reached at 100 nM, and the maximal response for MPA (~1.3-fold) at that concentration was lower than for Dex (~2.3-fold) (Figure 3). Note that the fold induction of apoptosis with MPA under these conditions varies between experiments from about 1.3- to 1.7-fold (Figures 1,2,3), most likely due to biological variability between donors. Even though no dose-dependent significant trend for changes in apoptosis was observed for NET-A or P4, a small

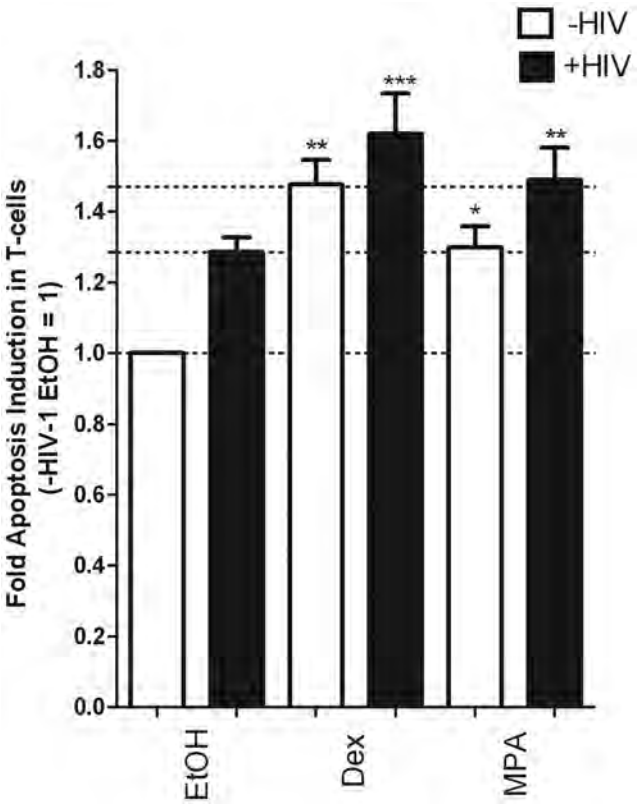

Figure 8. HIV-1-mediated apoptosis is enhanced in the presence of Dex and MPA. PBMCs were activated in the presence of PHA and rhIL-2 for 3 days at 37°C as described. Pseudotyped HIV-1 virus or control medium without virus was added to the cells, followed by incubation for a further 3 days to allow infection. Cells were then treated with vehicle (EtOH) or 100 nM Dex or MPA for an additional 24 hrs. Acquisition and analysis was carried out as described in the methods. The histogram shows pooled results from two independent experiments with samples from three donors. Statistical significance was determined by one-way ANOVA with Dunnett's post-test, where *, **, and *** indicate $p<0.05$, 0.01 and 0.005 respectively. Error bars represent standard deviation.
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response (~1.1-fold) appeared to occur for P4 at 1 μM. These results are similar to dose-responses observed with these ligands for transcriptional regulation via the GR, with Dex acting as a full agonist and MPA as a partial agonist for the GR at concentrations between 1–100 nM, but with NET-A showing no agonist activity and P4 very weak to partial agonist activity in some contexts only at micromolar concentrations [31,34]. Having established that both Dex and MPA induce apoptosis in the $CD4^+$ T-cells in a dose-dependent manner, we next sought to determine if Dex and MPA can enhance Vpr-mediated apoptosis.

Dex Enhances Vpr-mediated Apoptosis in a GR-dependent Manner

It is well established that Vpr is a potent inducer of apoptosis in a number of different cell lines and primary cells. Therefore, we determined whether exogenous C-terminal Vpr peptide could induce apoptosis via the GR in $CD4^+$ T-cells. As expected, Vpr peptide significantly induced apoptosis by approximately 1.8-fold in the $CD4^+$ T-cells (Figure 4). This apoptotic induction was decreased in the presence of RU486, a potent GR antagonist, indicating that the GR was involved in Vpr-mediated apoptosis (Figure 4). We next determined whether Dex could enhance Vpr-mediated apoptosis through the GR in $CD4^+$ T-cells. Cells were incubated with Vpr peptide in the absence and presence of Dex. Vpr and Dex alone induced apoptosis in $CD4^+$ T-cells, although statistical significance could not be established, most likely due to the small responses (Figure 5). Furthermore, when cells were treated with Dex and Vpr in combination, a significant increase in apoptosis was observed as compared to Vpr or Dex alone. To establish whether the GR was involved in combined effects of Vpr- and Dex-mediated apoptosis, cells were treated in the absence and presence of RU486. RU486 alone had no effect on apoptosis (Figures 4 and 5). Apoptosis by Dex and Vpr alone was decreased in the presence of RU486, although statistical significance could not be established. Importantly, the 3-fold increase in apoptosis observed when cells were treated with Dex and Vpr in combination was significantly decreased in the presence of RU486 (Figure 5). Although RU486 is also a PR and MR antagonist [29], our data discount a role for these receptors in apoptosis in these cells (Figure 2). Taken together, the data suggest that the GR is required for Vpr- and Dex-mediated apoptosis, and suggests that the GR is required for Vpr enhancement of Dex-mediated apoptosis.

MPA, but not NET-A or P4, Enhances Vpr-mediated Apoptosis in a GR-dependent Fashion

Having shown that Dex treatment further increases Vpr-mediated apoptosis in a GR-dependent fashion, we next sought to investigate whether MPA, similarly to the full GR-agonist Dex, has the capability to enhance Vpr-mediated apoptosis. Under the experimental conditions used in figure 6, we observed statistically significant [85] trends only for MPA ($p = 0.012$) in the presence of Vpr, as well as for P4 in the absence of Vpr ($p = 0.005$), showing increased apoptosis with increasing concentrations of ligand (Figure 6A). Interestingly, a response was observed for MPA in the presence of Vpr at concentrations as low as 1 nM. A maximal increase of approximately 6-fold was obtained at a concentration of 1 μM MPA (Figure 6A). This was in contrast to cells treated in the absence of Vpr, where MPA appeared to have no dose-dependent effect on apoptosis (Figure 6A). The lack of apoptotic activity by MPA alone in these experiments compared to figures 1,2,3 was likely due to the conditions required to treat the cells with Vpr or control peptide, which masks the smaller effects of MPA alone. The dose response results are consistent with the results in Figure 6B, showing a statistically significant increase in Vpr-mediated apoptosis with MPA, but not NET-A or P4, using concentrations of ligands that nearly or fully saturate the GR.

Towards establishing a role for the GR in the MPA response, further experiments were performed with RU486, in the absence and presence of Vpr peptide (Figure 7). In the absence of prior incubation with peptide buffer (see methods), MPA significantly increased apoptosis compared to untreated $CD4^+$ T-cells (Figure 7A), as previously shown (Figures 1,2,3). Importantly, although RU486 alone had no effect on apoptosis, this GR agonist could reverse MPA-mediated apoptosis in the $CD4^+$ T-cells (Figure 7A) in a statistically significant manner. Vpr alone significantly induced apoptosis in $CD4^+$ T-cells, and this response was decreased in the presence of RU486 (Figure 7A). Vpr and MPA in combination enhanced apoptosis by about 3-fold in a statistically significant manner, which was decreased by RU486 (Figure 7B). These results strongly suggest that MPA and Vpr alone or in combination, enhance apoptosis in $CD4^+$ T-cells via a mechanism involving the GR. The lack of an effect by the natural PR ligand P4 or the synthetic progestin NET-A on Vpr-induced apoptosis is consistent with the requirement for GR

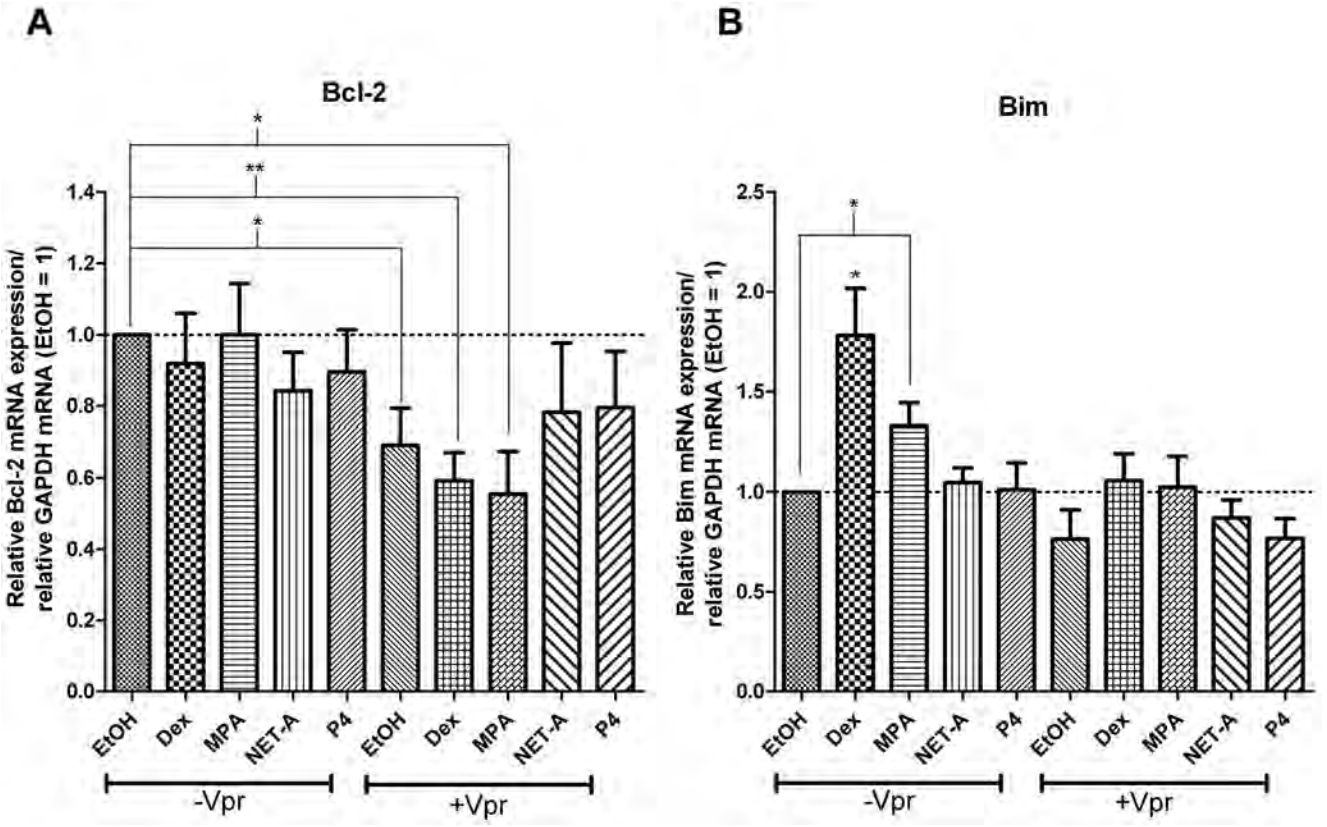

Figure 9. GC and Vpr differentially regulate key genes involved in apoptosis. PBMCs were treated with or without 5 μM Vpr peptide as described previously and treated with or without 100 nM Dex, MPA, NET-A or P4 for 24 hrs. After treatment, RNA was extracted, reverse transcribed, and Bcl-2 (A) or, Bim (B) mRNA expression was measured by real time PCR, normalising to GAPDH expression levels. The histogram shows pooled results from two independent experiments with samples from three donors. Statistical significance was determined by one-way ANOVA with Dunnett's post-test, where *, **, and *** indicate $p<0.05$, 0.01 and 0.005 respectively. Error bars represent standard deviation.
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agonist or strong partial agonist activity of a ligand to modulate Vpr-mediated apoptosis in $CD4^+$ T-cells.

Dex and MPA Enhance HIV-1-mediated Apoptosis in $CD4^+$ T-cells

Having shown that Dex and MPA enhance Vpr-mediated apoptosis using peptide studies, we next determined whether this effect could be elicited by intact HIV-1 pseudovirus. PBMCs were first activated with PHA and rhIL-2. Cells were then infected with pseudotyped HIV-1 virus for 3 days before being treated with the test compounds as indicated for an additional 24 hrs. The apoptotic phenotype was detected by flow cytometry as described above. However, we could not detect $CD4^+$ T-cellsin this assay, which was most likely owing to decreased expression of the $CD4^+$ receptor following T-cell activation and subsequent infection [96]. Thus, the results are representative of the T-cell population that was gated from the forward and side scatter plot. The responses observed from this PBMC population most likely represent the T-cell population only, because monocytes (which would scatter with the T-cells) are resistant to ligand- and Vpr-mediated apoptosis (data not shown; [97]). Consistent with results obtained in figures 1,2,3 and 5,6,7, stimulation with Dex and MPA resulted in a statistically significant increase in apoptosis (Figure 8). HIV-1 infection also increased apoptosis, which is consistent with results obtained with Vpr peptide (Figures 4,5,6,7) and in the literature [98–100]. Importantly, Dex and MPA stimulation further enhanced HIV-1 mediated apoptosis. In summary the data presented here indicate that Dex and MPA have the ability to increase T-cell apoptosis in the presence of HIV-1.

Dex and Vpr Differentially Regulate Pro- and Anti-apoptotic Genes

The mechanism of apoptotic induction by the GR and Vpr in the presence of GR ligands most likely involves the transcriptional regulation of pro- and anti-apoptotic genes [35,53]. To this end we set out to identify key genes that could be regulated by both Vpr and the GR. PBMCs were treated with or without 5 μM Vpr peptide in the presence or absence of 100 nM Dex, MPA, NET-A or P4 for 24 hrs (Figure 9 A and B). mRNA levels of potential target genes were determined by using real time PCR with specific primers to Bcl-2 or Bim. In the presence of Vpr alone or Vpr in combination with Dex or MPA, mRNA expression of the anti-apoptotic factor Bcl-2 was significantly repressed compared to vehicle-treated cells, although Dex, MPA, NET-A or P4 had no significant effect in the absence of Vpr (Figure 9A). However, Dex and MPA alone significantly increased the expression of the pro-apoptotic factor Bim by approximately 1.7-fold and 1.3-fold, respectively, whereas Vpr alone had no significant effect (Figure 9B). Both NET-A and P4 alone or in combination with Vpr peptide had no effect on Bcl-2 or Bim mRNA levels (Figure 9A and B). Additionally we found that Vpr and Dex alone or in combination had no effect on the pro-apoptotic genes Bcl-2-associated death promoter protein (Bad) and phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) (data not shown).

Taken together the data suggest that Vpr regulates different genes involved in the apoptotic pathway as compared to Dex and MPA, with Dex/MPA up-regulating Bim gene expression and Vpr decreasing Bcl-2 gene expression. Furthermore, the other steroid receptor-selective agonists (Ald, E2, Mib and R5020) did not affect expression of the genes investigated, indicating that the responses were most likely mediated by the GR (data not shown).

Discussion

In this study we investigated the effects and molecular mechanisms of the injectable progestin contraceptives, MPA and NET-A, in $CD4^+$ T-cells on apoptosis, in the absence and presence of the HIV-1 protein Vpr. It has previously been shown that GCs and Vpr induce apoptosis in T-cells [35,53], that Vpr modulates GR function [70] and that MPA, but not NET-A, acts as a partial agonist for the GR [33,34,95]. We thus hypothesized that, similarly to GCs, MPA but not NET-A may also increase apoptosis in $CD4^+$ T-cells, which may be further enhanced in the presence of Vpr. Consistent with this hypothesis, we show that the GR agonists Dex and F, as well as MPA, but not NET-A or P4, induce apoptosis in $CD4^+$ T-cells. The predicted GR ligand response profile, and the inability of other steroid receptor-selective agonists to induce apoptosis in these cells, strongly suggests that the GR is the predominant receptor eliciting this effect. The relative responses by progestins are consistent with a lack of involvement of the PR, since MPA, P4 and NET are all potent PR agonists and hence apoptotic effects via the PR would be expected to be similar for these ligands, contrary to what is observed. On the other hand, MPA and NET-A have similar partial agonist activity via the AR and thus AR-mediated apoptotic effects via these ligands would be expected to be similar. Further support for the role of the GR is the finding that only ER, MR and GR mRNAs were detected in the PBMCs and only the GR protein but no other steroid receptor proteins were detected by Western blot analysis. MPA does not bind to and has no activity via the ER [29], while it binds very weakly but has no agonist activity on endogenous genes via the MR [101] and hence the progestins are unlikely to exert any apoptotic effects via these steroid receptors in PBMCs. Interestingly the AR, ER and MR have been shown to inhibit apoptosis in skeletal cells, breast cancer cells, neuronal cells and/or cardiomyocytes, when activated by their receptor-selective agonists, an effect that is most likely cell-specific [102,103–105]. Our results showing no detectable PR or AR, but ER, MR and GR expression in PBMCs are consistent with the literature [49,106,107]. Furthermore, MPA-induced apoptosis could be inhibited in the presence of the GR antagonist RU486. A role for the GR in mediating apoptosis by MPA and not NET-A or P4 is consistent with the relative binding affinities, potencies (concentration for half maximal response) and efficacies (maximal response) for transcriptional regulation by these ligands via the GR [31–34,95].

As expected, Vpr alone induced apoptosis in $CD4^+$ T-cells, which was further increased dose-dependently in the presence of Dex or MPA. Remarkably, MPA appeared to enhance Vpr-mediated apoptosis at a concentration as low as 1 nM (Figure 6A) which is lower than the peak and plateau levels observed in the serum of female patients using DMPA [87]. These findings suggest that the presence of MPA during HIV-1 infection would further potentiate the effects of Vpr on apoptosis in T-cells. As hypothesized due to their weak GR activity, NET-A or P4 did not induce apoptosis alone or in combination with Vpr. The increase in apoptosis observed with MPA, Dex or Vpr alone or Dex and MPA in combination with Vpr was decreased by the GR antagonist RU486, indicating that the GR is required for these effects. This is the first report to our knowledge showing that the GR is required for Vpr-mediated apoptosis in primary T-cells. The physiological significance of these findings with Vpr require further investigation. From the literature, it is unclear at what concentration Vpr occurs in the serum of infected individuals. One report has detected Vpr in the serum of infected patients at the same concentrations as circulating viremia [56], whereas another suggests that Vpr is present at a concentration of 0.7 nM [108]. Higher concentrations of Vpr peptide as used in this study (5 μM) and by others (1–10 μM) are required to induce apoptosis *in vitro* [61,62,99,109]. The intracellular concentrations of Vpr protein delivered and/or expressed in specific T-cells during chronic infection are unknown and likely to be much higher than serum concentrations reflecting Vpr diluted in the total volume of blood in the body. Thus whether the concentrations of Vpr peptide used in this study are physiologically relevant is not possible to ascertain at present. Interestingly, it has been reported that low concentrations of Vpr protect T-cells from apoptosis [110]. These authors suggested that the levels of Vpr during infection may vary in a manner that may be crucial to maintaining viral virulence and increased pathogenesis. Thus, it is possible that the levels of Vpr vary both in specific cellular environments and during different stages of disease progression such that at low levels of Vpr, apoptosis of T-cells does not occur to favour viral replication, whereas at other stages of the disease, increased Vpr levels may favour apoptosis and T-cell death.

To investigate whether the results with Vpr peptide are consistent with a role for Vpr delivered in the context of the whole virus we treated PBMCs with or without HIV-1 pseudotyped virus in the absence and presence of MPA and Dex. The results showed that MPA and Dex increase apoptosis induced by the HIV-1 pseudovirus particles. This result is consistent with potentiation by GR ligands of apoptosis in the presence of HIV-1 proteins. However, it does not exclude the possibility that other proteins besides Vpr are involved in the response in the context of HIV-1 pseudovirus particles.

Even though the literature suggests that Vpr directly targets the mitochondria during apoptosis, there is evidence that Vpr is predominately localized to the nucleus [66,111–113]. It is possible that a small percentage of Vpr translocates to mitochondria, but requires the transcription of pro-apoptotic genes in the nucleus to fully commit to apoptosis. For this reason, Vpr may regulate host gene expression to induce apoptosis. To determine which genes are involved in Vpr-mediated apoptosis in the presence of GR ligands we investigated key genes that have previously been shown to be regulated by either GCs or Vpr. The anti-apoptotic factor Bcl-2 was previously identified as a key mediator of apoptosis because its overexpression in a murine lymphoma cell line protected cells from GC-induced apoptosis [114]. Bcl-2 has been shown to be down-regulated by Vpr in a human promonocytic cell line [97,109]. Key genes that are upregulated by GCs include Bim in human and murine leukaemia cell lines as well as primary murine thymocytes [115]. As shown previously [109], the Vpr peptide down-regulated the anti-apoptotic gene Bcl-2. In contrast, we show that Dex and MPA, but not NET-A or P4, increased the expression of the pro-apoptotic genes Bim. Both Vpr and Dex alone or in combination had no effect on the pro-apoptotic genes NOXA and Bad (data not shown). Furthermore, no other steroid receptor-selective agonist enhanced or decreased expression of Bim or Bcl-2, indicating that the GR was the only steroid receptor that increased Bim expression (data not shown). Surprisingly Vpr and Dex did not act in concert to regulate gene expression of any genes tested. The evidence presented here suggests that the GR

and Vpr differentially regulate either pro- or anti-apoptotic genes, most likely resulting in a potent apoptotic response over a prolonged period of time. In the absence of Vpr, apoptosis is favoured by GCs or the progestin MPA, by induction of the pro-apoptotic gene Bim, whereas in the absence of GCs or MPA but the presence of Vpr, apoptosis is favoured by the repression of the anti-apoptotic gene Bcl-2. It is likely that the differential regulation of apoptotic genes by GCs/MPA and Vpr contributes to increased pathogenicity of the virus and T-cell depletion. We cannot however rule out the possibility that GCs/MPA and/or Vpr regulate other genes involved in the apoptosis pathway, or that Vpr induces apoptosis through direct interaction with the mitochondrial membrane or that the extrinsic and intrinsic pathways act together in inducing apoptosis in the $CD4^+$ T-cells.

Taken together, these findings are consistent with a role for MPA in repressing systemic immune function by increasing apoptosis in $CD4^+$ T-cells in the absence of HIV-1 infection, and an increase in this effect in the presence of HIV-1 infection. Furthermore the findings suggest that this occurs via a mechanism involving the GC-like properties of MPA, via GR-mediated changes in transcription of apoptotic genes, which are involved in the intrinsic apoptotic pathway. The extent to which these systemic immunosuppressive effects are physiologically relevant requires further investigation in clinical models, but the dose response results suggest that the apoptotic effects of MPA could occur within the peak nanomolar physiological concentration range measured in serum samples of women on DMPA. Additionally, the results in Figure 6 suggest that even when the MPA concentrations drop to about 2.6 nM a few weeks after injection of DMPA, MPA could potentiate apoptotic effects of Vpr in HIV-1 infected patients during chronic infection. The apoptotic effects of MPA in the absence of HIV-1 infection could have a role in acquisition of the virus owing to compromised immune responses, whereas the effects of DMPA in the presence of HIV-1 could have a role in disease progression and depletion of the T-cell population. These results for MPA are consistent with results showing a slower cellular immune response rate in DMPA-treated animals [40] and decreased T-cell numbers in patients treated with high concentrations of MPA for breast cancer [41].

There is not much information available regarding the effects of DMPA contraceptive usage on T-cell populations in women. Synthetic hormones in combined oral preparations, which usually do not contain MPA, were found not to affect absolute numbers or percentages of lymphocytes, T-cells and subsets of T-cells [49,116], consistent with a lack of an effect of contraceptives other than MPA on T-cell apoptosis. However, our results are consistent with the findings showing accelerated loss of $CD4^+$ T cells and death rate in women on DMPA infected with HIV-1 compared to non-contraceptive users [12]. The results in this study are also consistent with results for women using MPA in HRT, who exhibited a decrease in total lymphocyte count [117], the percentage of T-cells [117,118] and the percentage of T-helper (Th) lymphocytes [117].

Our finding that NET-A does not exhibit these GR-mediated apoptotic effects like MPA and F, suggests that choice of progestin contraceptive could significantly affect susceptibility to and progression of infectious diseases, such as HIV-1 and AIDS. The finding that P4 at concentrations less than 1 μM does not induce apoptosis in T-cells suggests that P4 concentrations in the luteal phase of the menstrual cycle [29] are unlikely to affect immune function via apoptosis of T-cells. These findings highlight the fact that not all progestins are the same [29,31,33,119] and that choice of progestin in hormonal therapy needs to be carefully considered. The choice of progestin for contraception may be particularly important for young women of child bearing age in the developing world in high risk areas for HIV-1 infection, where MPA usage as an injectable contraceptive is high [120].

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Author Contributions

Performed 80% of the experiments: MT. Conceived and designed the experiments: JPH MT CA ADT RMR. Performed the experiments: MT CA ADT RMR. Analyzed the data: JPH MT CA ADT RMR. Wrote the paper: JPH MT.

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