Cross talk between the glucocorticoid receptor and the progesterone receptor in modulation of progestin responses and HIV-1 infection

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Abstract

Current epidemiological data showing that the use of the injectable contraceptive progestin Depot-medroxyprogesterone acetate (DMPA) is associated with increased HIV-1 acquisition is controversial. However, animal and *ex vivo* data reveal plausible biological mechanisms whereby MPA may increase HIV-1 acquisition. Relatively high levels of endogenous progesterone (P_4) found in the luteal phase of the menstrual cycle have also been linked to increased HIV-1 acquisition in animal, clinical and *ex vivo* models. One of the central hypotheses of the present study was that the mechanism of MPA-induced increase in HIV-1 infection occurs via a different mechanism to that of the luteal phase. Furthermore, MPA has been shown to activate both the glucocorticoid receptor (GR) and its target, the progesterone receptor (PR) isoform B (PR-B), which are both transcription factors and regulate genes involved in immune function. Both the GR and PR are expressed in the cervix, the primary site of heterosexual HIV-1 infection. PR is regulated by endogenous estrogen (E_2), of which the concentrations fluctuate throughout the menstrual cycle, and GR expression also varies in response to stress hormones, leading to conditions of varied relative levels of GR/PR. The immune-related consequences of changing the relative levels of GR and PR-B are not well understood. Therefore another hypothesis of this study was that changing the relative levels of GR/PR-B modulates HIV-1 infection and immunomodulatory gene expression in response to the GR/PR agonist, MPA. Since GR and PR-B recognize similar DNA target sequences and may regulate the same genes at the same time, the final hypothesis of the present study was that GR and PR-B reciprocally modulate each other's activity, through possible association.

To investigate the effects of exogenous hormones on HIV-1 infection and mechanisms thereof, peripheral blood mononuclear cells (PBMCs) and TZM-bl cervical cells were used as model systems for HIV-1 infection. These cells were stimulated with P_4 and E_2 at concentrations mimicking the menstrual cycle phases or with levels of MPA at the upper range of peak serum levels detected in DMPA users. Cells were infected with the R-tropic HIV-1 infectious molecular clone, HIV-$1_{Bal\_Renilla}$ and luciferase assays were used to measure HIV-1 infection. Levels of HIV-1 CD4 receptor and CCR5 co-receptor protein or mRNA were measured by flow cytometry or qPCR, respectively, while activation of CD4+ T cells using the activation marker CD69 was measured by flow cytometry in PBMCs. To investigate the effects of changing GR/PR-B levels on HIV-1 infection and immune gene regulation, GR/PR levels were altered in End1/E6E7 immortalized endocervical and HeLa/TZM-bl cervical carcinoma cells by GR siRNA knockdown with or without the simultaneous over-expression of PR-B, and cells were stimulated with MPA or the GR agonist Dexamethasone. mRNA expression

of key immunomodulatory genes in End1/E6E7 and HeLa cells was measured by qPCR. The modulation of GR activity by PR-B was assessed by promoter-reporter assay in COS1 and U2OS cells over-expressing GR and PR and stimulated with GR- and/or PR-specific ligands. Association of GR and PR-B was measured by co-immunoprecipitation in COS1 and MCF-7 cells, while co-localization of GR and PR-B was measured by confocal microscopy and super-resolution structured illumination microscopy in COS1 cells.

MPA significantly increased HIV-1 infection in both PBMCs and TZM-bl cells, while luteal phase hormones did so to a lesser extent. However, MPA but not luteal phase hormones increased the ratio of CD4+/CD8+ T cells in PBMCs. MPA but not luteal phase hormones also increased CCR5 protein expression on CD4+ T cells in PBMCs and total CCR5 mRNA expression in TZM-bl cells. In addition, MPA but not luteal phase hormones increased activation of CD4+ T cells in PBMCs. Using a GR antagonist or GR siRNA, it was shown that the GR but not PR-B is required for MPA-, but not luteal phase hormone-induced increased HIV-1 infection in PBMCs and TZM-bls. The presence of PR-B altered the anti-inflammatory, GR-mediated regulation of some key immunomodulatory genes, including GILZ and IL-6, in End1/E6E7 and HeLa cells in response to MPA. In general, basal (unliganded) expression of immunomodulatory genes exhibited a pro-inflammatory profile in the presence of PR-B. Co-immunoprecipitation assays showed that GR and PR-B appeared to associate. Confocal microscopy suggested GR and PR co-localized in the nucleus in response to GR- and/or PR-specific ligands, while super-resolution microscopy showed that co-localization occurred in select regions within the nucleus.

Taken together, MPA increases HIV-1 infection in a manner different from that of luteal phase hormones, most likely involving increased CD4+ T cell frequency (CD4+/CD8+ ratio), activation and increased expression of CCR5 on CD4+ T cells, and requiring the GR. Furthermore, PR-B modulates GR-mediated immune function gene regulation, via potential association and region-specific nuclear co-localization. This suggests that the relative levels of GR/PR may play an important role in determining the inflammatory and immune responses and HIV-1 infection in HIV-1 target cells, both in DMPA users and women not using hormonal contraception.

Plagiarism Declaration

I, Alexis Joanna Bick, hereby declare that the work within this thesis is my original work, unless otherwise acknowledged, and that neither this thesis nor part thereof has been submitted for another degree at any other university.

Signature: Date: 19/02/2018

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Finally, a very special thank you to my boyfriend James Hall, who has had a seemingly endless supply of encouragement, love, pride and support. Thank you for loving me.

Table of Contents

List of abbreviations

AF	activation function domain
AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
AP-1	activator protein 1
APS	ammonium persulphate
AR	androgen receptor
ARE	androgen response element
ATCC	American type culture collection
bp	base pair
BPE	bovine pituitary extract
CCR5	C-C chemokine receptor type 5
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CO_2	Carbon dioxide
Co-IP	co-immunoprecipitation
CORT	cortisol
cs	charcoal-stripped
CTD	carboxy terminal domain
CVL	cervico-vaginal lavage
CXCR4	C-X-C chemokine receptor type 4
DBD	DNA binding domain
DEPC	diethylpyrocarbonate
DEX	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMPA	depot-medroxyprogesterone acetate
DNA	deoxyribose nucleic acid
E_2	17β-estradiol
EC_{50}	effective concentration required for 50% of maximal response
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERE	estrogen response element
EtBr	ethidium bromide
EtOH	ethanol
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FRT	female reproductive tract
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde phosphate dehydrogenase
GBS	GR binding sequence
GC	glucocorticoid
GILZ	glucocorticoid induced leucine zipper
GR	glucocorticoid receptor

GRE	glucocorticoid response element
GRIP-1	glucocorticoid receptor interacting protein 1
h	hour/s
HAT	histone acetyltransferase
HCl	hydrochloric acid
HDAC	histone deacetylase
HIV-1	human immunodeficiency virus type 1
HPV	human papillomavirus
HREC	human research ethics committee
HRP	horseradish peroxidase
HRT	hormone replacement therapy
HSP90	heat shock protein 90
HSV	herpes simplex virus
IFN	interferon
IκBα	inhibitor of NFκB type α
IL	interleukin
i.m.	intra-muscular
K_D	equilibrium dissociation constant
kDa	kiloDalton
K_i	dissociation constant
KSFM	keratinocyte serum-free medium
LBD	ligand binding domain
LH	luteinizing hormone
LNG	levonorgestrel
LTR	long terminal repeat
LUC	luciferase
M	Molar
MAPK	mitogen activated protein kinase
MDM	monocyte-derived macrophage
min	minute/s
MKP1	mitogen activated protein kinase phosphatase 1
mL	millilitre
mM	milliMolar
MMTV	mouse mammary tumour virus
MOPS	4-morpholine-propanesulfonic acid
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
mRNA	messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NCoR	nuclear receptor co-repressor
NET	norethisterone
NET-A	norethisterone acetate
NET-EN	norethisterone enanthate
NFκB	nuclear factor kappa B
nGRE	negative GRE
nM	nanoMolar
NTD	amino-terminal domain
p24	HIV-1 viral core protein

P_4	progesterone
PAGE	polyacrylamide gel electrophoresis
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PR	progesterone receptor
PRE	progesterone response element
qRT-PCR	quantitative reverse transcription PCR
R5020	promegestone
RANTES	regulated upon activation, normal T cell expressed and secreted
RBA	relative binding affinity
RLU	relative light units
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
s	seconds
s.c.	sub-cutaneously
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	steroid receptor coactivator
STI	sexually transmitted infection
TAT	tyrosine aminotransferase
TBS	TRIS-buffered saline
TBST	TRIS-buffered saline-tween
TNFα	tumour necrosis factor alpha
µL	microlitre
UTR	untranslated region
VpR	HIV-1 viral accessory protein R
v/v	volume per unit volume
WHO	World Health Organization
w/v	weight per unit volume

Thesis Outline

This thesis comprises of six chapters and five appendices. There are three results chapters and supplementary results are included in the appendix.

Chapter 1: Literature Review. This chapter outlines the current literature on mechanisms of GR- and PR-mediated gene regulation, the immune responses of the FRT mucosae, and HIV-1 infection in response to endogenous sex hormones and hormonal contraception with the injectable MPA. The rationale behind the research conducted is also discussed and the main hypotheses are stated.

Chapter 2: Materials and Methods. This chapter describes in detail the methods used to obtain the results in the subsequent chapters.

Chapter 3: Results: Both MPA used in injectable contraception and exogenous hormones mimicking the luteal phase of the menstrual cycle increase HIV-1 infection, but via potentially different mechanisms. This chapter investigates HIV-1 infection in peripheral blood and an infectable cervical cell line model in response to MPA or hormones representing the luteal and follicular phases of the menstrual cycle, and analyzes potential biological mechanisms of infection through regulation of key markers relevant to HIV-1 infection.

Chapter 4: Results: MPA-induced and basal HIV-1 infection and immunomodulatory gene expression change when the relative levels of GR and PR are altered. This chapter explores the effects of molecular manipulation of relative GR/PR levels in cervical cell lines on HIV-1 infection, expression of markers relevant to HIV-1 infection and immunomodulatory gene expression.

Chapter 5: Results: Modulation of GR activity by PR potentially occurs via GR-PR association and co-localization. This chapter investigates potential interactions and cellular co-localization of the GR and PR in the absence and presence of hormone ligands.

Chapter 6: Discussion. This chapter critically discusses the results obtained in the previous three chapters and contextualizes the findings from this thesis within the fields of biological mechanisms of HIV-1 infection and cross-talk between the GR and PR.

Chapter 7: Conclusions and Future Perspectives. This chapter draws concluding remarks from the observations presented in this thesis and proposes new avenues of research arising from this work.

Appendix A: Supporting data from other researchers in the Hapgood laboratory. This appendix consists of supporting data carried out by other researchers in the present author's laboratory, and

shows the endogenous steroid receptor levels of the various model systems (End1/E6E7, HeLa, TZMBL, PBMCs, cervical explants) used in this study.

Appendix B: Supporting data from the present author. This appendix includes supplementary data carried out by the present author and consists of pilot experiments, important controls and the investigation of possible model cell line strategies in which relative GR/PR levels could be altered.

Appendix C: Gating strategy used for flow cytometry analysis. This appendix consists of flow diagrams indicating how samples from a representative PBMC donor were gated for marker-negative cell populations and analysed for flow cytometry.

Appendix D: Cervical explant donor information. This appendix lists the endogenous hormone levels, STI status and menstrual cycle phase of the donors whose cervical explant tissue was used for quantification of endogenous GR/PR protein levels.

Appendix E: Approval letters from the Human Research Ethics Committee. This appendix contains scanned copies of the approval letters received from the University of Cape Town's Human Research Ethics Committee for the doctoral research as a sub-study of an existing study.

References. This alphabetical list includes all the publications cited in this thesis.

Parts of this work were presented by the candidate at the following meetings:

- Nuclear Receptors and Disease, Cold Spring Harbor, New York, USA, October-November 2014
- South African Society for Biochemistry and Molecular Biology (SASBMB) Congress, Goudini Spa, South Africa, July 2014
- British Council Mucosal surfaces workshop, Cape Town, South Africa, January 2015

Some of the candidate's flow cytometry data from Chapter 3 and TZM-bl GR siRNA infection data from Chapter 4 were used in the following publication:

Michelle F. Maritz*, Roslyn M. Ray*, **Alexis J. Bick***, Michele Tomasicchio, John G. Woodland, Yashini Govender, Chanel Avenant and Janet P. Hapgood. **Medroxyprogesterone acetate, unlike norethisterone, increases HIV-1 replication in human peripheral blood mononuclear cells and an indicator cell line, via mechanisms involving the glucocorticoid receptor, increased CD4+/CD8+ ratios, T-cell activity and CCR5 levels.** Submitted to *PLoS ONE* in December 2017.
* co-first authors

Information on serum concentrations of MPA from Chapter 1 was compiled by the candidate for the following publication, in preparation:

Alexis J. Bick, Renate Louw-du Toit, Donita Africander, Salndave B. Skosana and Janet P. Hapgood. **Pharmacokinetics, metabolism and serum concentrations of progestins used in contraception.**

Chapter 1

Literature Review

1.1 HIV-1 prevalence in South Africa

With 38.8 million people worldwide living with HIV/AIDS in 2015, HIV-1 infection, incidence and prevalence remain a global health concern (Collaborators et al. 2016). From about 1.8 million new infections recorded globally in 2015, approximately 75% of these occurred in Sub-Saharan Africa (Collaborators et al. 2016), thereby burdening this region with the highest incidence of HIV-1 and AIDS (Fig. 1.1.). South Africa in particular has the highest HIV-1 prevalence, with about 7 million infected people in 2015 (UNAIDS South Africa , Avert 2016). HIV-1 incidence in South Africa is in the 98-99th percentile, in the range of 707-1043 per 100 000 people (Fig. 1.1.).

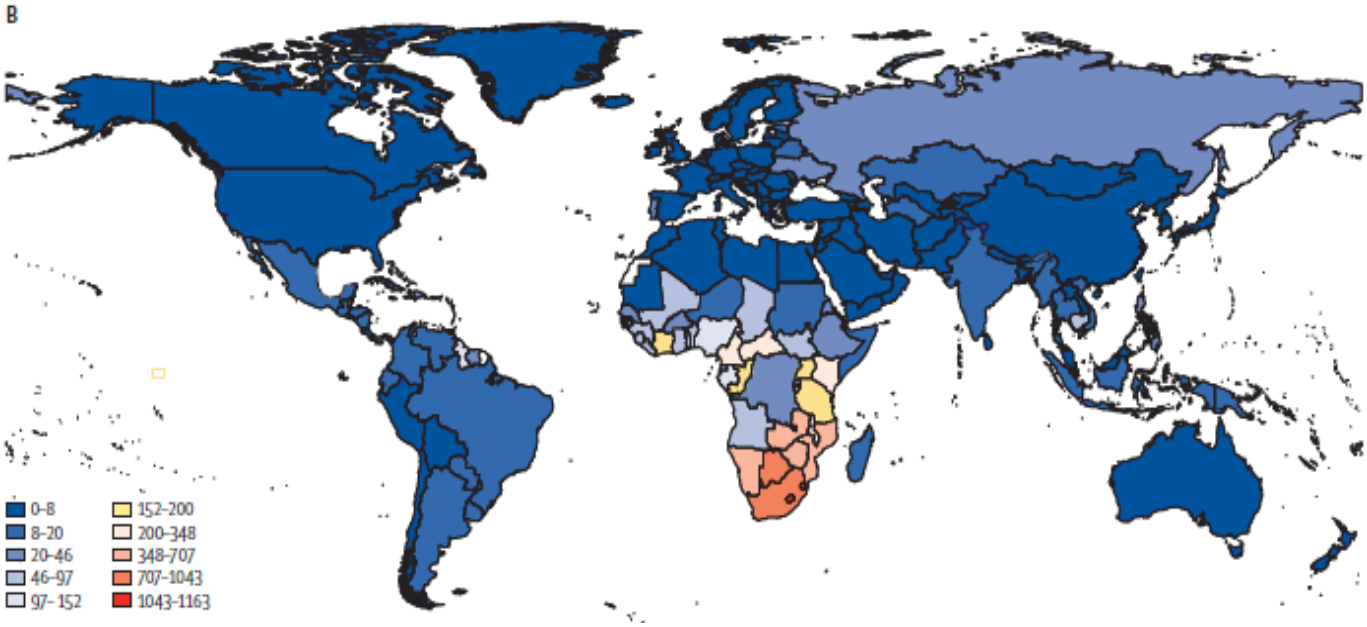


Figure 1.1. Global incidence of HIV-1 per 100 000 people by country in 2015. Incidence was calculated as cumulative new HIV infections during the year divided by the total mid-year population. Colour blocks indicate the 0–50th, 50–70th, 70–80th, 80–90th, 90th–92nd, 92nd–94th, 96–98th, 98–99th, and 99–100th percentiles. Several islands, all in blue colours, have been excluded from the map, including Singapore, Fiji, Malta and the Caribbean. Taken from (Collaborators et al. 2016).

Globally more than half of new infections occur in women (Joint United Nationals Programme on HIV/AIDS (UNAIDS) 2016). This trend is also observed in South Africa, where the prevalence of HIV-1 is higher in women than men (Shisana et al. 2014). This difference is highest for women of reproductive age, between ages 20-34 (Shisana et al. 2014) (Fig. 1.2).

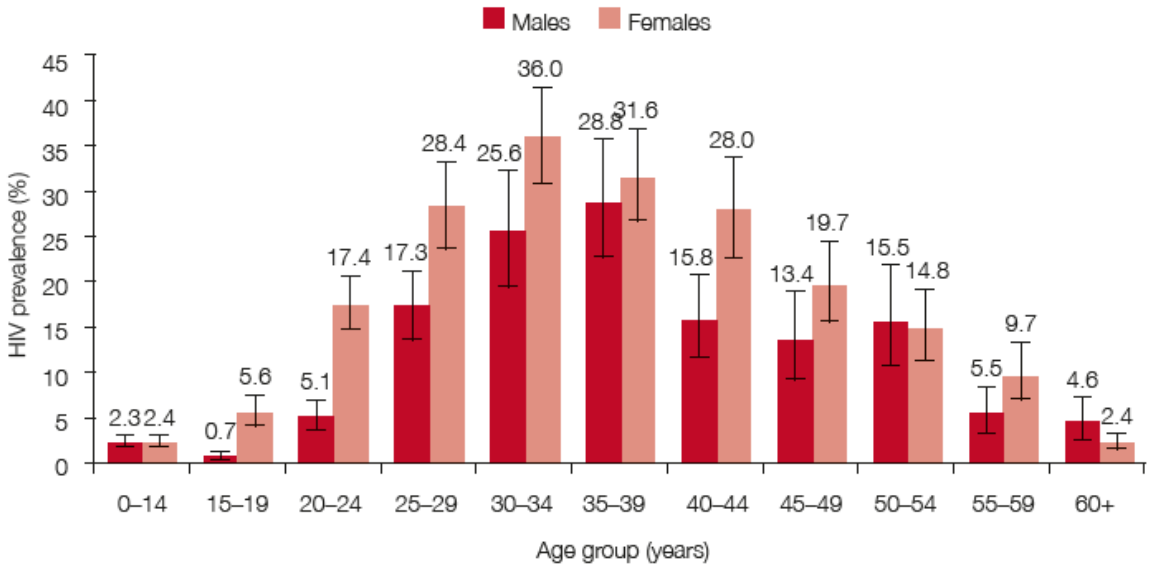


Figure 1.2. HIV-1 prevalence in South African men and women in 2012. HIV-1 prevalence is indicated as percentage. Prevalence is higher in females than males for all age groups except 50-54 and over 60 years. The highest prevalence occurs in females aged 30-34 years. Taken from (Shisana et al. 2014).

Men and women differ considerably in their immune responses and susceptibility to infectious diseases (Klein and Flanagan 2016, Roved et al. 2017). This phenomenon is largely a consequence of different endogenous sex steroid hormones, estrogen (E_2) and progesterone (P_4) in women and testosterone in men. In addition to regulating reproduction, female sex hormones regulate immune function in the female reproductive tract.

1.2 The female reproductive tract (FRT)

The FRT consists of several organs co-ordinating in function to regulate reproductive processes including egg maturation and release, implantation of fertilized eggs, foetal development and maintenance of pregnancy, parturition and in the absence of pregnancy, menstruation. These processes are intricately regulated by the endogenous sex steroid hormones, E_2 and P_4. The FRT also functions to protect against bacterial, viral and yeast pathogens (Wira et al. 2005b). During heterosexual intercourse, the FRT is the first line of defence against sexually transmitted infections, including HIV-1, by providing both a physical barrier and a protective immune microenvironment (Wira et al. 2005b, Wira et al. 2005c, Hickey et al. 2011).

1.2.1 Structure

The organs of the FRT are classified as upper FRT (ovaries, fallopian tubes, uterus) or lower FRT (vagina, cervix, Fig. 1.2.1) (Miller and Shattock 2003, Lee et al. 2015, Wira et al. 2015). The cervix is further divided into the endocervix (uterine side) and ectocervix (vaginal side, Fig. 1.2.1) and is believed to be the primary site of HIV-1 infection (Pudney et al. 2005).

The mucosal surfaces lining the FRT are comprised of stratified squamous epithelium in the vagina and ectocervix, and columnar epithelium in the endometrium and endocervix (Fig. 1.2.1) (Hladik and Hope 2009, Nguyen et al. 2014, Wira et al. 2014a). The mucosal epithelium is structurally supported by the underlying lamina propria and stromal connective tissue. Epithelial barrier integrity is promoted by tight junctions between cells (Kaushic 2009, Kaushic 2011). An additional physical barrier is the secretion of thick mucus by the cervix (Fig. 1.2.1) (Hickey et al. 2011, Wira et al. 2015).

The cervical transformation zone, also known as the squamo-columnar junction, is the area in which the cells change from multi-layer squamous cells to monolayer columnar cells (Fig. 1.2.1). This thin layer of columnar epithelium and the large number of CD4+ T cells populating the transformation zone make the cervix an important site for initial HIV-1 infection (Pudney et al. 2005, Haynes and Shattock 2008, Wira et al. 2010, Lee et al. 2015).

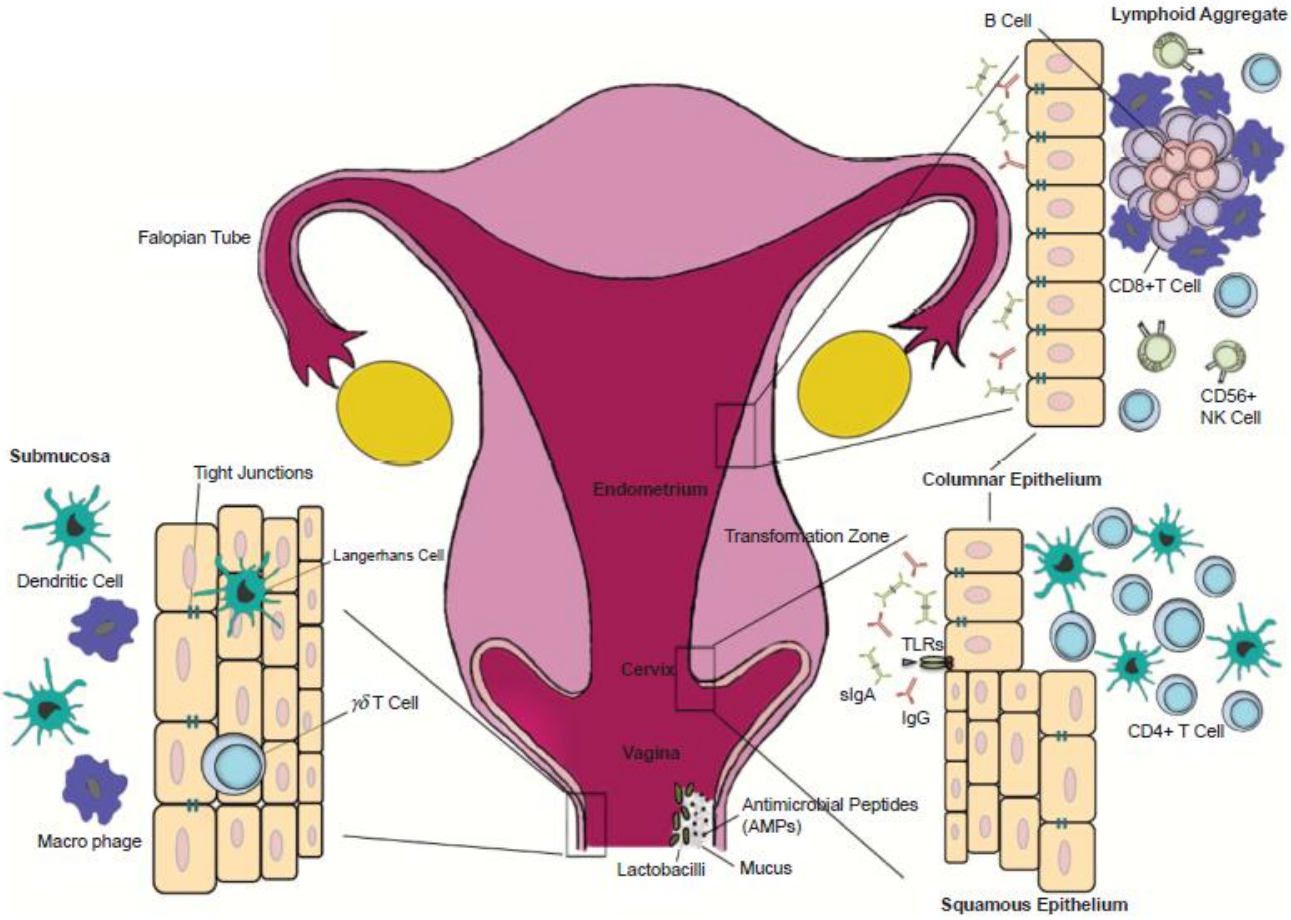


Figure 1.2.1. Anatomical structure, epithelium and immune cells of the FRT. Ovaries are shown in yellow, with connective tissue in light pink and the FRT lumen in dark pink. Compartments of the lower FRT (vagina and ectocervix) are lined with squamous epithelium, while the upper FRT (endocervix and uterine endometrium) is lined with columnar epithelium. The transformation zone occurs where epithelial cells change from squamous to columnar. Immune cells shown include HIV-1 target CD4+ T cells. Innate immunity in the vagina is represented by antimicrobial peptides, *Lactobacilli* and mucus. Taken from (Nguyen et al. 2014).

1.2.2 HIV-1 infection in the FRT

Infection with the retrovirus HIV-1 in the FRT occurs through sexual transmission. Despite low transmission rates (1:200-1:2000), more new cases arise through vaginal intercourse than other types of transmission (Hladik and McElrath 2008, Hladik and Hope 2009). HIV-1 crosses through genital epithelial cells at regions of compromised barrier integrity via shearing, microabrasions, impaired tight junction function, ulcerations and/or other sexually transmitted infections (Hladik and Hope 2009, Kaushic 2009, Nazli et al. 2010, Kaushic 2011, Rodriguez-Garcia et al. 2013b). HIV-1 uptake via transcytosis in genital epithelial cells has been demonstrated (Ferreira et al. 2015), however HIV-1 does not actively replicate in epithelial cells (Dezzutti et al. 2001, Dezzutti and Hladik 2013, Ferreira et al. 2015).

Infection occurs through viral envelope proteins binding to host CD4 receptors (Sattentau et al. 1986) on the surface of target cells, which are typically CD4+ T cells, macrophages or dendritic cells (DCs) (Givan et al. 1997, Howell et al. 1997, Hladik and Hope 2009, Nguyen et al. 2014, Wira et al. 2015). Importantly, women have greater frequencies of systemic CD4+ cells than men (Maini et al. 1996). Upon CD4 receptor binding, viral envelope proteins undergo a conformational change to permit binding to one of two host co-receptors: either C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) (Cullen 2001). The preference of the transmitting virus to utilize either CCR5 or CXCR4 classifies the virus as R5-tropic or X4-tropic, respectively, or even dual-tropic (Berger et al. 1998). Usually a single virus variant is transmitted to the new host, and the transmitting virus is predominantly R5-tropic, although a small percentage of infections occur through X4-tropic viruses (Carrington et al. 1999, Keele et al. 2008, Salazar-Gonzalez et al. 2009, Grivel et al. 2011). R5-virus transmission is likely to be augmented by the high frequency of CCR5-expressing immune cells in the FRT (Patterson et al. 1998, Shacklett and Greenblatt 2011).

Following co-receptor binding, a second conformational change facilitates viral fusion to the host membrane, after which the virion is shed and viral RNA undergoes reverse transcription, integration into the host genome and transcription of viral genes (Haseltine and Wong-Staal 1988, Briggs and Krausslich 2011). Infected cells then carry the virus to other local T lymphocytes or migrate to nearby lymph nodes and the circulatory vascular system (Hladik and Hope 2009).

1.2.3 Immune function of mucosal surfaces

In order to perform the dual functions of promoting reproductive processes and protecting against pathogens, the FRT has evolved multiple innate and adaptive immune responses, in addition to the protective barrier function of mucosal epithelium (Wira et al. 2005b, Wira et al. 2010, Wira et al. 2011).

The innate immune response of the FRT provides the initial rapid defence against pathogens and consists of several non-specific mechanisms. These include the production of the bactericidal enzyme lysozyme, mucus and antimicrobial peptides (Fig. 1.2.1) such as secretory leukocyte protease inhibitor (SLPI) and human beta-defensin 2 (HBD2) through toll-like receptor (TLR)-mediated signalling in epithelial cells (Wira et al. 2005b). SLP1 and HBD2 exhibit potent anti-HIV activity (Hocini et al. 2000, Quinones-Mateu et al. 2003, Weinberg et al. 2006). In addition local flora such as *Lactobacillus* species producing lactic acid and H_2O_2 maintain a low pH in the vagina that is hostile to invading pathogens (Fig. 1.2.1) (Reis Machado et al. 2014, Vitali et al. 2017). The innate immune response is also regulated by the actions of specific immune cells, specifically plasmacytoid dendritic

cells (pDCs), neutrophils, macrophages and natural killer (NK) cells, which function in a general manner to destroy invading pathogens (Wira et al. 2005b).

Cells involved in adaptive immunity include T cells and antibody-producing B cells. The adaptive immune response occurs through several co-ordinated processes, starting with pathogen antigen recognition by antigen-presenting cells (APCs) which present to T cells thereby inducing their activation and subsequent cytokine and antibody production (Wira et al. 2005b). Immune protection is mediated by humoral (antibody production by B cells) and/or cell-mediated (pathogen destruction by T cells) functions (Wira et al. 2005b). In this way, the adaptive immune system generates an immunological memory to pathogenic antigens that will promote a rapid response upon subsequent challenges (Hapgood et al. 2018). APCs such as DCs link the adaptive and innate responses.

T cells are arguably the adaptive immune cells most relevant to HIV-1 infection and are the major component (30-60%) of immune cells expressed in all FRT tissues (Givan et al. 1997). T cells are broadly categorized as CD8+ cytotoxic T cells, which kill infected cells, and CD4+ helper T cells, which support the function of other immune cells and are primary targets for HIV-1 infection (Hapgood et al. 2018). CD4+ and CD8+ T cells are found in the lamina propria and squamous epithelium of the vagina, ectocervix and endocervix (Miller and Shattock 2003, Pudney et al. 2005, Hladik and Hope 2009, Hafner et al. 2013, Reis Machado et al. 2014), with many of these cells also expressing the CCR5 co-receptor (Patterson et al. 1998, Shacklett and Greenblatt 2011).

Cytokines and chemotactic cytokines (chemokines) are soluble signalling molecules that are secreted by many cell types from both the innate and adaptive immune systems, including FRT epithelial cells, natural killer (NK) cells and T cells (Fahey et al. 2005, Wira et al. 2005b, Wira et al. 2005c). Cytokines are involved in a number of cellular functions including recruiting circulating immune cells to migrate to the FRT and inducing their maturation and differentiation (Wira et al. 2005b, Wira et al. 2005c). Common cytokines and chemokines expressed in FRT immunity include interleukins (ILs) 1β, 6, 8, tumour necrosis factor α (TNFα), regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP) 1β, MIP 3α, monocyte chemotactic protein (MCP) 1, granulocyte-macrophage colony-stimulating factor (GM-CSF), and migration inhibitory factor (MIF). Cytokines and chemokines are generally classified as pro-inflammatory molecules that promote inflammatory functions, or anti-inflammatory cytokines which serve to reduce inflammation.

Importantly, the innate and adaptive immune responses in the upper and lower FRT are regulated by the female sex hormones, E_2 and P_4, and therefore the factors and cells involved in these immune responses are intricately varied during the menstrual cycle (see section 1.3.2) (Kutteh et al. 1998,

Beagley and Gockel 2003, Wira et al. 2005a, Wira et al. 2005b, Ochiel et al. 2008, Wira et al. 2010, Weinberg et al. 2011, Wira et al. 2011, Dunbar et al. 2012, Oertelt-Prigione 2012, Hafner et al. 2013, Rodriguez-Garcia et al. 2013a, Rodriguez-Garcia et al. 2013b, Goode et al. 2014, Nguyen et al. 2014, Wira et al. 2014a, Wira et al. 2014b, Tan et al. 2015, Wira et al. 2015).

1.3 The menstrual cycle

The female menstrual cycle is a series of hormonally controlled events that regulate the reproductive system in premenopausal women, and is typically 28 days long. The menstrual cycle is classically charted from the first day of menstruation and is divided into two ovarian phases: follicular and luteal, which are separated by ovulation (Fig. 1.3). The corresponding uterine phases are the proliferative and secretory phases (Fig. 1.3).

1.3.1 Hormonal regulation and biological events of the menstrual cycle

The menstrual cycle is regulated by endogenous sex steroid hormones: the gonadotropic hormones, gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), prostaglandins and the ovarian hormones, E_2 and P_4. These hormones form part of the hypothalamic-pituitary-gonadal (HPG) axis. The fluctuations in sex hormones during the menstrual cycle cause a series of biological events in the ovaries and uterus (Fig. 1.3).

GnRH secreted in a pulsatile manner from the hypothalamus stimulates pulsatile secretion of LH and FSH from the pituitary gland (Owen 1975, Chabbert Buffet et al. 1998, Mihm et al. 2011). During the follicular phase, FSH promotes E_2 secretion from the developing dominant follicle in ovarian granulosa cells, and E_2 levels peak during the mid-cycle (Moghissi et al. 1972, Owen 1975, Wira et al. 2015). At the same time the endometrial tissue proliferates in response to increasing E_2 levels (Owen 1975, Chabbert Buffet et al. 1998, Mihm et al. 2011). The steep rise in LH levels, or LH surge, is required for ovulation to occur, whereby an ovum is released from the mature Graafian follicle (Speroff and Vande Wiele 1971, Wira et al. 2015) (Fig. 1.3). LH and FSH levels are regulated by both positive and negative feedback in response to estrogens and P_4 (Speroff and Vande Wiele 1971, Chabbert Buffet et al. 1998, Mihm et al. 2011).

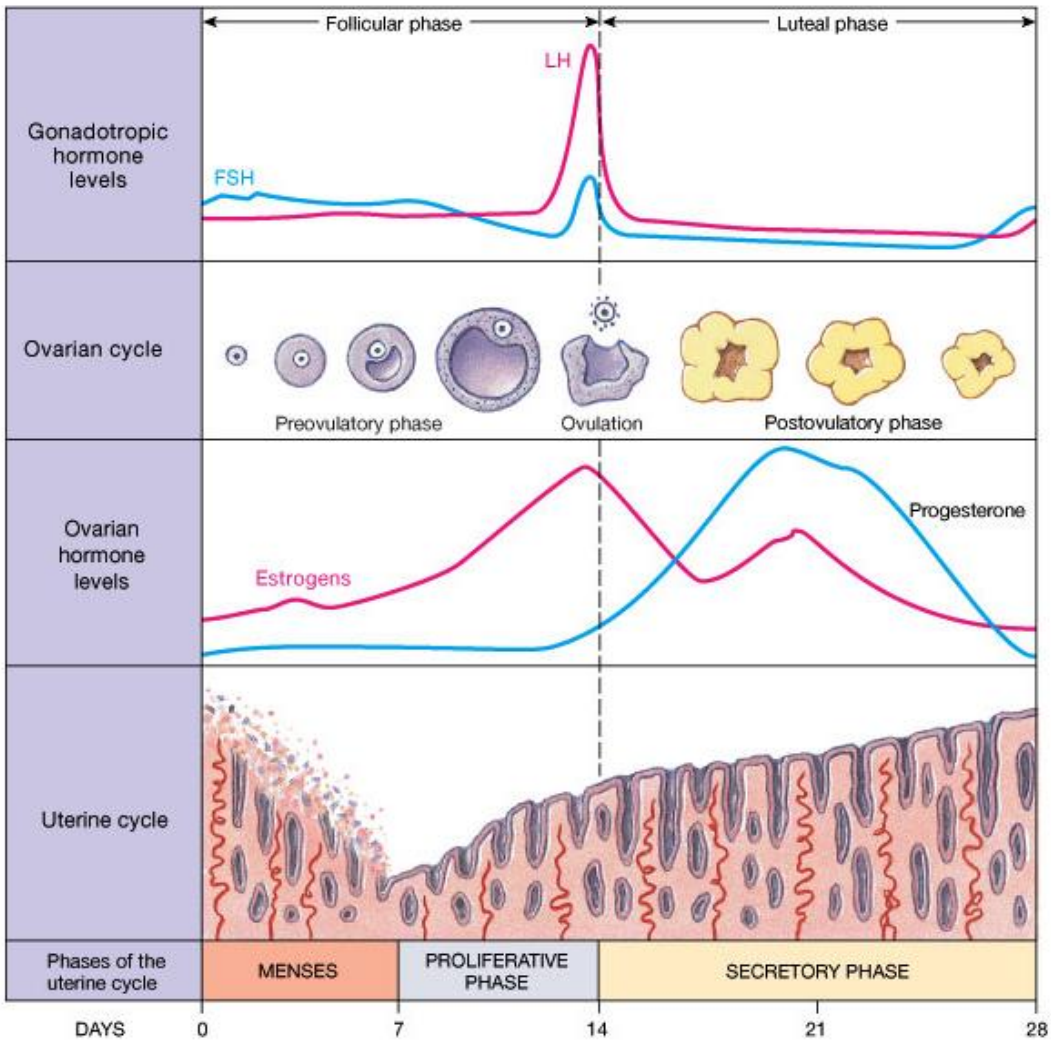


Figure 1.3. Biological events, phases and hormone levels of the female menstrual cycle. The cycle begins at menses/menstruation where the uterine lining is shed and corresponding hormone levels are low. During the follicular/proliferative phase, ovarian follicles develop as FSH and E_2 levels increase, culminating in the LH surge that precedes ovulation. The uterine lining proliferates in anticipation of embryo implantation. In the absence of fertilization, P_4 levels rise during the luteal/secretory phase and the ovum degenerates. LH: luteinizing hormone; FSH: follicle stimulating hormone. Taken from http://science.taskermilward.org.uk/mod1/KS4Biology/B1/Topic%201%20lessons/B1_14.htm

During the luteal phase, endometrial blood vessels proliferate and coil, and endometrial stromal cells differentiate (known as decidualization), in anticipation of implantation of a fertilized embryo (Chabbert Buffet et al. 1998, Mihm et al. 2011, Hapgood et al. 2018). During this period from about Day 20-24, known as the implantation window, the uterus is receptive toward implantation (Chabbert Buffet et al. 1998).

Following ovulation, the corpus luteum (empty follicle and some surrounding cells) secretes P_4, which increases in concentration until P_4 levels peak at the mid-luteal phase (Moghissi et al. 1972, Chabbert Buffet et al. 1998, Farage et al. 2009, Wira et al. 2015). If fertilization has occurred, the corpus luteum continues to secrete P_4 under control of LH which is induced by human chorionic gonadotropin (hCG) produced by the developing trophoblast, thereby maintaining pregnancy (Kumar and Magon 2012). In the absence of fertilization, however, the corpus luteum degrades (called luteolysis) and stops secreting P_4 (Mihm et al. 2011, Wira et al. 2015). Thereafter E_2 and P_4 levels decline from the mid-luteal phase, culminating in the degradation and shedding of the uterine lining during menstruation, and thereafter the cycle repeats (Chabbert Buffet et al. 1998, Mihm et al. 2011, Wira et al. 2015).

In addition to LH, FSH, E_2 and P_4 and prostaglandins, the functions of additional hormonal regulators such as inhibins and activins provide further complexity to the hormonal regulation of the menstrual cycle (Chabbert Buffet et al. 1998, Farage et al. 2009, Mihm et al. 2011, Messinis et al. 2014).

E_2 and P_4 are master regulators of female reproduction and the menstrual cycle, with P_4 regulating mammary gland development, and the establishment and maintenance of pregnancy, while E_2 controls ovulation, gonadotropic hormone release and other metabolic, neurological and cardiovascular functions (Hapgood et al. 2018). The biological effects of E_2 and P_4 are mediated by binding and activating their respective steroid receptors (SRs), the estrogen receptor (ER) and progesterone receptor (PR) (Chabbert Buffet et al. 1998, Stanczyk et al. 2013, Wira et al. 2015).

The serum levels of E_2 peak during the follicular phase, while P_4 levels peak during the luteal phase (Speroff and Vande Wiele 1971, Thorneycroft et al. 1971, Moghissi et al. 1972, Owen 1975, Altemus et al. 1997, Chabbert Buffet et al. 1998, Sturgeon et al. 2004, Farage et al. 2009, Kuhl 2011, Weinberg et al. 2011, Hafner et al. 2013, Thurman et al. 2016). Thus the follicular phase is regarded as E_2-dominant, while the luteal phase is classified as the P_4-dominant phase (Speroff and Vande Wiele 1971, Thurman et al. 2016). The serum concentration of endogenous E_2 ranges from 50-1500 pM during the follicular phase and 50-1100 pM across the luteal phase of the menstrual cycle (Stricker et al. 2006) (Fig. 1.3). Endogenous P_4 concentrations range from 0.4-1.6 nM during the follicular phase to a wide range of 1-72 nM during the luteal phase (Altemus et al. 1997, Stricker et al. 2006, Hafner et al. 2013, Thurman et al. 2016).

During pregnancy, serum P_4 levels are even higher and range from 400-600 nM (Kuhl 1990, Sheffield et al. 2009, Africander et al. 2011b) but can reach as high as 1 μM (Goebelsmann 1986) to 4-10 μM at the umbilical cord (Dawood and Helmkamp 1977, Goebelsmann 1986). Serum E_2 levels during pregnancy range from 22-110 pM (Lindberg et al. 1974). In postmenopausal women, serum E_2 and P_4 levels remain quite low, with E_2 levels at about 15 pM (Edlefsen et al. 2010, Hafner et al. 2013) and

P_4 levels at about 230 pM (Edlefsen et al. 2010). The wide ranges in reported concentrations are likely to reflect the large inter-individual biological variation between women and studies. Nevertheless, serum E_2 and P_4 levels fluctuate in a specific pattern during the menstrual cycle, and therefore will regulate different biological responses at different stages of the cycle.

1.3.2 Immune function and susceptibility to infection across the menstrual cycle

The FRT is uniquely equipped to respond to sexually transmitted pathogens, foreign sperm and a foetus that is immunologically dissimilar (Wira et al. 2005b). It is hypothesized that the period 7-10 days following ovulation (i.e. during the luteal phase) provides a "window of vulnerability" for infection, in which sex hormones suppress immunity in order to support fertilization and implantation (Wira and Fahey 2008, Wira et al. 2010, Rodriguez-Garcia et al. 2013b, Wira et al. 2014b).

In support of this hypothesis are multiple studies, indicated below, showing that immune cell frequency and function, innate immunity factors, co-receptor expression, cytokine production, gene expression and HIV-1 infectivity vary with fluctuating sex hormone levels across the menstrual cycle. The frequency of immune cells differs between peripheral blood and FRT tissues, and also between the upper and lower FRT (Givan et al. 1997, Wira and Fahey 2008, Hafner et al. 2013, Nguyen et al. 2014, Wira et al. 2015). Regardless of their localization, their proportions and/or functions have been shown to be sensitive to changes in female sex hormone levels in several reports.

In peripheral blood samples from naturally cycling women, the proportion of CD4+ T cells was higher during the luteal phase in some reports (Faas et al. 2000, Weinberg et al. 2011) but higher during the follicular phase in another report (Lee et al. 2010). Some studies showed that CD8+ T cell numbers were higher during the luteal phase (Faas et al. 2000, Weinberg et al. 2011), but another study showed no change in the proportion of CD8+ T cells or B cells (Lee et al. 2010). The proportion and activity of NK cells in peripheral blood was increased during the luteal phase in some studies (Faas et al. 2000, Lee et al. 2010, Lee et al. 2015) but during the follicular phase in other studies (Souza et al. 2001, Yovel et al. 2001). Some reports have shown no change in total numbers of peripheral leukocytes (Tikare et al. 2008) or lymphocytes (Mathur et al. 1979, Lopez-Karpovitchs et al. 1993, Bouman et al. 2001, Rajnee et al. 2010) across the menstrual cycle, while others found increased total leukocytes (Bouman et al. 2001, Begum and Ashwini 2012, Nowak et al. 2016) or lymphocytes (Rosemary et al. 2014) during the luteal phase. In contrast, another report showed higher leukocyte numbers during the follicular phase (Rajnee et al. 2010). Numbers of monocytes were reported to be higher during the luteal phase (Mathur et al. 1979, Northern et al. 1994). In addition, in ovariectomized rhesus macaques treated with E_2 or P_4 pellets, where P_4 serum levels reached mid-

luteal concentrations, decreased frequencies of CD4+ and CD8+ T cells and those expressing the activation markers CD69 or CD25 were observed in PBMCs after P_4 treatment (Attanasio et al. 2002). Collectively some of these studies suggest that the menstrual cycle may have effects in immune cells from PBMCs, while some do not.

Immune cells from different FRT compartments in naturally cycling women are also sensitive to endogenous hormone levels across the menstrual cycle. The presence of higher numbers of important immune cells, such as CD4+ T cells and cells expressing the CCR5 co-receptor, at the time of HIV-1 exposure might increase the chance of infection (Wira et al. 2014b). In the endometrium, for example, there have been reports that the number of CD68+ macrophages (Bonatz et al. 1992) and the size of lymphoid aggregates (consisting of B cells and CD8+ T cells) in endometrial sections (Yeaman et al. 1997) were increased during the luteal phase. However, cytotoxic T cell function was lower in hysterectomy samples from the luteal phase (White et al. 1997). Other research using hysterectomy samples observed lower expression of CD4 and CCR5 in uterine epithelial cells from the luteal phase (Yeaman et al. 2003) and higher CD4 expression during the follicular phase but no change in CCR5 expression in ectocervical epithelial cells (Yeaman et al. 2004). Other reports have shown that the luteal phase exhibits higher frequency of endocervical CCR5+ CD4+ T cells from cytobrush samples (Byrne et al. 2016), frequency of CD4+CCR7^{hi} memory T cells in cervico-vaginal lavage (CVL) samples (Swaims-Kohlmeier et al. 2016), frequency of CCR5+CD3+ cells, CCR5+CD14+ cells and CCR5 mRNA in PBMCs and vaginal biopsies (Sheffield et al. 2009), frequency of regulatory T cells in PBMCs (Weinberg et al. 2011), and levels of CCR5 mRNA from endometrial biopsies (Dominguez et al. 2003) compared to the follicular phase. However, some studies have shown no differences in immune cell frequencies in vaginal and/or cervical explants in humans (Givan et al. 1997, Poppe et al. 1998, Patton et al. 2000, Pudney et al. 2005, Thurman et al. 2016) or from tissue sections in rhesus macaques (Ma et al. 2001). Studies using exogenous P_4 stimulation have also yielded differing results. In one study, ~100 nM P_4 increased CCR5 mRNA levels in CD14+ monocytes from PBMCs (Patterson et al. 1998), while in another study, 10-100 nM P_4 reduced CCR5 protein in PBMCs from both HIV-1 positive and negative women (Cabrera-Munoz et al. 2012). High P_4 levels (100 nM-10 μM) inhibited IL-2-induced CCR5 protein expression in activated T cells (Vassiliadou et al. 1999), while a combination of menstrual cycle hormones (2 nM E_2, 200 pM P_4, 4 nM testosterone) did not change CD4, CCR5 or CXCR4 levels in the Jurkat T cell line (Ragupathy et al. 2013). However the latter study used a concentration of E_2 higher than the physiological range. Taken together, some studies show different effects on immune cell frequency or CCR5 levels with different menstrual cycle phases, in different FRT compartments, while some do not. It is therefore difficult to say conclusively whether or not menstrual cycle status has an effect, and it might be that there is no significant effect on immune function. The different findings between studies for both PBMCs and

FRT compartments may reflect differences in endogenous E_2/P_4 concentrations, tissue composition, immune activation status, sampling time, sample group and/or other variables.

The menstrual cycle is also thought to influence the expression of innate immune factors (Hafner et al. 2013). For example, cervical mucus is thickest and most viscous during the luteal phase (Wira et al. 2015). Furthermore in cervico-vaginal samples, levels of the antibodies immunoglobulin (Ig) A and IgG, IL-6, IL-8, SLP1, HBD2 and lactoferrin are lowest at the mid-cycle around ovulation and highest during the follicular phase (Angstwurm et al. 1997, Ahmed et al. 2001, Keller et al. 2007, Hel et al. 2010, Wira et al. 2010, Hafner et al. 2013, Wira et al. 2015), although other studies indicated higher peripheral blood IL-6 levels during the luteal phase (Konecna et al. 2000, Sikora et al. 2015). Another report showed no change in IL-6 levels but increased IL-1α, IL-1β and TNFα after ovulation (i.e. luteal phase) in purified monocytes (Willis et al. 2003). There is also evidence that APC function is modulated by sex hormones during menstrual cycle phases (Wira and Rossoll 1995, Beagley and Gockel 2003, Ochiel et al. 2008, Hel et al. 2010, Hafner et al. 2013). Furthermore, epithelial thickness in the vagina is greater when E_2 levels are high during the follicular phase and ovulation (Hel et al. 2010, Rodriguez-Garcia et al. 2013b). These data are consistent with recent transcriptomics and/or proteomics studies in human cervical tissue (Yildiz-Arslan et al. 2016) or pigtail macaque vaginal tissue (Vishwanathan et al. 2015) showing that the luteal phase was associated with higher expression of genes, proteins and/or pathways involved in inflammation and immune cell regulation.

Differential susceptibility to HIV-1 infection during the menstrual cycle is a major concern for women's health. Studies in non-human primate models have indicated that rhesus (Sodora et al. 1998) and pigtail (Vishwanathan et al. 2011) macaques are more susceptible to SIV infection in the luteal than the follicular phase. Similarly, SHIV infection in pigtail macaques occurred more frequently in the premenstrual phase (Kersh et al. 2014). In an *ex vivo* cervical tissue explant model, productive HIV-1 infection was associated with the luteal phase (Saba et al. 2013). One report showed increased X4- but not R5-tropic HIV-1 replication in activated PBMCs stimulated with high levels of P_4 (1 μM) ex vivo (Huijbregts et al. 2013). Other studies using exogenous hormone stimulation have shown that a combination of menstrual cycle hormones (2 nM E_2, 200 pM P_4, 4 nM testosterone) increased HIV-1 replication in the Jurkat T cell line (Ragupathy et al. 2013), but luteal phase hormones (100 nM P_4 plus 1 nM E_2) or high P_4 levels (100 nM-10 μM) decreased HIV-1 replication in PBMCs (Asin et al. 2008) or activated T cells (Vassiliadou et al. 1999), respectively. The differences between studies using endogenous versus exogenous hormones may critically depend on species, concentrations of hormones, composition of mixed cell populations, sampling time, duration of exogenous hormone stimulation and/or other experimental differences. Nevertheless it appears that S/HIV infection is higher during the P_4-high luteal phase.

There is also evidence that menstrual cycle phase can influence HIV-1 shedding, where free virus is deposited into cervico-vaginal fluid. Several studies analyzing HIV-1 RNA cervico-vaginal lavages (CVLs) in normally cycling HIV-1 positive women suggest that HIV-1 shedding is increased during the luteal compared to the follicular phase (Reichelderfer et al. 2000, Benki et al. 2004, Curlin et al. 2013) or periovulatory phase (Money et al. 2003), although other reports suggest no effect (Mostad et al. 1998, Villanueva et al. 2002, Sheth et al. 2014). Thus the risk of female-to-male transmission may be higher for HIV-1 positive women in the luteal phase.

During pregnancy, P_4 levels are 10-40 times higher than in the luteal phase (see section 1.3.1). High P_4 levels play an important role in maternal immunosuppression in order to tolerate foetal growth (Szekeres-Bartho et al. 1983, Hel et al. 2010, Weinberg et al. 2011, Oertelt-Prigione 2012). This occurs through an immune shift from a Th1 response (cytotoxic CD8+ T cell functions) to a Th2 response (B cell-mediated antibody production) (Jones et al. 2008, Hapgood et al. 2018). Pregnant women have an increased susceptibility to HIV-1 infection (Gray et al. 2005, Mugo et al. 2011). Taken together, there is a growing body of evidence to suggest a link between high P_4 levels, either during the luteal phase or during pregnancy, and suppressed immune function as well as increased susceptibility to infection.

Consistent with this, there is evidence that suggests E_2 is protective against HIV-1 infection via multiple potential mechanisms involving immune function (Wira et al. 2014a). For example, exogenous E_2 (10-50 nM) is protective against HIV-1 infection in purified CD4+ T cells and macrophages (Rodriguez-Garcia et al. 2013a, Tasker et al. 2014) and against SIV in macaques (Smith et al. 2000b, Smith et al. 2004). Additionally, exogenous E_2 thickens the vaginal epithelium in macaque models, while P_4 is associated with epithelial thinning (Smith et al. 2000b, Smith et al. 2004, McNicholl et al. 2014, Radzio et al. 2014)). Furthermore HIV-1 uptake was lower in human genital epithelial cells when stimulated with 1 nM E_2 compared to 100 nM P_4 (Ferreira et al. 2015). In another study, E_2-stimulated macaques (0.02 mg/kg) exhibited lower expression of the immune markers CCR5 and integrin α4β7 on CD4+ T cells than DMPA-treated macaques (Goode et al. 2014). Cell line studies showed that exogenous E_2 repressed cytokine production induced by TLR3 in endometrial epithelial cells at 10 nM (Lesmeister et al. 2005), and in uterine epithelial cells, 50 nM E_2 reduced transepithelial resistance (TER), increased SLP1, decreased HBD2 (Wira et al. 2010, Patel et al. 2013, Wira et al. 2014a) and repressed IL-1β-mediated inflammatory responses (Schaefer et al. 2005). E_2 is also protective against Herpes simplex virus (HSV)-2 infection in mice models and immortalized vaginal Vk2/E6E7 cells (Gillgrass et al. 2005, Gillgrass et al. 2010, Kaushic et al. 2011, Anipindi et al. 2016, Lee et al. 2016). However since many of these studies used a supraphysiological concentration of 50 nM E_2, these effects may not necessarily be physiologically relevant *in vivo*, where E_2 concentrations range from 50 pM- 1.5 nM (see section 1.3.1).

Taken together the clinical and *in vitro* data suggest that the cycling female sex hormones may play a role in the regulation of local and systemic immune responses, and therefore susceptibility to infections, with P_4 generally suppressing immunity and increasing susceptibility and E_2 generally protecting against infection.

1.4 Hormonal contraception

Women of reproductive age can protect themselves against unintended pregnancies by the use of contraception. Further benefits of contraception include reduced maternal and infant mortality and morbidity and a decreased need for unsafe abortions (World Health Organization 2017). Non-hormonal methods of contraception include condoms, copper T intrauterine devices (Cu-IUDs), withdrawal and male sterilization through vasectomy. Hormonal contraceptives control the levels of the sex steroid hormones, E_2 and/or P_4, in women. P_4 levels are maintained at low or undetectable concentrations (<636 pM) (Kirton and Cornette 1974, Fotherby et al. 1984, Jain et al. 2004). Typically hormonal contraceptives are administered as synthetic progestin-only or a combination of estrogen (usually estradiol) plus progestin. There are various doses and methods of administration of hormonal contraceptives, including as combined oral contraceptives (COCs), intramuscular or subcutaneous injections, intrauterine devices (IUDs), intra-vaginal rings (IVRs), subdermal implants and patches (Sitruk-Ware 2007).

Injectable contraceptives are the most commonly used method of hormonal contraception in Sub-Saharan Africa (Fig. 1.4.1) (United Nations Department of Economic and Social Affairs Population Division 2015), with about 38% of contraceptive users (an estimated 16.5 million women between the ages of 15-49) using injectable contraceptives in 2014 (United Nations 2015).

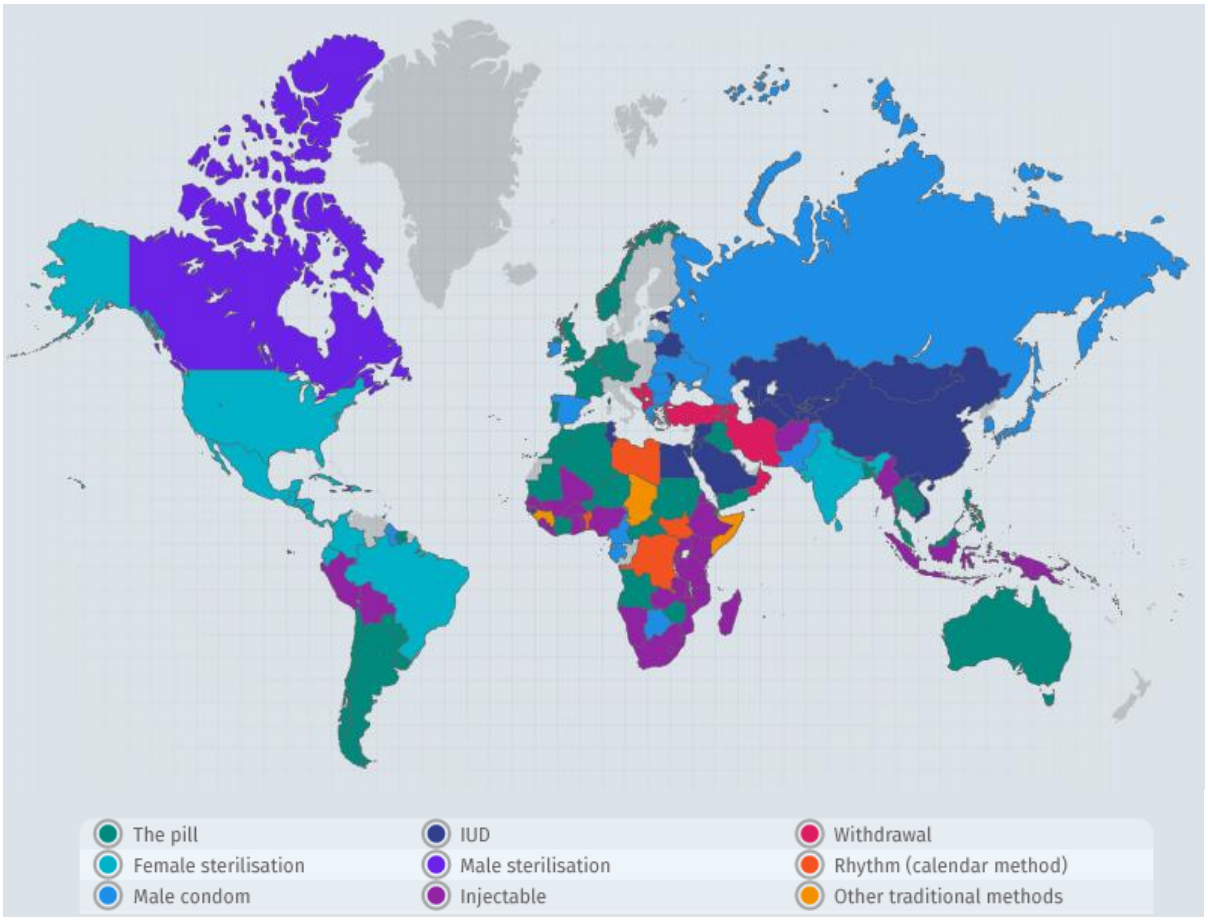


Figure 1.4.1. Top methods of hormonal and non-hormonal contraception used per country. Non-hormonal (male condom, IUD, male sterilization or vasectomy, female sterilization or fallopian tube ligation, rhythm, withdrawal) and hormonal (the pill, injectable) methods of contraception are indicated by different colours. Injectable contraceptives (purple) are the most commonly used in Sub-Saharan Africa, including South Africa, and other regions such as Peru, Bolivia (South America), Indonesia, Burma (South-East Asia) and Afghanistan (Middle East). No information is available for Greenland and some other countries (grey). Data is from http://www.un.org/en/ development/desa/population/publications/dataset/contraception/wcu2015.shtml and image is adapted from https://onlinedoctor.superdrug.com/birth-control-around-the-world/.

There are two main intramuscular injectable progestin-only contraceptives: 150 mg medroxyprogesterone acetate (MPA) administered at three monthly intervals as Depo-Provera or Depot-MPA (DMPA or DMPA-IM) and 200 mg norethisterone enanthate (NET-EN), administered at two-month intervals as Nur-Isterate (Affandi 2002, Hapgood et al. 2018). NET is only used in some countries, like South Africa (Smit et al. 2001, Morrison et al. 2012, Hapgood et al. 2018). A new lower-dose subcutaneous formulation of 104 mg MPA (DMPA-SC), marketed as Sayana Press, is also a three-monthly injectable contraceptive that is currently being introduced in several developing African and non-African countries (Spieler 2014, Hapgood et al. 2018). The long intervals between injections and relatively easy concealment make injectable contraceptives a popular choice in rural

areas where access to healthcare frequently requires travelling long distances, and where contraceptive use may be culturally opposed.

The main biological mechanisms of action behind progestin contraceptives are thickening cervical mucus, thereby hindering sperm mobility; inhibiting ovulation by inhibiting the mid-cycle LH surge; and inducing secretory changes in E_2-primed endometrium (Kuhl 1990, Bassol et al. 1995, Petta et al. 1998, Rivera et al. 1999, Kuhl 2005, Sitruk-Ware 2006, Sitruk-Ware and Nath 2010, Kuhl 2011, Mueck and Sitruk-Ware 2011). Interestingly, while multiple genes are regulated by progestins, the exact target genes that account for progestogenic effects are unknown (Hapgood et al. 2018). However, the anti-estrogenic effects of progestins are mediated by the PR and its effect on both E_2 and the ER. Progestin-bound PRs suppress ER gene expression and increase E_2 metabolism by up-regulating 17β-hydroxysteroid dehydrogenase (HSD) expression (Kuhl 2005, Africander et al. 2011b, Kuhl 2011). The ER also plays a role in the response to progestins, given that E_2-activated ER regulates PR gene expression (Kuhl 2005, Africander et al. 2011b, Kuhl 2011). Thus progestins inhibit pregnancy through multiple genotropic and biological mechanisms.

1.4.1 MPA and other progestins

The therapeutic use of the endogenous hormone P_4 is limited owing to its short biological half-life as a result of its rapid metabolism (Kuhl 2005, Kuhl 2011, Stanczyk et al. 2013). Consequently, synthetic progestins with improved bioavailability were developed to mimic the functions of endogenous P_4. Progestogens (that is, endogenous progesterone and synthetic progestins) are defined as pro-gestational hormones that maintain pregnancy (Sitruk-Ware 1986). The first progestin, ethisterone, was synthesized in 1938, and MPA later in 1957 (Kuhl 2011).

Progestins are generally classified by generations according to when they were synthesized: thus MPA and NET are regarded as first-generation progestins, while levonorgestrel (LNG), etonogestrel (ETG) and nestorone (NES) are second, third and fourth generation progestins, respectively (Sitruk-Ware 2004, Sitruk-Ware 2006, Sitruk-Ware and Nath 2010, Africander et al. 2011b, Stanczyk et al. 2013). Newer generations of progestins are typically used in newer administration technologies, for example, IUDs (LNG), implants (ETG, LNG) and IVRs (NES) (Sitruk-Ware 2007).

Progestins are also used in hormone replacement therapy (HRT) in postmenopausal women, but are frequently combined with estrogens (Africander et al. 2011b, Stanczyk et al. 2013). The progestin protects against unwanted endometrial proliferation, while the estrogen protects against

cardiovascular diseases and risk of breast cancer (Rossouw et al. 2002, Beral and Million Women Study 2003, Sitruk-Ware 2004, Africander et al. 2011b, Stanczyk and Bhavnani 2014).

MPA, currently used in both contraceptives and HRT, has been in use for more than 30 years (Stanczyk and Bhavnani 2014). As a contraceptive, MPA is most commonly administered as a three-monthly 150 mg intramuscular injection, although other doses and routes of administration have been used, for example high oral doses of 100-1000 mg in breast cancer patients (Bick et al.) (unpublished). Analysis of published peak serum MPA concentrations following intramuscular administration shows that there is wide inter-individual and inter-study variation in MPA serum levels (Fig. 1.4.1.1) (Bick et al.) (unpublished). Within the first four weeks post-administration, most studies report serum MPA levels of 10-40 nM, however some studies show MPA serum levels as high as 100 nM (Fig. 1.4.1.1) (Bick et al.) (unpublished). MPA serum levels plateau after one month at concentrations of about 2.6 nM (Mishell 1996). The minimum threshold concentration needed for contraceptive efficacy of most progestins is poorly defined (Cherala et al. 2016), but concentrations of MPA maintained above 0.5 nM (0.2 ng/ml) for three months is considered highly effective (Polis et al. 2017). No information is available on MPA concentrations in target FRT tissues. However, it could be that MPA accumulates in target tissues *in vivo* and therefore local concentrations could be higher than in peripheral blood.

Peak MPA serum levels are difficult to compare between studies due to differences in study design, such as method of quantification, time of sampling post-injection, and number of injections, or differences in the population of women assessed, such as ethnicity, body weight and lactation status (Hapgood et al. 2018). Considering method of quantification, most recent studies (20 years or less) tend to use liquid chromatography/tandem mass spectrometry-based methods while older studies (20-40 years old) typically use radioimmunoassay methods with or without prior organic solvent extraction. However, there does not appear to be a pattern suggesting that MPA concentrations are higher or over-estimated in older than younger studies (Fig. 1.4.1.1). This suggests that the large range in published serum concentrations is likely due to biological variation between women and between studies.

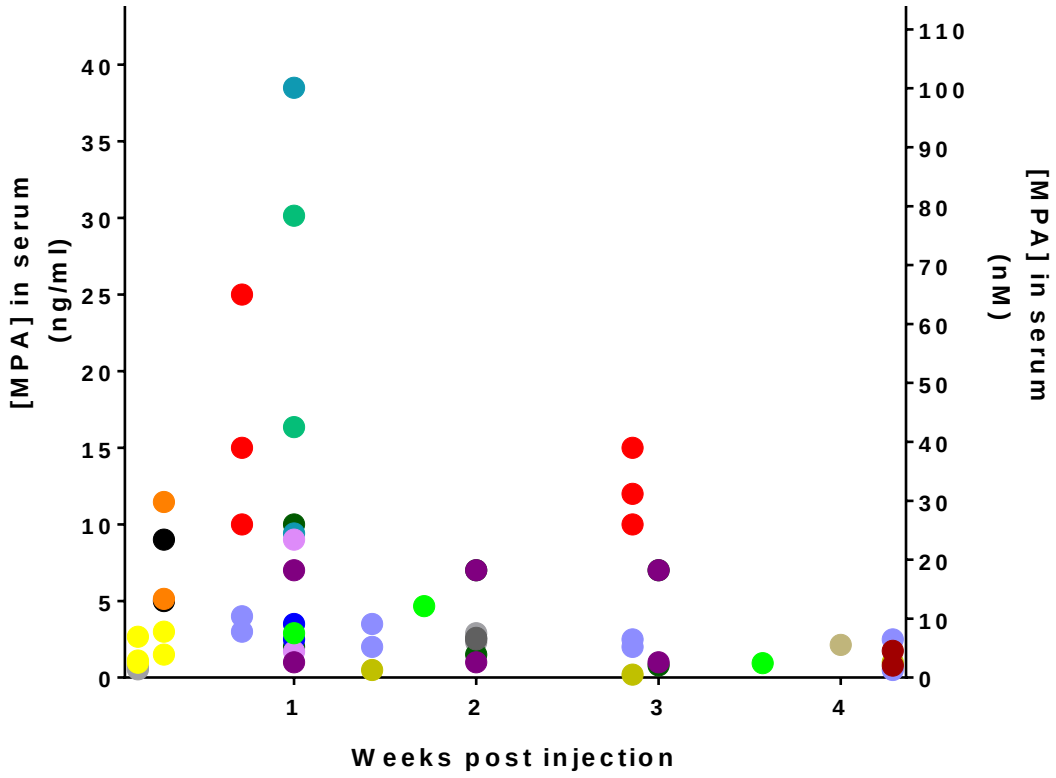


Figure 1.4.1.1. MPA serum concentrations in the first 30 days following 150 mg intramuscular injection of DMPA. MPA serum concentrations, shown as both ng/ml (left axis) and nM (right axis), were compiled from 17 published studies, each represented by a different colour, indicated in the legend table below.

Symbol	Reference	Symbol	Reference	Symbol	Reference
	(Jeppsson et al. 1982)		(Bonny et al. 2014)		(Koetsawang 1977)
	(Ortiz et al. 1977)		(Shrimanker et al. 1978)		(Fang et al. 2004)
	(Virutamasen et al. 1996)		(Fotherby et al. 1980b)		(U.S. Food and Drug Administration 2003)
	(Fotherby et al. 1980a)		(Bassol et al. 1984)		(Fotherby and Koetsawang 1982)
	(Kirton and Cornette 1974)		(Nanda et al. 2016)		(Nanda et al. 2008)
	(Jeppsson and Johansson 1976)		(Mauck et al. 1999)		

Progestins are synthesized either as derivatives of progesterone (such as MPA) or testosterone (such as NET) and are therefore slightly different in structure (Schindler et al. 2003, Africander et al. 2011b, Kuhl 2011, Stanczyk et al. 2013, Hapgood et al. 2014a) (Fig. 1.4.1.2).

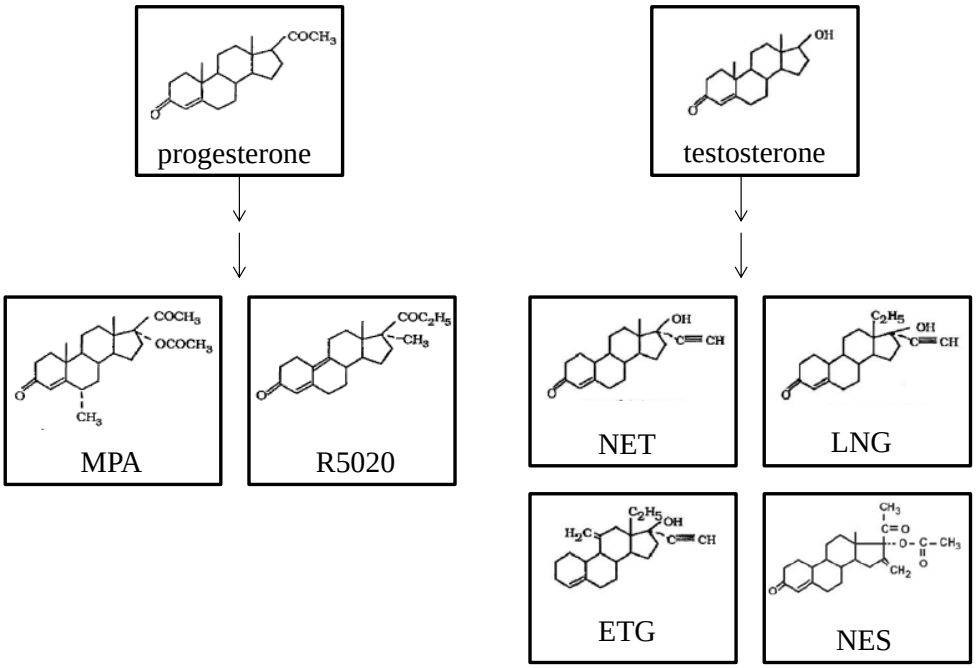


Figure 1.4.1.2. Chemical structures of progestins derived from progesterone or testosterone. MPA: medroxyprogesterone acetate, R5020: promegestone, NET: norethisterone, LNG: levonorgestrel, ETG: etonogestrel, NES: nestorone. Arrows indicate synthetic derivatives of the parent compound. Images are from (Schindler et al. 2003) and (Kuhl 2011).

Progestins were designed to act like P_4 and therefore act as agonist ligands for the PR (Hapgood et al. 2014a). However due to variations in their structure (Fig. 1.4.1.2), different progestins have been shown to exhibit varying degrees of off-target glucocorticoid, androgenic and mineralocorticoid activities (Schindler et al. 2003, Stanczyk 2003, Africander et al. 2011b, Kuhl 2011, Africander et al. 2013, Stanczyk et al. 2013, Africander et al. 2014, Hapgood et al. 2014a, Louw-du Toit et al. 2017, Hapgood et al. 2018). These effects are due to differential relative binding affinities (RBAs) to other SRs, the glucocorticoid receptor (GR), androgen receptor (AR) and mineralocorticoid receptor (MR), respectively. These SRs are ligand-activated transcription factors that regulate expression of target genes. However the binding affinity does not necessarily correlate with biological effects (Africander et al. 2011b, Hapgood et al. 2014a, Hapgood et al. 2018), for example, the antagonist RU486 binds both GR and PR with high affinity but does not elicit a biological response. Therefore the biological response to a given progestin will depend on a number of variables: the concentration of available

receptors, the concentration of the progestin and other competing hormone ligands, the extent of progestin binding to serum proteins, the concentration of other signalling factors and the cell type (Africander et al. 2011b, Stanczyk et al. 2013, Hapgood et al. 2014a, Hapgood et al. 2018).

The biocharacter of a ligand is determined from two pharmacological properties, potency and efficacy, using dose response analysis (Fig. 1.4.1.3). Efficacy is defined as the maximal effect or response generated by a ligand for its receptor (Africander et al. 2011b, Hapgood et al. 2014a, Hapgood et al. 2018) (Fig. 1.4.1.3). Thus efficacy is used to classify a ligand as a full agonist, which can elicit a maximal response, or a partial agonist, which elicits a less than maximal response (Africander et al. 2011b, Hapgood et al. 2014a, Hapgood et al. 2018) (Fig. 1.4.1.3). Potency, or EC_{50}, is defined as the concentration of the ligand required to generate 50% of the maximal response (Africander et al. 2011b, Hapgood et al. 2014a, Hapgood et al. 2018) (Fig. 1.4.1.3). The potency depends on the cell type, gene promoter and receptor concentration and is variable between experiments (Africander et al. 2011b, Hapgood et al. 2014a). Therefore the potency of different progestins is compared relative to a reference ligand, which is usually set to 100% (Hapgood et al. 2018). The RBAs and biological activities via different SRs of P_4, MPA and NET are indicated in Table 1.4.1

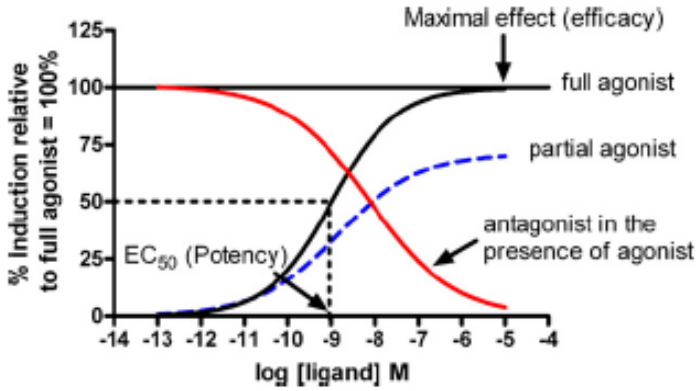


Figure 1.4.1.3. Schematic sigmoidal dose response curve indicating the efficacy (maximal response) and potency (EC_{50}) of a full and partial agonist via a given receptor. A full agonist (solid black curve) elicits a maximal response (efficacy) which is usually set to 100% (solid black line), while a partial agonist (dotted blue curve) generates a less than maximal response. The effect can be, for example, mRNA levels or reporter gene activity. An antagonist (solid red curve) binds to the receptor and inhibits effects induced by agonists. The potency (EC_{50}) is the ligand concentration on the x-axis which corresponds to 50% of the maximal response on the y-axis. Taken from (Africander et al. 2011b).

All progestins were designed to be full agonists for the PR, and exhibit greater potency than P_4 for the PR (Africander et al. 2011b, Kuhl 2011, Hapgood et al. 2014a). However, MPA binds to the glucocorticoid receptor (GR) with higher affinity than the endogenous glucocorticoid cortisol

(Kontula et al. 1983, Fuhrmann et al. 1995), unlike NET which has almost no GR binding and no GR activity (Koubovec et al. 2005, Ronacher et al. 2009). MPA can act as a potent partial to full agonist for the GR, depending on the cellular context, while P_4 is a partial agonist with low potency (Bamberger et al. 1999, Koubovec et al. 2005, Ronacher et al. 2009).

The differences in structure, SR affinity and consequently the off-target effects via activation of SRs other than the PR imply that progestins cannot be grouped together as a single class, with MPA as a notable outlier (Stanczyk 2003, Hapgood et al. 2004, Stanczyk et al. 2013).

Table 1.4.1. Biological activities of some progestogens and their RBAs for different SRs

Progestin	PR		AR			GR		MR	
	RBA %	Progesto-genic	RBA %	Andro-genic	Anti-androgenic	RBA %	Gluco-corticoid	RBA %	Anti-mineralo-corticoid
Progesterone	100	+	80	±	(+)	5	±	6	+
Medroxyprogesterone acetate	65-98	++	151	±	-	74	+	0. 13	±
Norethisterone/ Norethisterone acetate	27-34	++	134	+	-	0.8	-	0.15	±

RBAs are indicated relative to the reference ligand set to 100% (PR: P_4, AR: dihydrotestosterone, GR: dexamethasone, MR: aldosterone). Data from cell lines, pre-clinical studies and animal models are from (Stanczyk 2003, Koubovec et al. 2004, Koubovec et al. 2005, Ronacher et al. 2009, Africander et al. 2011b, Kuhl 2011, Africander et al. 2013, Stanczyk et al. 2013, Africander et al. 2014, Hapgood et al. 2014a). -, not effective; (+), weakly effective; +, effective; ++, strongly effective; ±, literature inconsistent. Data for ER not shown. Table is modified from (Hapgood et al. 2018).

Given that the GR mediates the expression of important immune function and inflammatory genes (Zhou and Cidlowski 2005, Baschant and Tuckermann 2010, Chinenov et al. 2013) (see sections 1.5.2-1.5.3), the off-target activation of the GR by MPA is likely to have alarming effects on immune function in DMPA users.

1.4.2 Association between DMPA use and HIV-1 acquisition

The topics of DMPA use, increased HIV-1 acquisition risk and likely biological mechanisms of increased infection (see section 1.4.3) have been extensively and exhaustively evaluated in a recent review (Hapgood et al. 2018). The parts of that review most relevant to the present study are summarized here.

Globally, regions with the highest injectable contraceptive use overlap geographically with the areas of highest HIV-1 prevalence (Butler et al 2013). A multitude of observational and epidemiological studies has recently emerged to assess the potential association between MPA use and HIV-1 acquisition, with conflicting results. Some studies showed no effect (Kleinschmidt et al. 2007, Morrison et al. 2007, Myer et al. 2007, Reid et al. 2010, Morrison et al. 2012, Lutalo et al. 2013, Wall et al. 2015), while others showed increased HIV-1 acquisition risk for DMPA users (Leclerc et al. 2008, Morrison et al. 2010, Heffron et al. 2012, Wand and Ramjee 2012, McCoy et al. 2013, Crook et al. 2014, Noguchi et al. 2015, Byrne et al. 2016). Several reasons have been put forward to explain the different findings between studies, including confounding variables such as reporting of condom usage (Myer et al. 2007, Ralph et al. 2015), sexually transmitted infection (STI) status (HSV-2, bacterial vaginosis, *Neisseria gonorrhoea*, *Chlamydia trachomatis*, *Trichomonas vaginalis* and syphilis), age, socio-demographic variables, and limitations of study design and analysis (Polis et al. 2013b, Noguchi et al. 2015, Polis et al. 2016). Some studies combine injectable contraceptives together and do not distinguish between DMPA and NET-EN (Reid et al. 2010, Heffron et al. 2012, Balkus et al. 2016, Polis et al. 2016).

Importantly, the most recent meta-analyses of higher quality observational studies have indicated that DMPA use is associated with a significant increased risk of HIV-1 acquisition (Heffron et al. 2012, Polis and Curtis 2013, Polis et al. 2013a, Polis et al. 2014, Morrison et al. 2015, Ralph et al. 2015, Polis et al. 2016). The latest meta-analysis shows a significant 40% increased risk, with a 95% confidence interval ranging from 23-59% (Polis et al. 2016).

Studies assessing the risk of other hormonal contraceptives have shown no association between HIV-1 acquisition risk and the injectable, NET-EN although studies are limited (Polis et al. 2014, Morrison et al. 2015, Ralph et al. 2015, Polis et al. 2016) and there is no or insufficient data on the risk for the lower dose DMPA-SC (Polis et al. 2017, Hapgood et al. 2018). Despite this, in 2017 the World Health Organization (WHO) changed the medical eligibility criteria of all progestin-only injectable contraceptives (including DMPA-IM, DMPA-SC and NET-EN) from Category 1 (injectable contraceptive use has no restriction) to Category 2 (where the benefits of using this method "generally outweigh the theoretical or proven risks") (World Health Organization 2017). In their guidance statement, the WHO recommends that women considering injectable contraceptive methods should be counselled about the concerns over increased HIV-1 acquisition and how to minimise their risk of exposure (World Health Organization 2017).

A recently initiated clinical trial, Evidence for Contraceptive Options and HIV Outcomes (ECHO), may provide more information on the relative HIV-1 incidence and contraceptive efficiency for

DMPA-IM compared to the LNG implant (Jadelle) and the Cu-IUD, although this study has no control group of women not using contraception (ECHO 2016).

The current meta-analyses have also indicated that there is no association between HIV-1 acquisition and use of combined oral contraceptives (COCs) (Polis and Curtis 2013, Polis et al. 2013a, Polis et al. 2014, Morrison et al. 2015, Ralph et al. 2015, Polis et al. 2016). Accordingly, the medical eligibility criteria of COCs remain at Category 1 (World Health Organization 2017). The evidence supporting a policy change from MPA use to other progestins such as NET or LNG is a subject of ongoing research (Hapgood et al. 2004, Hapgood 2013, Noguchi et al. 2015, Hapgood et al. 2018). It is recognized that cessation of contraceptive use would increase rates of mother and child mortality and morbidity (Jain 2012), thus there is an urgent need to promote informed contraceptive choices in areas where HIV-1 risk is high (Hapgood 2013).

In support of the clinical observational data are *in vitro*, *ex vivo* and non-human primate studies suggesting MPA increases HIV-1/SIV replication. For example, primate studies frequently use pre-treatment with DMPA to enhance SIV infection (Abel et al. 2004, Trunova et al. 2006, Genesca et al. 2007). Furthermore MPA has been shown to increase HIV-1 infectious molecular clone replication in activated human PBMCs (Huijbregts et al. 2013) and single-cycle HIV-1 pseudovirus replication in non-activated PBMCs (Sampah et al. 2015), and increase HIV-1 transcytosis in primary genital epithelial cells *in vitro* (Ferreira et al. 2015).

Taken together, the association between DMPA use and HIV-1 acquisition risk in clinical observational studies raises concerns about a potential causal relationship and provides impetus to investigate the possible mechanisms by which MPA could increase HIV-1 susceptibility.

1.4.3 Mechanisms of increased HIV-1 infection by MPA

Several plausible biological mechanisms have been proposed to explain how MPA could enhance HIV-1 acquisition, with increasing lines of evidence to support each mechanism (Blish and Baeten 2011, Murphy et al. 2014, Irvin and Herold 2015, Hapgood et al. 2018). These include:

1. Changing the FRT defensive barrier, structural integrity and permeability
2. Changing levels of soluble defence molecules secreted in the FRT, such as mucus, antimicrobials, antibodies, cytokines and chemokines
3. Changing the frequency/expression of the HIV-1 co-receptor CCR5 on key immune cells

4. Changing the frequency/activation of adaptive immune cells that reside in or migrate to the FRT, or circulating in the peripheral blood, such as T cells and monocytes
5. Changing the function of innate immune cells, such as NK cells, pDCs, macrophages
6. Changing viral replication in target cells
7. Changing the functions of the vaginal microbiome and enhanced risk of STIs
8. Changing uptake of the virus across the epithelial barrier

Some of these biological mechanisms are represented in Fig. 1.4.3 (Hapgood et al. 2018) and may contribute to a compromised local immune system that facilitates the transmission of HIV-1 in the FRT.

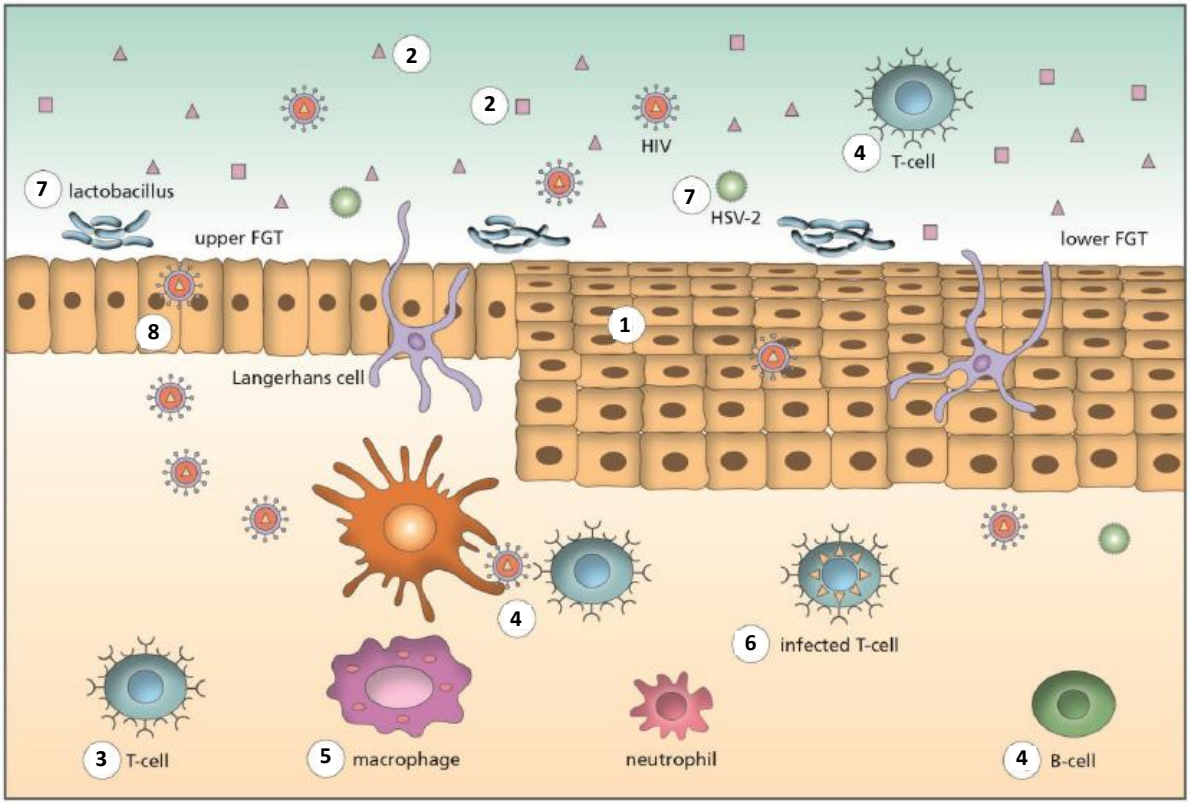


Figure 1.4.3. Proposed mechanisms by which MPA can increase HIV-1 acquisition in the FRT. Increased HIV-1 infection in the FRT of DMPA users can occur through multiple mechanisms, including (1) MPA reducing epithelial barrier integrity, via decreasing expression of proteins relevant to barrier function, (2) MPA decreasing levels of innate immune factors such as antimicrobials, and changing levels of secreted inflammatory mediators, (3) MPA increasing the frequency of HIV-1 target cells such as CCR5-expressing T cells, (4) MPA decreasing the function of adaptive immune cells, such as APCs and B cells, (5) MPA changing the frequency and/or function of innate immune cells, such as macrophages, (6) Increased replication in infected target cells, such as CD4+ T cells, (7) MPA changing the vaginal microbiome such as *Lactobacillus* species, and increasing HSV-2 infection, and (8) MPA increasing transcytosis across single-layer epithelium. Figure adapted from (Hapgood et al. 2018).

Importantly, several studies have investigated the dose-dependent effects of MPA using PBMCs and cell lines *in vitro*. The potency of MPA has been reported as 0.5-100 nM for modulating immune function gene expression in cell lines (Hapgood et al. 2014b, Louw-du Toit et al. 2014, Irvin and Herold 2015) and PBMCs (Kleynhans et al. 2011, Huijbregts et al. 2013, Hapgood et al. 2014b, Huijbregts et al. 2014), inhibiting some immune cell functions in PBMCs (Huijbregts et al. 2014, Quispe Calla et al. 2015), and inducing apoptosis in CD4+ T cells (Tomasicchio et al. 2013). Together these data suggest that the *in vitro* effects of MPA are relevant to physiological doses of MPA in the serum of DMPA users.

Limited studies using other progestins such as LNG and NET showed no effects on T cell function and/or immune function gene expression (Huijbregts et al. 2013, Tomasicchio et al. 2013, Hapgood et al. 2014b, Huijbregts et al. 2014).

One of the proposed mechanisms by which MPA can increase susceptibility to HIV-1 infection is through thinning of vaginal epithelium, which would place the virus in closer physical proximity to underlying target cells. DMPA use in non-human primate and rodent models has been shown to decrease the thickness of vaginal epithelium in several studies (Parr et al. 1994, Hild-Petito et al. 1998, Mauck et al. 1999, Kaushic et al. 2003, Kaushic 2011, Butler et al. 2015, Dietz Ostergaard et al. 2015, Butler et al. 2016, Hapgood et al. 2018). However, this effect is modest or undetectable in humans (Mauck et al. 1999, Miller et al. 2000, Chandra et al. 2013, Bahamondes and Bahamondes 2014, Bahamondes et al. 2014, Mitchell et al. 2014, Irvin and Herold 2015, Hapgood et al. 2018). There is however some evidence that MPA modulates the vaginal microbiome functions (Kaushic 2011, Achilles and Hillier 2013, Brooks et al. 2016, Roxby et al. 2016), for example the study showing that DMPA users had decreased H_2O_2-producing *Lactobacilli* (Mitchell et al. 2014).

Another proposed mechanism for enhanced HIV-1 acquisition by MPA is increased STI risk. Some reports suggest DMPA use is associated with increased risk of HSV-2 (Baeten et al. 2001, Gillgrass et al. 2003, Grabowski et al. 2015, Borgdorff et al. 2016), *Chlamydia* (Morrison et al. 2004) and bacterial vaginosis (Cherpes et al. 2008b, Achilles and Hillier 2013), although the available meta-analyses suggest no conclusive association (Mohllajee et al. 2006, Morrison et al. 2009).

Another mechanism by which MPA can increase HIV-1 susceptibility is through changes in the local FRT or systemic immunity, which would affect the function of the FRT to protect against infections. This is significant since higher proportions of susceptible CD4+ and CCR5+ target cells in the FRT at the time of HIV-1 exposure could facilitate infection (Wira et al. 2014b). However, the available clinical, animal and *ex vivo* studies appear to be inconsistent, with some studies but not others showing an effect for MPA on CCR5 expression and/or target cell frequency. For example, a study

showed that PBMCs from DMPA users have increased CCR5 expression in CD4+ and CD8+ T cells, despite no increase in the frequency of CD4/8+CCR5+ cells, compared to women using no hormonal contraception (Sciaranghella et al. 2015). In FRT samples, injectable contraceptive (predominantly DMPA) users express increased CCR5+CD4+ T cells in the cervix (Byrne et al. 2016) and increased CCR5-expressing leukocytes in the vagina (Chandra et al. 2013) than normally cycling women. In a rhesus macaque model, DMPA-treated animals exhibited a higher frequency of CCR5+CD4+ cells in vaginal tissue than E_2-treated macaques (Goode et al. 2014). However, other studies did not observe increased CCR5+ CD4+ T cells in the endocervix or endometrium (Smith-McCune et al. 2017) or increased vaginal CD3+, HLA-DR+ or CCR5+ cells (Mitchell et al 2014). A study using PBMCs stimulated *ex vivo* with exogenous MPA also did not observe an effect on CCR5/CXCR4 in CD4+ T cells (Sampah et al. 2015).

Similarly, effects of MPA on innate immune factors such as antimicrobials (HBD2, SLP1) and antibodies (IgG, IgA) have been shown in some studies but not others. For example, in CVL samples from DMPA users, HBD2 levels were decreased in some studies (Morrison et al. 2014, Fichorova et al. 2015), but appeared to increase in another study (Francis et al. 2016). Decreased FRT SLP1 levels were observed in some (Li et al. 2008) but not other (Morrison et al. 2014, Deese et al. 2015, Fichorova et al. 2015, Francis et al. 2016, Roxby et al. 2016) studies. MPA use decreases production and transepithelial transport of IgG and IgA (Hel et al. 2010, Hapgood et al. 2018), although increased levels were observed in CVL samples from DMPA users in a recent study (Francis et al. 2016). Taken together, there appears to be large variability between studies, with some studies showing effects for MPA while others do not. This could suggest that overall there may be no effect, and that differences between studies are due to differences in MPA serum concentrations, time of sampling, duration on MPA, immune cell activation and other factors between individuals.

Whether MPA elicits a pro-inflammatory or immunosuppressive (anti-inflammatory) effect in the FRT of DMPA users is controversial. Some studies have reported increased expression of pro-inflammatory cytokines and chemokines such as IL-6 and IL-8 in DMPA users (Morrison et al. 2014, Deese et al. 2015, Francis et al. 2016), while others showed suppressed expression of pro-inflammatory mediators in DMPA users or other models (Trunova et al. 2006, Hughes et al. 2008, Kleynhans et al. 2011, Hapgood 2013, Huijbregts et al. 2013, Kleynhans et al. 2013, Huijbregts et al. 2014, Walong et al. 2016), or no effect in DMPA users (Arnold et al. 2016, Weinberg et al. 2016). Decreased levels of pro-inflammatory mediators in DMPA users could suppress host immunity to HIV-1 infection (Hapgood 2013), whereas increased levels of pro-inflammatory mediators in DMPA users could result in increased recruitment of HIV-1 target cells (Morrison et al. 2014).

However, in multiple *ex vivo* studies, MPA suppresses T cell and pDC function in humans and macaques (Trunova et al. 2006, Cherpes et al. 2008a, Hughes et al. 2008, Kleynhans et al. 2011, Vicetti Miguel et al. 2012, Kleynhans et al. 2013, Huijbregts et al. 2014, Weinberg et al. 2016). Furthermore, in cell line models and PBMCs, exogenous stimulation with MPA, unlike NET or P_4, decreased expression of pro-inflammatory genes (such as IL-6, IL-8, RANTES, IL-12) and increases expression of anti-inflammatory genes (such as GILZ, IL-10) (Kleynhans et al. 2011, Huijbregts et al. 2013, Govender 2014, Govender et al. 2014, Hapgood et al. 2014b, Louw-du Toit et al. 2014, Ray 2015, Grandi et al. 2016).

These data imply that MPA is immunosuppressive in multiple systems (Hapgood et al. 2018). Of particular interest to this study is the observation that the immunosuppressive effects of MPA occur via the GR in some cell line studies. GR activation will influence the regulation of multiple genes in many physiological systems and could promote activation of multiple signalling pathways via cross-talk with other members of the SR family.

1.5 Steroid hormone receptors (SRs)

The family of SRs belongs to the nuclear receptor superfamily and consists of the glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR), estrogen receptor (ER) and mineralocorticoid receptor (MR) (Evans 1988, Lu et al. 2006, Huang et al. 2010). These receptors function as ligand-activated transcription factors (TFs) and share similar structures (Fig. 1.5) (Griekspoor et al. 2007, Africander et al. 2011b). All SRs consist of a moderately conserved ligand-binding domain (LBD) at the carboxy-terminal domain (CTD), a highly conserved DNA-binding domain (DBD), hinge region and variable amino terminal domain (NTD) (Fig. 1.5) (Africander et al. 2011b, Stanczyk et al. 2013). Both the LBD and NTD contain activator function (AF) domains, which play a role in transcriptional activity of the SR (Lavery and McEwan 2005). The sequence homology of the LBD between SRs means that promiscuous ligand binding can occur and as such, hormone ligands can elicit off-target effects by binding to other SRs. This effect is particularly pronounced for the GR and PR, since they share 55% LBD amino acid sequence homology and 90% DBD homology (Evans 1988, Stanczyk et al. 2013) and both recognize the same DNA sequences (Strahle et al. 1987, Beato et al. 1989).

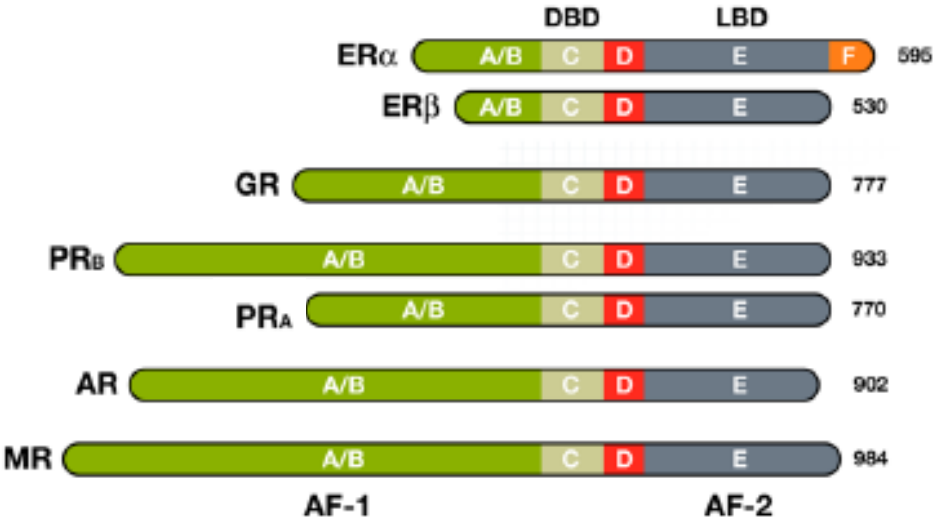


Figure 1.5. The structures of nuclear steroid receptors. SRs contain several functional domains, including NTD (A/B), DBD (C), hinge region (D) and LBD (E) at the CTD. ERα also has an additional CTD domain (F). Numbers indicate the protein length in amino acids. Adapted from (Griekspoor et al. 2007).

1.5.1 Function, regulation and expression of the GR and PR

The GR is a key regulator of the immune system by mediating anti-inflammatory responses to glucocorticoids (GCs) such as endogenous cortisol or the synthetic GC, Dex. GCs are important for metabolic, bone, renal, central nervous system, reproductive and immune function, with predominantly immunosuppressive effects (Baschant and Tuckermann 2010, Cruz-Topete and Cidlowski 2015).

The PR plays an important role in regulation of the female reproductive system, including mammary gland development, and mediates responses to progestogens. GCs acting via the GR, and progestogens acting via the PR, carry out their effects through mediating gene expression in target tissues. The PR is only expressed in some tissues, including the female reproductive tract, brain, mammary gland and pituitary gland (Lu et al. 2006). Conversely, GR is ubiquitously expressed (Lu et al. 2006) and is essential for life as GR knockout mice are not viable (Cole et al. 1995).

There is a single gene for GR, but multiple isoforms exist as a result of alternative splicing (Pujols et al. 2002, Lu and Cidlowski 2005, Zhou and Cidlowski 2005, Duma et al. 2006, Cao et al. 2013, Kadmiel and Cidlowski 2013, Cruz-Topete and Cidlowski 2015, Hapgood et al. 2016, Scheschowitsch et al. 2017). The 95 kDa GR isoform, GRα, is the most predominantly expressed. The GR is regulated both post-transcriptionally (Turner et al. 2010, Turner et al. 2014) and undergoes

post-translational modifications (PTMs), such as phosphorylation (Faus and Haendler 2006, Chen et al. 2008, Beck et al. 2009, Anbalagan et al. 2012). GR activity is regulated by phosphorylation at key residues (Avenant et al. 2010a, Avenant et al. 2010b).

The gene for PR is regulated by E_2 and ERα (Aronica and Katzenellenbogen 1991, Schultz et al. 2003) and contains two distinct E_2-regulated promoters, resulting in the transcription of two isoforms of PR, A and B (Kastner et al. 1990). These translate into two proteins, PR-A (94 kDa) and PR-B (114 kDa) which are identical except for the additional 164 amino acids on the N-terminus of PR-B (Fig. 1.5) (Kastner et al. 1990, Giangrande et al. 1997). This extra region harbours a third AF domain, rendering PR-B more transcriptionally active than PR-A (Sartorius et al. 1994). Like the GR, PR also undergoes PTMs (Bagchi et al. 1992, Lange et al. 2000, Faus and Haendler 2006, Anbalagan et al. 2012, Abdel-Hafiz and Horwitz 2014). PR-A and PR-B can form homo- and heterodimers with each other, as well as mixed ligand heterodimers (Sartorius et al. 1994, Leonhardt et al. 1998, Scarpin et al. 2009, Jacobsen and Horwitz 2012). The PR isoforms have also been shown to have differential cell- and promoter-specific effects on transcription (Wen et al. 1994, Giangrande and McDonnell 1999, Lim et al. 1999, Graham and Clarke 2002, Tung et al. 2006, Jacobsen and Horwitz 2012). Interestingly, PR-A represses the activity of PR-B (Tung et al. 1993) as well as other SRs including the GR (Vegeto et al. 1993) and MR (McDonnell et al. 1994). Consequently, the ratio of PR-A:PR-B can also influence the response to progestins on PR target genes, as demonstrated in breast cancer and myometrial cells (Graham et al. 2005, Jacobsen et al. 2005, Khan et al. 2012, Tan et al. 2012).

1.5.2 Classic mechanisms of action of SRs

Traditionally, SRs including the GR and PR regulate transcription through classical mechanisms of transactivation (up-regulation) and transrepression (down-regulation). A schematic model representing transactivation and transrepression is shown in Figure 1.5.2 (Nixon et al. 2012). In the absence of ligand, GR is mostly cytoplasmic, while PR is located in the nucleus (Renoir et al. 1990, Griekspoor et al. 2007), although there is evidence that some of the unliganded GR resides in the nucleus (Pekki et al. 1992), and this effect may depend on GR levels (Hapgood et al. 2016). The GR is retained in the cytoplasm in a chaperone complex which includes heat shock protein (HSP) 90 (Oakley and Cidlowski 1993, Griekspoor et al. 2007, Kadmiel and Cidlowski 2013). A small proportion of HSP90-bound PR is located in the cytoplasm (Passinen et al. 1999).

Upon ligand binding to the LBD, the GR undergoes a conformational change, dissociates from the chaperone complex and translocates to the nucleus (Zhou and Cidlowski 2005, Griekspoor et al. 2007). Here, classical transactivation is achieved by the ligand-bound SR DBD binding directly to its

hormone response elements (HREs) in target genes, that is, glucocorticoid response elements (GREs) for GR or progesterone response elements (PREs) for PR. GR typically binds to GREs as a dimer, (Zhou and Cidlowski 2005), and recruits co-activators and chromatin remodelling factors to activate transcription (Kadmiel and Cidlowski 2013, Cruz-Topete and Cidlowski 2015). In addition to the above mechanism, PR can bind to PREs or PRE ½ sites as a monomer (Jacobsen and Horwitz 2012).

The most well-established mechanism of transrepression is tethering, whereby the SR binds to another transcription factor via protein-protein interactions and inhibits its activity. This has been demonstrated for activator protein 1 (AP1) and nuclear factor kappa B (NFκB). Since NFκB is a key mediator of inflammatory gene expression, both GR and PR can modulate inflammatory responses by repressing NFκB-driven gene regulation through direct binding to NFκB subunits (Kalkhoven et al. 1996, De Bosscher et al. 2000, Nissen and Yamamoto 2000). The SR recruits co-repressors and chromatin remodelling factors to condense chromatin and inhibit transcription (Chinenov et al. 2013). Transrepression can also occur through direct GR binding to negative GREs (nGREs) (Surjit et al. 2011).

However, recent studies show that the mechanisms behind GR-mediated gene regulation are much more complex (Schoneveld et al. 2004, Newton et al. 2010, Nixon et al. 2013, Cruz-Topete and Cidlowski 2015, Newton et al. 2017, Scheschowitsch et al. 2017, Weikum et al. 2017a, Weikum et al. 2017b). For example, the GR can mediate transactivation through direct binding to a simple GRE, a composite GRE or a GRE ½ site as well as through tethering and co-factor-assisted tethering (Scheschowitsch et al. 2017). Furthermore transrepression can occur through multiple mechanisms including direct binding to a composite GRE, or nGRE, competition with other factors, sequestering TFs, tethering and co-factor-assisted tethering (Scheschowitsch et al. 2017). The GR can also repress gene expression through transactivation mechanisms (King et al. 2012, Vandevyver et al. 2013, Nacht et al. 2017, Weikum et al. 2017a). Furthermore, GR-mediated gene regulation is modulated by the adjacent sequences and chromatin landscape (Meijsing et al. 2009, Burd and Archer 2013, Sacta et al. 2016, Telorac et al. 2016, Love et al. 2017), the GR dimerization interface (Watson et al. 2013), and ability to form homo- or heterodimers (Nixon et al. 2013, Robertson et al. 2013a, Schiller et al. 2014, Lim et al. 2015) or even tetramers (Presman et al. 2016). Recently the term GR binding site (GBS) has been introduced, to refer to a short DNA sequence that binds GR with high affinity *in vitro*, while GRE refers to a longer DNA sequence that generates a GR response *in vivo* (Weikum et al. 2017b).

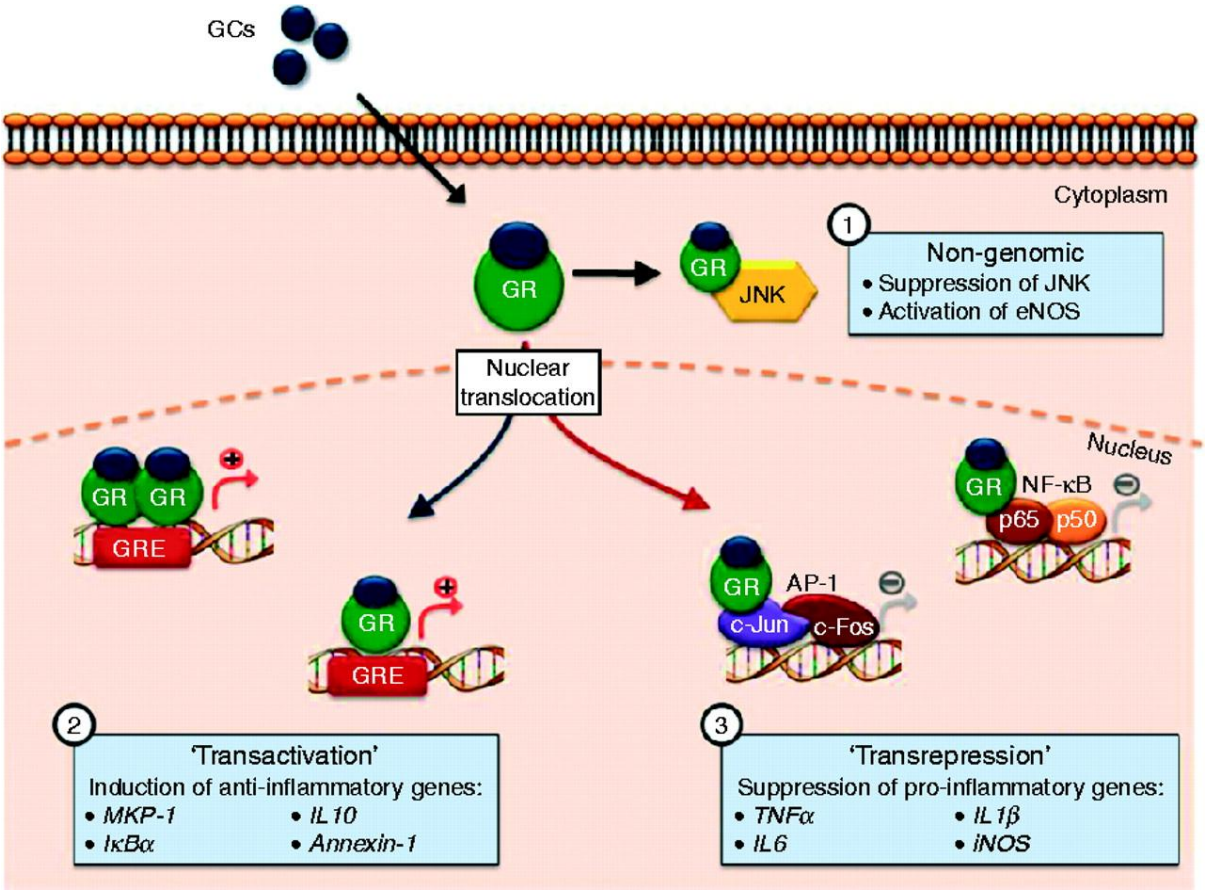


Figure 1.5.2. Classical model of transactivation and transrepression mechanisms mediated by the ligand-activated GR. The lipophilic steroid diffuses across the cell membrane into the cytoplasm, where it binds to the SR (in this case, the GR). The GR can activate cellular responses via non-genomic mechanisms, such as suppression of JNK (1). The ligand-activated GR also translocates into the nucleus, where it binds directly the GREs in target transactivation genes thereby activating their transcription (2), or as a monomer tethered to pro-inflammatory transcription factors NFKB/AP-1 to repress target transrepression genes (3). MKP-1: mitogen activated protein kinase phosphatase 1; IκBα: inhibitor of NFκB; IL10: interleukin 10; TNFα: tumour necrosis factor α; iNOS: inducible nitric oxide synthase. Taken from (Nixon et al. 2012).

Consistent with its role in mediating anti-inflammatory responses to GCs, the GR has been established to transactivate anti-inflammatory genes and repress pro-inflammatory genes, notably cytokines and chemokines (Chinenov et al. 2013). For example, GR mediates the up-regulation of the anti-inflammatory genes interleukin (IL)-10 (Visser et al. 1998), glucocorticoid-induced leucine zipper (GILZ) (Wang et al. 2004), mitogen activated protein kinase phosphatase 1 (MKP1) (Kassel et al. 2001) and inhibitor of NFκB (IκBα) (Heck et al. 1997). GR has also been shown to mediate the repression of the pro-inflammatory genes IL-6 (De Bosscher et al. 2003) and IL-8 (Mukaida et al. 1994).

Owing to the role of the PR in mediating the reproductive functions and mammary gland development, many identified PR-regulated target genes are involved in these processes, such as prolactin receptor (PRLR) and β-casein, although PR also regulates the expression of genes involved in cell growth and proliferation, such as epidermal growth factor (EGF), transforming growth factor β (TGFβ), insulin-like growth factor binding protein 1 (IGFBP1), cyclin-dependent kinase inhibitor 1A (p21), receptor activator of NFκB ligand (RANKL) and v-myc myelocytomatosis viral oncogene homolog (avian) (MYC) (Moore et al. 1997, Jacobsen and Horwitz 2012, Moore et al. 2012, Obr and Edwards 2012, Wetendorf and DeMayo 2012, Yin et al. 2012, Lain et al. 2013). Some PR-regulated genes are regulated by both PR isoforms, like p21, while others are preferentially regulated by PR-B, such as FK506 binding protein 5 (FKBP5), or by PR-A such as IGFBP1 (Jacobsen and Horwitz 2012).

Thus the mechanism of gene regulation by the GR and PR is a complex process with multiple players, including transcriptional machinery, chromatin remodelers, co-activators and co-repressors, with capacity for regulation at each step.

1.5.3 Cross-talk between SRs

The biological responses mediated by SRs can also involve non-genomic, transcription-independent and/or ligand-independent mechanisms of action (Leonhardt et al. 2003, Nilsen and Brinton 2003, Stellato 2004, Boonyaratanakornkit and Edwards 2007, Boonyaratanakornkit et al. 2008, Gellersen et al. 2009, Africander et al. 2011b, Hapgood et al. 2016, Hapgood et al. 2018). These effects generally involve the modulation of signalling cascades through the phosphorylation/de-phosphorylation action of kinases and phosphatases. Non-genomic SR activities allow for rapid responses without changing SR levels and promote the integration of multiple physiological functions (Hapgood et al. 2016).

This capacity for SRs to modulate the activity of other transcription factors gives rise to cross-talk (McKay and Cidlowski 1998, Lange 2004, De Bosscher et al. 2006, Kassel and Herrlich 2007, Beck et al. 2009, Bagamasbad and Denver 2011, Ratman et al. 2013, Hapgood et al. 2016). Cross-talk can refer to a positive or negative, direct or indirect modulation of the activity of other TFs (Kassel and Herrlich 2007). Indirect modulation can occur through activation of signalling pathways converging on SR target genes, while direct modulation can occur through direct interactions between SRs thereby modulating their activity. This cross-talk therefore contributes to the complexity of biological responses to multiple hormones *in vivo*.

Multiple studies show mechanisms of direct cross-talk between the GR and ER. For example, in MCF-7 breast cancer cells, Dex modulates ER transcriptional activity (Karmakar et al. 2013) and similarly E_2 inhibits GR mRNA and protein expression (Krishnan et al. 2001) while ER mediates proteasomal degradation of GR (Kinyamu and Archer 2003). Some studies have also shown that ER/E_2 can modulate GR/GC-mediated responses on inflammatory genes (Feldman et al. 2007, Cvoro et al. 2011, Eritja et al. 2012, Whirledge and Cidlowski 2013). Furthermore GR has been shown to interact with ERα (Karmakar et al. 2013). In addition, the GR has been shown to form a heterodimer with the MR (Liu et al. 1995, Trapp and Holsboer 1996, Ou et al. 2001, Savory et al. 2001) and the AR (Chen et al. 1997).

There is also substantial evidence of cross-talk between the PR and ER. Early studies showed that stimulation with E_2 increased PR protein expression in human breast cancer (MCF-7) and rat uterine cells (Horwitz and McGuire 1978, Aronica and Katzenellenbogen 1991). Subsequently it has been demonstrated at the genomic level that ER is recruited to EREs within the PR promoter sequence, thereby inducing its transcription (Petz et al. 2002, Schultz et al. 2003, Flötotto et al. 2004, Petz et al. 2004). In a mechanism of reciprocal regulation, PR regulates (frequently represses) ER-mediated transcriptional activity via association between ER and PR and/or regulation of signalling pathways and/or recruitment of repressors (Kraus et al. 1995, Lee and Gorski 1996, Ballare et al. 2003, Vallejo et al. 2005, De Amicis et al. 2009, Mohammed et al. 2015, Diep et al. 2016). One study also showed that progestin metabolites modulate ER protein levels in MCF-7 cells (Pawlak and Wiebe 2007).

Of importance to this study is the extent of cross-talk between the GR and the PR, owing to their mutual activation by the progestin MPA. Several genes have been shown to be ligand-dependently regulated by both GR and PR, including the mouse mammary tumour virus (MMTV), tyrosine aminotransferase (TAT), chicken lysozyme, rabbit uteroglobin and human metallothionein (MetIIA) genes (von der Ahe et al. 1985, Strahle et al. 1987, Jacobsen and Horwitz 2012). Microarray studies using Dex, R5020 or P_4 stimulation showed that these hormones regulate the expression of both overlapping and unique gene sets, in endometrial cancer Ishikawa H cells and T47D breast cancer cells (Wan and Nordeen 2002, Davies et al. 2006). In these studies it is presumed that R5020-induced gene regulation is via the PR, however the genes regulated in response to P_4 could be via the GR and/or PR.

The GR- and PR-mediated regulation of the MMTV promoter, which contains 4 GRE/PREs, has been well characterized. Despite both binding to the MMTV promoter, the GR and PR still exhibit selective gene regulation in different contexts (Cato et al. 1986, Cato et al. 1988, Nordeen et al. 1989, Archer et al. 1994a, Archer et al. 1994b, Archer et al. 1995, Mymryk et al. 1995, Le Ricousse et al. 1996, Lambert and Nordeen 1998, Nelson et al. 1999). Reasons for this selectivity include differential

interaction with co-factors (Szapary et al. 2008) including preferential use for different steroid receptor coactivator (SRC) coactivators (SRC2 for GR and SRC1 for PR), (Li et al. 2003), chromatin structure (Archer et al. 1994b, Archer et al. 1995, Mymryk et al. 1995, Smith et al. 1997), surrounding sequences (Cato et al. 1988, Chalepakis et al. 1988), and the low amino-terminal end homology (Song et al. 2001). Crucial to this study, different levels of receptors within a given cell would also contribute to different GR/PR-mediated responses on the same gene (Strahle et al. 1989).

1.5.4 Variations in SR levels in the FRT

Cross talk between the GR and PR is likely to be highly important in the FRT, if both of these receptors are expressed, because these receptors could simultaneously mediate the regulation of immune function and/or reproductive genes.

Previous research has indicated that PR protein is differentially expressed in FRT tissues throughout the menstrual cycle. However these studies generally used immunohistochemistry techniques that may be prone to antibody cross-reactivity. In general, higher PR protein levels were observed during the follicular phase compared to the luteal phase in multiple FRT tissues, including the fallopian tube and myometrium (Pino et al. 1984, Englund et al. 1998, Tone et al. 2011), vagina (Konishi et al. 1991) and endocervical columnar cells (Sanborn et al. 1976). In the endometrium, higher PR mRNA or protein levels were observed during the follicular phase in some (Lessey et al. 1988, Ingamells et al. 1996, Mylonas et al. 2007) but not all (Snijders et al. 1992, Marshburn et al. 2005) studies. In the ectocervix, higher PR levels were detected in ectocervical epithelium during the luteal phase compared to the follicular phase (Konishi et al. 1991). The PR isoforms are generally expressed at similar levels in reproductive tissues (Scarpin et al. 2009) but there is evidence that PR-A levels are preferentially decreased in the endometrium during the luteal phase (Mangal et al. 1997, Mote et al. 1999). GR protein has been detected in VEN-100 primary endocervical cells (Govender 2014) and ectocervical explants (Ray 2015) by other researchers in the present author's laboratory, consistent with the notion that GR is reportedly ubiquitously expressed in all tissues (Lu et al. 2006).

Collectively these studies show a trend that PR levels are lower during the luteal phase compared to the follicular phase in some FRT tissues. Interestingly, different PR levels are observed in the ectocervix compared to the endocervix during the luteal phase. This implies that throughout the menstrual cycle there will be conditions where the relative levels of GR/PR are higher or lower. Changes in the relative levels of GR/PR in the cervix, the primary site for HIV-1 infection, may therefore influence immunomodulatory gene expression and potentially also susceptibility to infection.

1.6 Thesis rationale and hypotheses

The current epidemiological research showing that use of DMPA is associated with increased HIV-1 acquisition is still controversial, despite the most recent meta-analyses showing increased risk (Morrison et al. 2015, Ralph et al. 2015, Polis et al. 2016). However, biochemical and other basic research show that MPA increases HIV-1 infection *ex vivo* (Huijbregts et al. 2013, Sampah et al. 2015) and SIV/SHIV infection in non-human primate models (Trunova et al. 2006, Smith et al. 2015, Butler et al. 2016). Several studies have also linked the luteal phase of the menstrual cycle – characterized by relatively high levels of endogenous P_4 in the luteal phase compared to the follicular phase – with increased HIV-1 or SHIV acquisition in various models (Marx et al. 1996, Vishwanathan et al. 2011, Saba et al. 2013, Kersh et al. 2014, McNicholl et al. 2014, Radzio et al. 2014). Since MPA is a derivative of P_4 and was designed to imitate the physiological properties of P_4, MPA may prevent pregnancy in a manner similar to the high-P_4 state of the luteal phase. However, very limited research is available comparing contraception with MPA and menstrual cycle phases, or P_4 alone, within the same study (Huijbregts et al. 2013, Byrne et al. 2016, Francis et al. 2016). This leads to the interesting question whether the increase in HIV-1 acquisition is comparable between DMPA and the luteal phase and whether infection occurs via similar or different molecular mechanisms, possibly based on the differential GR activity of P_4 and MPA. **Therefore, the first central hypothesis of the current study is that both MPA and luteal phase hormones increase HIV-1 infection, but through potentially different mechanisms**.

In order to address this hypothesis, human PBMCs from female donors were used as a model for systemic HIV-1 infection. PBMCs are a useful model since they contain a multitude of immune cells relevant to HIV-1 infection (Kleiveland 2015). These include CD4+ T cells, CD8+ T cells and CD14+ monocytes and cells expressing the CCR5 co-receptor. CD4+ T cells are the predominant target cells for HIV-1 infection, since the CD4 receptor is used by the virus for initial entry (Sattentau et al. 1986). However, HIV-1 infection has also been demonstrated in other cells such as macrophages (Givan et al. 1997, Howell et al. 1997, Hladik and Hope 2009, Nguyen et al. 2014, Wira et al. 2015). Since CD8+ T cells are cytotoxic towards HIV-1, the CD4/CD8 ratio is an important parameter to consider the balance between frequency of infectable cells (CD4+) and cells combating infection (CD8+). Monocytes are important to study since they play a role in the establishment of infection and disease progression and secrete cytokines that modulate T cell function (Campbell et al. 2014). Levels of the co-receptor CCR5 are crucial determinants of efficient viral entry (Sciaranghella et al. 2015). Therefore CCR5 expression and the frequency of CCR5-expressing cells were also investigated. In addition the activation status of target immune cells was investigated as a possible mechanism by which MPA could affect HIV-1 infectivity. Activated target cells are more susceptible to HIV-1 infection, because they are primed to recruit infectable target cells (McKinnon and Kaul 2012).

Therefore a marker for activation, CD69, was measured in PBMCs. CD69 is an early activation marker that is expressed within 4 hours and for up to 14 days thereafter (Ziegler et al. 1994, Caruso et al. 1997, Nielsen et al. 1998, Clausen et al. 2003).

TZM-bl human cervical epithelial cells were used as a model of infectable cells in which to study possible biological mechanisms of infection. These cells have been engineered to stably express CD4, CCR5 and CXCR4, and a luciferase gene linked to the HIV-1 LTR promoter (Platt et al. 1998) and are therefore readily infectable. Furthermore as a single cell type, TZM-bl cells do not undergo activation like T cells, allowing additional insight into CCR5 regulation independent of T cell-specific signalling. HIV-1 infection was assessed using the laboratory-adapted R-tropic HIV-1 infectious molecular clone, HIV-$1_{BaL\_Renilla}$ using luciferase assays as a proxy for HIV-1 replication. This clone was used in order to represent the transmitting founder virus, which is predominantly R5-tropic and therefore utilizes the CCR5 co-receptor for initial infection (Carrington et al. 1999, Keele et al. 2008, Salazar-Gonzalez et al. 2009, Grivel et al. 2011). Combinations of E_2/P_4 were used to mimic the luteal phase (10 nM P_4/400 pM E_2) or the follicular phase (1 nM P_4/400 pM E_2) of the menstrual cycle, which are within the range of reported serum E_2/P_4 levels (Stricker et al. 2006). The concentrations of MPA used (100 nM) represents the upper levels detected in the serum of DMPA users (Hapgood et al. 2018).

While MPA and P_4 act via the PR, MPA also binds to the GR with high affinity (Africander et al. 2011b) and thus can control the expression of GR-regulated genes. Since the GR is a key mediator of the immune system and inflammatory responses, MPA-induced changes in GR-regulated gene expression could have important consequences for immune function and therefore susceptibility to infection in the FRT. The present author's laboratory has shown that MPA, like the GC Dex but unlike NET or P_4, at physiologically relevant concentrations (excluding during pregnancy), changes the expression of key immunomodulatory genes in a GR-dependent manner, in both cell lines and primary cells (Koubovec et al. 2004, Verhoog 2010, Africander et al. 2011a, Verhoog et al. 2011, Govender 2014, Govender et al. 2014, Hapgood et al. 2014b, Louw-du Toit et al. 2014, Ray 2015). These important GR-regulated immunomodulatory genes include the transactivation gene, GILZ, and the transrepression gene, IL-6. GILZ is an anti-inflammatory protein that plays an important role in mediating the effects of GCs (Ronchetti et al. 2015). GILZ expression is induced by GCs and its GR-mediated regulation occurs through by direct GR binding to multiple GREs in its promoter (Wang et al. 2004, Ayroldi and Riccardi 2009). IL-6 is one of the pro-inflammatory cytokines that drive inflammatory responses (Feghali and Wright 1997, Ershler and Keller 2000). IL-6 expression is activated by the transcription factors NFκB and AP-1 and therefore GR-mediated repression of IL-6 is believed to occur via GR tethering to subunits of NFκB and inhibiting its activity (Heck et al. 1997, De Bosscher et al. 2000, Nissen and Yamamoto 2000, Tao et al. 2001, De Bosscher et al. 2003).

However both PR and GR recognize similar DNA target sequences (such as GRE/PREs) and both inhibit the activity of NFκB (Kalkhoven et al. 1996, Davies et al. 2004, Hardy et al. 2006). Following this, it is possible that PR could regulate GR-mediated gene expression in some contexts, depending on relative receptor levels. GR is ubiquitously expressed whereas PR is expressed in some tissues, including reproductive tissues, and is dependent on regulation by E_2, which fluctuates with the menstrual cycle. Therefore in the FRT there are likely to be states in which the relative levels of GR and PR change in response to endogenous and exogenous sex hormones. The consequences of changing GR/PR levels on immune function and HIV-1 susceptibility have not been studied. **The second central hypothesis of this study is therefore that changing the relative levels of GR/PR can influence HIV-1 infection and the regulation of immunomodulatory genes.**

In order to investigate this hypothesis, human cervical cell line models were used to represent the FRT and conditions of changing GR/PR levels. These cell lines, which express high levels of endogenous GR, were the End1/E6E7 immortalized endocervical epithelial cells, HeLa cervical carcinoma cells and TZM-bl cervical carcinoma cells, which are modified HeLa cells. In these cells, the relative levels of GR/PR were changed by using siRNA to knock down the endogenous GR, with or without the simultaneous over-expression of PR-B, the more transcriptionally active PR isoform. While other cell line strategies of GR/PR over-expression and endogenous expression were also investigated, End1/E6E7, HeLa and TZM-bl cells were chosen for this study as models for investigating the effects of changing GR/PR levels in cervical cells. HeLa and End1/E6E7 cells have been shown by the present author's laboratory to have robust GR-mediated regulation of GILZ and IL-6 (Verhoog 2010, Govender 2014). While cell line studies have limitations, molecular techniques such as transfection used to change GR/PR levels were already established in the present author's laboratory and are generally more robust and reproducible than in primary cells. HIV-1 infection was assessed in TZM-bl cells using the R5-tropic IMC HIV-$1_{BaL\_Renilla}$ and luciferase reporter assays. Secretion of IL-6 was investigated as a possible soluble mediator that enhances infection. Previous *in vitro* work has demonstrated that exogenous IL-6 stimulation induces HIV-1 reverse transcriptase or LTR activity in monocyte-derived macrophages (MDMs) and promonocyte U1 cells (Poli et al. 1990, Kinter et al. 2001) and enhances infection of MDMs (Zaitseva et al. 2000). Some clinical studies have shown correlations between increased IL-6 levels and higher HIV-1 RNA levels or viral load (Spear et al. 2008, Roberts et al. 2012, Masson et al. 2014). Therefore the effect of changing relative GR/PR levels on IL-6 expression and the effect of IL-6 and/or other soluble mediators on HIV-1 infection was also investigated.

Transcriptional activity can be modulated by cross talk between steroid receptors (Bagamasbad and Denver 2011). There are multiple lines of evidence of cross talk between the GR-ER and the PR-ER, including direct interactions between the proteins. Therefore it is likely that mechanisms of cross-talk

exist between GR and PR. While both GR and PR can regulate the same promoters, such as MMTV, whether GR and PR associate together is not established. **The final hypothesis of the current study is that GR and PR reciprocally modulate each other's activity through a possible interaction.**

In order to investigate this hypothesis, cells expressing very low levels of endogenous GR and no PR (COS1 monkey fibroblast and U2OS human osteosarcoma cells) were used in assays where GR/PR were transiently over-expressed. Alternatively cells expressing high endogenous levels of both GR and PR (MCF-7 human breast cancer cells) were used. Co-immunoprecipitation assays in COS1 and MCF-7 cells were used to indicate a potential association between GR and PR in response to the GR/PR agonist MPA. In addition, the nucleocytoplasmic distribution of the GR and PR in COS1 cells over-expressing GR and PR was measured using immunofluorescence with confocal microscopy. Quantitative co-localization analysis was used to suggest close physical proximity of the GR and PR, using both confocal microscopy and structured illumination super-resolution microscopy. The ligand-dependency of this potential association was investigated by comparing equimolar concentrations (100 nM) of the GR-specific agonist Dex, the PR-specific agonist R5020 and MPA. Specific research questions are outlined in more detail at the start of each results chapter.

Chapter 2

Materials and Methods

All work in this thesis was carried out by the present author, with technical assistance for the following: fixed cells prepared by the candidate were analyzed for flow cytometry by Dr Michele Tomasicchio at the Lung Infection and Immunity Unit (University of Cape Town), and immunofluorescence slides prepared by the candidate were analyzed together with Mrs Susan Cooper at the Confocal and Light Microscope Unit (University of Cape Town) and Dr John Woodland at the Central Analytical Facility Fluorescence Microscopy Unit (Stellenbosch University).

2.1 Ethics and biosafety

This research formed part of a sub-study of HREC 210/2011, which was approved by the Human Research Ethics Committee at the University of Cape Town for use of human peripheral blood mononuclear cells from the Western Province Blood Transfusion Services and cervical explant tissue from Groote Schuur and Tygerberg Hospitals. Donors provided written informed consent.

This study, for a doctoral degree, was approved by the Human Research Ethics Committee at the University of Cape Town with reference number 076/2017.

All work was carried out according to the biosafety procedures established by the Health and Safety Committee of the Department of Molecular and Cell Biology at the University of Cape Town.

2.2 Compounds and antibodies

The following compounds were purchased from Sigma Aldrich, South Africa: (11b,16a)-9-fluoro-11,17,21- trihydroxy-16-methylpregna-1,4-diene-3,20-dione (dexamethasone, Dex); 6α-methyl-17α-hydroxy-progesterone acetate (medroxyprogesterone acetate; MPA); 4-pregnene-3,20-dione (progesterone; P_4); 17α-ethynyl-19-nortestosterone (norethisterone; NET); 17β-estradiol (E_2); 13β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one (levonorgestrel; LNG); 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one (Mifepristone; RU486); and 17,21-dimethyl-19-norpregna-4,9-dien-3,20-dione (Promegestone, R5020). All hormones were

prepared in absolute ethanol (EtOH), and added to cells such that final [EtOH] was 0.1% (v/v). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was also obtained from Sigma Aldrich, South Africa. IL-2 was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

For western blotting, antibodies against human GAPDH (0411; sc-47724) and GR (H-300; sc-8992) were obtained from Santa Cruz Biotechnology, USA, while anti-HA (12CA5) was from Roche Applied Science, South Africa, and anti-PR (NCL-LPGR-312) was obtained from Leica Biosystems (Novacastra, UK). Horseradish peroxidise-conjugated secondary antibodies, anti-mouse (sc-2005) and anti-rabbit (sc-2313), were purchased from Santa Cruz Biotechnology, USA.

For flow cytometry, antibodies against human CD4 phycoerythrin (PE)-Dazzle 594 (357412), anti-CD8 PE/Cy5 (300910), anti-CD69 PE/Cy7 (310912), anti-CCR5 PE (359106), anti-CD14 APC (325608), anti-CD3 fluorescein isothiocyanate (FITC) (300306) and the ZOMBIE Violet Fixable Viability Kit (423113) were purchased from Biolegend, USA. The isotype controls, anti-mouse IgG (APC, 555751) and anti-rat IgG (PE, 12-4321) were purchased from Becton Dickinson (BD) Sciences, USA. The anti-rabbit AlexaFluor488 (#A21206) antibody was purchased from Invitrogen, USA, while the donkey anti-mouse Cy3 (#715-166-150) and the donkey anti-rabbit Cy5 (#711-175-152) antibodies were obtained from Jackson ImmunoResearch, USA.

2.3 Plasmids

The luciferase reporter gene plasmid, pTAT-GRE-E1b-LUC (TAT-GRE-LUC), containing two glucocorticoid response elements (GREs) from the rat tyrosine amino transferase (TAT) gene under the control of the E1b promoter was a gift from G. Jenster (Erasmus University of Rotterdam, Rotterdam, Netherlands) (Sui et al. 1999). The empty vector, pcDNA3.1, containing a CMV promoter and no downstream DNA sequence, was obtained from Invitrogen, UK. The steroid receptor plasmid for the GR, pcDNA3-hGR, containing full-length untagged human GRα cloned into the pcDNA3 vector, was a gift from D. W. Ray (University of Manchester, UK) (Ray et al. 1999). The steroid receptor plasmid for the PR isoform PR-B, pMT-hPRB, containing full length untagged human PR-B cloned into the pMT vector, was a gift from S. Okret (Karolinska Institute, Sweden) (Cairns et al. 1993).The steroid receptor plasmid for PR-A, pSG5-hPRA, was a gift from F. Claessens (University of Leuven, Leuven, Belgium) (Kastner et al. 1990). The plasmid for HA-tagged GR-Interacting Protein 1 (GRIP-1), pHA-GRIP1 was a gift from M. R. Stallcup (University of Southern California, Los Angeles, CA).

2.3.1 Plasmid transformation and purification

Plasmids were transformed into *Escherichia coli* DH5α cells using heat shock as previously described (Sambrook and Russell 2006). Competent DH5α cells (100 µL) were incubated with 10 ng plasmid DNA on ice for 30 min, then at 42°C for 2 min and on ice for 2 min. Cells were subsequently mixed into 900 µL Luria broth (LB, 1% (*w/v*) tryptone, 0.5% (*w/v*) yeast extract, 0.5% (*w/v*) NaCl) and incubated at 37°C for 1 h with shaking. Transformed cells were then plated onto LB-agar plates (1% (*w/v*) tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) containing 100 µg/mL final concentration of the antibiotic ampicillin (Sigma Aldrich, South Africa) and incubated overnight at 37°C. Day cultures of 5 mL LB containing 100 µg/mL ampicillin were inoculated with single colonies and incubated at 37°C for 8 h with shaking. Plasmid glycerol stocks were prepared by mixing 500 µL 70% (v/v) glycerol with 500 µL transformed cell suspension and storage at -80°C. Overnight cultures of 200 mL LB containing 100 µg/mL ampicillin were inoculated with 200 µL day culture cell suspension and incubated at 37°C for 16 h with shaking. Plasmid DNA was subsequently purified using the PureYield plasmid maxi-prep kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. The purity and yield of the plasmid preparation were assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies).

2.3.2 Restriction enzyme digestion

The supercoiled conformation of plasmid DNA was assessed by agarose gel electrophoresis by comparison with the linearized plasmid after restriction enzyme digestion. Reactions of 300 ng DNA, 1 unit (U)/µL restriction enzyme (or equivalent volume of water for the undigested control) and 1 X FastDigest universal buffer containing sample application dye (Fermentas, Thermo Scientific, USA) were incubated for 10 min at 37°C. Digested and undigested samples were separated by electrophoresis on a 0.8% (w/v) 1 X Tris-Acetate-EDTA (TAE) agarose gel containing 10 µg/mL ethidium bromide (Sigma Aldrich, South Africa). Samples were subsequently visualized under ultraviolet light on a Syngene, G:Box (Vacutec, England) and images acquired using GeneSnap version 7.08 (SynGene, England). The identity of the plasmids was assessed by restriction enzyme digestion pattern.

2.4 Mammalian cell culture

Immortalized or cancerous human and simian cell lines, and human PBMCs and cervical tissue were used as model systems in this study.

2.4.1 Cell lines

The African green monkey kidney fibroblast (COS1), human osteosarcoma (U2OS) and human embryonic kidney (HEK293T) cell lines were purchased from the America Type Culture Collection (ATCC, USA). The human endocervical epithelial cell line immortalized with HPV-16 E6E7 (End1/E6E7) was received from Dr R Fichorova (Brigham & Women's Hospital, Boston, USA) (Fichorova et al. 1997). The breast carcinoma cell line, MCF-7 (Soule et al. 1973), was a gift from Dr S Prince (Human Biology, University of Cape Town), and the T47D breast carcinoma cell line (Keydar et al. 1979) was from Prof M I Parker (ICGEB, University of Cape Town). The human cervical carcinoma cell lines, HeLa and TZM-bl, were obtained 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). TZM-bl cells are modified HeLa cells stably expressing CD4, CXCR4 and CCR5 (Platt et al. 1998) and the luciferase and β-galactosidase reporter genes controlled by the HIV-1 LTR promoter (Wei et al. 2002).

Cells were cultured in humidified 37°C incubators containing 5% CO_2 in the P2 Mammalian Tissue Culture Facility in the Department of Molecular & Cell Biology at the University of Cape Town. All cell lines were maintained 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 (Sigma Aldrich, South Africa), 44 mM sodium bicarbonate (Sigma Aldrich, South Africa), 10% (v/v) foetal calf serum (FCS, Thermo Scientific, South Africa) and 100 IU/mL penicillin and 100 mg/mL streptomycin (Gibco, Invitrogen, UK), except for End1/E6E7 cells which were maintained in Keratinocyte Serum-free media (Gibco, Invitrogen, UK) supplemented with bovine pituitary extract (BPE) and 100 IU/mL penicillin and 100 mg/mL streptomycin (Gibco, Invitrogen, UK).

Transfections and stimulations were carried out in serum-free (SF) media and HIV-1 infection assays in TZM-bl cells were carried out in phenol red-free DMEM (Sigma Aldrich, South Africa) supplemented with 10% (v/v) charcoal-stripped FCS (cs-FCS, Thermo Scientific, South Africa) and 100 IU/mL penicillin and 100 mg/mL streptomycin (Gibco, Invitrogen, UK). For sub-culturing, cells were washed with warm 1 X phosphate-buffered saline (PBS, Sigma Aldrich, South Africa) and incubated at 37°C with 2 mL 0.25% trypsin/0.1% EDTA in PBS (Highveld Biological, South Africa) for 5 min. Trypsin was neutralized by the addition of full DMEM containing 10% FCS.

Cells were routinely checked for mycoplasma contamination by Hoescht staining (Freshney 1987) and fluorescence microscopy (Department of Molecular and Cell Biology, University of Cape Town), and only mycoplasma-negative cells were used for experiments.

2.4.2 PBMCs

All PBMCs were from female donors only and were isolated from whole blood as previously described (Tomasicchio et al. 2013). Buffy packs from healthy female HIV-negative donors, of unknown menstrual cycle phase, were obtained from the Western Province Blood Transfusion Services (Pinelands, South Africa). PBMCs were isolated by Histopaque (H1077 Hybri-Max, Sigma-Aldrich) density centrifugation using Leucosep tubes (Greiner Bio-One International, Austria). Briefly, 15 mL Histopaque was centrifuged in Leucosep tubes at 2500 rpm for 1 min at room temperature using the Heraeus Megafuge 40 centrifuge (Thermo Scientific, South Africa), while whole blood was diluted in unsupplemented Roswell Park Memorial Institute (RPMI) 1640 (Lonza, Switzerland) and PBS at a ratio of 2:1:1. The diluted blood (40 mL) was loaded onto the Leucosep tube and centrifuged for 15 min at 2500 rpm with lowest deceleration. PBMCs that had separated from red blood cells and serum into a white buffy coat were collected with a 1 mL pipette and washed twice with 1 X PBS containing 1% (v/v) cs-FCS by centrifugation at 1200 rpm for 5 min. Cell number and viability was determined by diluting the PBS-suspended PBMCs 1:10 with trypan blue (Lonza, Switzerland) and counting on a haemocytometer.

PBMCs were maintained in high-glucose (4.5 g/mL) RPMI medium (Lonza, Switzerland) containing 10% (v/v) cs-FCS (Thermo Scientific, 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 mg/mL streptomycin (Sigma-Aldrich, South Africa) and 30 U/mL final concentration IL-2 at 37°C in a humidified incubator containing 5% CO_2 at the P2+ Mammalian Tissue Culture Facility in the Department of Molecular & Cell Biology at the University of Cape Town.

2.4.3 Cervical explants

Cervical epithelial explant tissue was obtained after informed consent from HIV-negative, normal Pap smear, pre- and postmenopausal women undergoing hysterectomies for benign reasons, at either Groote Schuur or Tygerberg hospitals. After pathologist inspection to exclude abnormal or cancerous tissue, the sample was transported to the P2 Mammalian Tissue Culture Facility at the Department of Molecular and Cell Biology, where the explant was washed in warm 1 X PBS and processed by removal of stroma and fatty connective tissue and separation of endo- and ectocervix if necessary and if available. Cervical explants were then cut into small pieces and transferred into round-bottom 96-well plates (Greiner Bio-One International, Austria) containing full RPMI 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). Thereafter, cervical explants were stimulated and cultured in the P2+ facility, in a 37°C humidified incubator containing 5% CO_2. Blood samples collected from hysterectomy donors were tested for HSV-1 and HSV-2 status and for the endogenous levels of LH, FSH, E_2 and P_4 at the National Health Laboratory Services (NHLS, Groote Schuur Hospital, South Africa). Menstrual cycle phase was determined from these four hormones, based on the NHLS guideline hormone ranges (Appendix D, Table D2).

2.5 Transfections

Transient transfection of steroid receptor expression constructs and luciferase reporter vectors was carried out using XtremeGENE-9 transfection reagent (Roche Applied Science, South Africa), according to the manufacturer's protocol at a ratio of 2:1 (XtremeGENE-9: DNA). Transfection mixes containing DNA and XtremeGENE-9 were incubated in SF-KSF (End1/E6E7 cells) or SF-DMEM (all other cells) at room temperature for 30 min before dropwise addition to the cells. For over-expression of PR-B, HeLa/TZM-bl (1 X 10^5 cells/well) and End1/E6E7 (1.5 X 10^5 cells/well) cells were seeded in 12-well plates (Greiner Bio-One International, Austria) for 24 h, and transfected with 250 ng (End1/E6E7) or 500 ng (HeLa/TZM-bl) pMT-hPRB or equivalent pcDNA3 for 24 h before stimulation with the indicated ligands for a further 24 h prior to harvesting for RNA, protein or supernatant media. Positive controls for western blots were prepared by transfecting 1 X 10^5 cells/well COS1 cells with 1 μg pcDNA3-hGR, pMT-hPRB or pSG5-PRA for 24 h prior to harvesting.

2.5.1 Promoter-reporter assays

Promoter-reporter assays using the luciferase reporter gene were carried out as described previously (Africander et al. 2011a) with a few modifications. Cells were seeded as indicated in the figure legends and allowed to adhere overnight in 24-well plates. Transfection mixtures containing 47 ng pTAT-GRE-LUC and either 125 ng or 250 ng steroid receptor expression vector (GR or PR-A or PR-B) or empty vector (pcDNA3.1) were prepared using XtremeGENE-9 transfection reagent (Roche Applied Science, South Africa) and added dropwise onto cells. After 24 h, cells were stimulated in SF-media with the indicated ligands for a further 24 h, after which cells were washed in cold 1 X PBS and harvested by shaking in 50 μL 1 X reporter lysis buffer (Promega, Madison, WI, USA) with a freeze-thaw step. Luciferase activity was assessed using the Luciferase Assay System (Promega, Madison, WI, USA) by measuring relative light units (RLU) in 10 μL cell lysate after addition of 50 μL luciferin substrate in a Modulus microplate luminometer (Turner Biosystems, Sunnyvale, CA,

USA). Luciferase activity was calculated as RLU relative to total protein content per well as measured spectrophotometrically using the Bradford assay (Bradford 1976).

2.5.2 Small interfering RNA (siRNA) transfections

Transfection with siRNA was carried out as previously described (Govender et al. 2014, Louw-du Toit et al. 2014) with a few modifications. Cells were seeded as indicated in the figure legends and allowed to adhere overnight in 12 well plates. Cells were transfected with 10 nM final concentration either validated GR HS_NR3C1_5 (GR5, referred to in this study as siGR) (catalogue no. SI02654757, Qiagen, South Africa) siRNA targeting the human GR or validated non-silencing control (NSC) scrambled sequence siRNA (catalogue no. 1027310, Qiagen, South Africa) using 3.5 μL HiPerfect transfection reagent (Qiagen, South Africa) in 46 μL OptiMEM medium with GlutaMAX^{TM} (Gibco-BRL Life Technologies) in a dropwise manner after incubation at room temperature for 15 min. Cells were incubated with the siRNA transfection mixtures for 48 h before stimulation with the indicated ligands for a further 24 h prior to harvesting for RNA, protein or supernatant media. GR protein knock down was confirmed by western blotting.

2.6 Protein assays

2.6.1 Protein isolation

2.6.1.1 Cell lines

At the end of the experiment (transfection, knockdown, stimulation, etc.), cells seeded in 12 well plates were washed in ice cold 1 X PBS. Total protein was then harvested by scraping in 50 μL 2 X 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, 0.1% (w/v) bromophenol blue). After transfer to microfuge tubes, samples were boiled for 10 min at 100°C then stored at -20°C. Secreted protein was harvested by collecting growth media supernatants from adhered End1/E6E7 or HeLa cells in cryovials, which were stored at -80°C.

2.6.1.2 PBMCs

At the end of the experiment, PBMCs were pelleted by centrifugation at 1200 rpm for 5 min using the Heraeus Megafuge 40 centrifuge (Thermo Scientific, South Africa), transferred to microfuge tubes and washed in ice cold 1 X PBS. Dry pellets were stored at -80°C until protein was harvested using N-[Tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid (TAPS) buffer [0.1 M TAPS, pH 9.5, supplemented with 1 mM final concentration phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL Aprotinin (Roche Applied Science, South Africa), 5 μg/mL Leupeptin (Roche Applied Science, South Africa) and 1 X Complete Mini protease inhibitor tablet (1 tablet/ 10 mL buffer; Roche Applied Science, South Africa)]. Samples were incubated on ice and vortexed every 10 min for 1 h prior to centrifugation at 12000 x g at 4°C for 15 min. Supernatants were transferred to new microfuge tubes in which 5 X SDS sample buffer was added to a final volume of 1 X (v/v). Samples were then boiled for 7 min at 100°C and stored at -20°C.

2.6.1.3 Cervical explants

On the day of surgery, individual pieces of cervical explant tissue (approximately $3mm^3$) were placed in cryovials containing about 200 μL All-Protect® (Qiagen, Netherlands) and were stored at -80°C until use. Explants were thawed at room temperature and excess All-Protect® was removed by wiping on tissue paper using sterile forceps. Explants were then transferred to 2 mL microfuge tubes containing two 7 mm stainless steel beads (Qiagen, Netherlands) and 100 μL radio-immunoprecipitation (RIPA) buffer (50 mM TRIS-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing 1 mM final concentration PMSF and 1 X Complete Mini protease inhibitor tablet (1 tablet/ 10 mL buffer; Roche Applied Science, South Africa). Total protein was harvested mechanically using the TissueLyser LT (Qiagen, Netherlands) at 50 Hz in 4 x 1 min steps, cooling on ice in between steps. Samples were centrifuged for 20 min at 12000 x g at 4°C, then transferred to new microfuge tubes. Total protein was quantified using the BCA protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL, USA) and 5 X SDS sample buffer was added to a final volume of 1 X (v/v) to the remaining sample. Samples were boiled at 100°C for 7 min and stored at -20°C.

2.6.2 Western blotting

Western blotting was carried out as previously described (Avenant et al. 2010a). Equal amounts of cell lysate (a minimum of 40 μg protein for explant samples) were loaded onto 8% SDS-polyacrylamide gels and separated by electrophoresis at 75 V for 20 min then 120 V for 1 h in 1 X running buffer (25 mM TRIS-HCl, 250 mM glycine and 0.1% (*v/v*) SDS pH 8.4) using a Bio-Rad Mini Protean II electrophoresis system (Bio-Rad, South Africa). Proteins were then blotted onto Hybond-ECL nitrocellulose membrane (AEC-Amersham, South Africa) for 1 h at 180 mA in cold 1 X transfer buffer (25 mM TRIS, 200 mM glycine, 20% (*v/v*) methanol). Membranes were subsequently blocked for 1 h at room temperature by shaking in 4% (*w/v*) ECL advance blocking powder (AEC-Amersham, South Africa) in 1 X TRIS-buffered saline (50 mM TRIS, 150 mM NaCl; TBS) containing 0.1% (*v/v*) Tween (TBS-Tween; TBST). Primary antibodies were diluted in 4% ECL-TBST (Table 2.1) and incubated on membranes overnight with shaking at 4°C. Membranes were then washed three times in 1 X TBST for 5 min and incubated with secondary antibodies diluted in 5% (w/v) skim milk powder in 1 X TBST (Table 2.1) for 1 h at room temperature with shaking. After three 5 min washes in 1 X TBST, membranes were placed in 1 X TBS prior to 1 min incubation with Pierce ECL-chemiluminescent western blotting substrate (Thermo Scientific, USA). Proteins were visualized by autoradiography using Amersham Hyperfilm™ MP high performance autoradiography film (AEC-Amersham, South Africa). Densitometric quantification of film was carried out using AlphaEaseFC™ Software, version 3.1.2 (Alpha Innotech, USA).

Table 2.6.2 Antibodies used in Western blotting procedure

Primary Antibody	**Size (kDa)**	**Dilution**	**Secondary Antibody**
GRα	95	1: 4000 (cell lines) 1: 2000 (primary cells)	Rabbit (1: 10 000)
PR-B/PR-A	114/94	1: 1000 (cell lines) 1: 500 (primary cells)	Mouse (1: 4000) (cell lines) (1:2000) (primary cells)
GAPDH	37	1:15 000	Mouse (1: 4000)

2.6.3 Co-immunoprecipitation (Co-IP) assay

The Co-IP assay was carried out as previously described (Avenant et al. 2010a) with a few modifications. COS1 cells were seeded at a density of 1.5 X 10^6 in 10 cm dishes for 24 h and were subsequently transfected with 3 μg of pMT-hPR-B and 3 μg of either pcDNA3.1 or pcDNA3-hGR using XtremeGene-9 transfection reagent (Roche Applied Science, South Africa) for a further 24 h. MCF-7 cells were seeded as for COS1 cells and were incubated for 48 h without transfection. Cells were then stimulated with 1 μM MPA or vehicle in SF-DMEM for 1 h, washed once with cold 1 X PBS and lysed by scraping in 500 μL CytoBuster™ protein extraction reagent (Novagen®) containing protease inhibitors (1 X Complete Mini protease inhibitor tablet [Roche, South Africa] per 10 mL). Cellular debris was pelleted by centrifugation at 16000 x g for 5 min at 4°C and 9 μL of supernatant were collected for inputs. From the supernatants of MPA-treated and vehicle control samples, equal volumes (200 μL each) were combined and incubated with 1.6 μg of donkey anti-rabbit IgG antibody (sc-2313, Santa Cruz Biotechnology) at 4°C overnight with rotation. The remaining supernatants were incubated with 1.6 μg anti-GR (H300, Santa Cruz Biotechnology) at 4°C overnight with rotation. Antibody-bound protein complexes were precipitated the following day by the addition of 20 μL pre-blocked PureProteome™ Protein A/G magnetic beads (LSKMAGG02, Millipore, Merck, South Africa) for 2 h with rotation at 4°C. The complexes were washed twice with 1 X PBS by centrifugation at 2000 x g for 1 min at 4°C. GR-bound proteins were then released from the beads by boiling in 25 μL 2 X SDS sample buffer for 5 min, then were separated using 6% SDS-PAGE and western blotting as described in 2.6.2. Samples were run on duplicate blots which were probed with anti-GR (H300, Santa Cruz Biotechnology) or anti-PR (NCL-LPGR-312, Leica Biosystems) antibodies.

2.6.4 IL-6 ELISA

The human IL-6 high sensitivity ELISA kit was purchased from eBioscience and followed according to the manufacturer's instructions (BMS213HS, San Diego, USA), except that supernatants from End1/E6E7 and HeLa experiments were diluted 1:40 in the sample diluent prior to the assay.

2.6.5 Flow cytometry

Fluorescence-activated cell sorting (FACS) was carried out as previously described (Tomasicchio et al. 2013) with a few modifications. PBMCs seeded at 4 X 10^6 in 5 mL Falcon tubes (Becton Dickson Scientific, South Africa) were stimulated with the indicated ligands for 7 days in full RPMI in an

incubator at 37°C. Cells were subsequently stained with anti-CD3 FITC, anti-CD4 PE-DAZZLE 594, anti-CD8 PE/Cy5, anti-CD14 APC, anti-CCR5 PE, anti-CD69 PE/Cy7 and the viability dye, ZOMBIE NIR (Biolegend, USA), for 15 min in the dark at room temperature. Fluorescence minus one (MFO) controls for CD14, CD69 and CCR5 were also prepared, including all the antibodies except CD14, CD69 and CCR5, respectively. Cells were washed with 1 X PBS then resuspended in 1 X Cell Fix solution (Becton-Dickinson, USA) and taken for analysis at the Lung Infection and Immunity Unit, Department of Medicine, University of Cape Town, using a BLSRII Becton-Dickinson flow cytometer (Becton-Dickinson, USA). Samples were analyzed from 50 000 cells using FlowJo software version 10.1 (Treestar Inc., Ashland, Ore). Lymphocytes were chosen from forward and side scatter plots and single cells were selected (Appendix C). Dead cells were excluded by selecting for ZOMBIE-negative cells (Appendix C). Negative gates were set using first the CD14 MFO control, then CCR5 and CD69 (Appendix C). Results were plotted as frequency, that is, a percentage of total, or expression, as median fluorescence intensity (MFI) per number of double-positive cells. Fold change was calculated relative to the vehicle control (EtOH) set to 1.

2.7 Gene expression assays

2.7.1 RNA isolation

RNA was isolated from both cell lines and PBMCs using Tri-Reagent® (T9429, Sigma-Aldrich, South Africa). PBMCs that were pelleted by centrifugation or adherent cells seeded in 12-well plates were incubated with 400 µL Tri-Reagent® for 5 min at room temperature. Lysed cells were transferred from the 12 well plates into microfuge tubes. After the addition of 80 µL chloroform, tubes were vortexed vigorously for 15 s then centrifuged at 20 000 x g at 4°C for 15 min. The aqueous phase was removed carefully by pipetting and transferred into a new microfuge tube, into which 200 µL isopropanol (Merck, South Africa) was then added. Samples were mixed by gentle inversion and incubated for 10 min at room temperature. RNA was pelleted by centrifugation at 4°C for 10 min at 20 000 x g. The supernatants were discarded and the RNA pellet was washed twice in 75 % (v/v) EtOH in diethyl pyrocarbonate (DEPC)-treated water (1:1000). Samples were air dried and RNA pellets were resuspended in 12 µL DEPC-treated water. RNA was precipitated by the addition of 24 µL 100% (v/v) EtOH and 1.2 µL 3M sodium acetate pH 5.5 followed by storage at -80°C for 30 min. RNA was then pelleted by centrifugation at 20 000 x g for 15 min at 4°C, washed in 75 % (v/v) EtOH in DEPC-treated water, air dried and resuspended in 12 µL DEPC-treated water. Following the clean-up procedure, RNA was quantified by spectrophotometry (NanoDrop Technologies) and integrity was measured by denaturing formaldehyde agarose gel electrophoresis (Sambrook and Russell 2006). Briefly, 500 ng RNA was mixed with sample loading buffer [12% (v/v) DEPC-treated water, 5% (v/v)

bromophenol blue, 7% (v/v) glycerol, 10% (v/v) 10X MOPS buffer (0.2 M MOPS in DEPC-treated water, 0.05 M sodium acetate, 0.01 M EDTA), 17% (v/v) 12.3 M formaldehyde and 49% (v/v) formamide] and 20 μg/mL EtBr. Samples were then electrophoresed on a 1% formaldehyde agarose gel [70% (v/v) DEPC-treated water, 10% (v/v) 10 x MOPS buffer, 20% (v/v) formaldehyde] in 1 X MOPS buffer (40 mM MOPS; 10 mM sodium acetate; 1 mM EDTA, pH 8.00) at 65V for 40 min. Samples were visualized under ultraviolet light on a Syngene, G:Box (Vacutec, England) and images acquired using GeneSnap version 7.08 (SynGene, England). RNA was stored at -80°C.

2.7.2 cDNA synthesis

cDNA was synthesized from 250 ng RNA using the Transciptor First Strand Synthesis cDNA kit (Roche Applied Science, South Africa) according to the manufacturer's instructions. This reverse transcription method is based on anchored oligo d(T) priming. Samples were stored at -20°C.

2.7.3 Quantitative real-time RT-PCR (qRT-PCR or qPCR)

Relative gene expression was determined by qPCR using 1 X SensiMix™ SYBR® no ROX master mix (QT650-05, Bioline, USA), forward and reverse primers (sequences and concentrations used are shown in Table 2.7.3) and 1 μL cDNA template in a 20 μL reaction. Samples were analysed on a RotorGene 3000 qPCR machine (Qiagen, Netherlands). Cycling conditions included an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 8 s, annealing (primer-specific annealing temperatures are indicated in Table 2.7.3) for 10 s and elongation for 8 sec at 72°C. Melting curve analysis and Ct determination were carried out by the RotorGene software (version 1.7). qPCR product samples were analysed by 2% agarose gel electrophoresis in 1 X TAE at 70 V for 1 h. The primer efficiency was determined by standard curves. mRNA transcript levels normalized to the housekeeping gene GAPDH were calculated using the Pfaffl method (Pfaffl 2001). Fold change was calculated relative to the vehicle control (EtOH) set to 1.

Table 2.7.3 Primers used for qPCR reactions, primer sequences, concentrations, annealing temperatures and product sizes

Primer	Sequence	Final conc.	Ta (°C)	Product size (bp)	Reference
GAPDH	F: 5'- TGAACGGGAAGCTCACTGG-3' R: 5'-TGTCAGTTGATAAAACCGCTGCC-3'	200 nM	60	307	(Ishibashi et al. 2003)
GILZ	Quantitect Primer QT00091035	1 X	60	69	Qiagen, Netherlands
IL-6	F: 5' - TCTCCACAAGCGCCTTCG - 3' R: 5' - CTCAGGGCTGAGATGCCG - 3'	250 nM	60	193	(Wolf et al. 2002)
IL-8	F: 5' - TGCCAAGGAGTGCTAAAG - 3' R: 5' - CTCCACAACCCTCTGCAC - 3'	250 nM	60	197	(Wolf et al. 2002)
RANTES	F: 5' - TACCATGAAGGTCTCCGC - 3' R: 5' - GACAAAGACGACTGCTGG - 3'	250 nM	60	199	(Wolf et al. 2002)
IL-10	F: 5'-AAAGGCATCTACAAAGCCA-3' R: 5'-TTGTCATGTAGGCTTCTATGTAGT-3'	500 nM	55	67	(Louw-du Toit et al. 2014)
IL-12p35	F: 5'-TGATGAGCTGATGCAGGC-3' R: 5'-ATCCGGTTCTTCAAGGGAG-3'	500 nM	56	73	(Louw-du Toit et al. 2014)
IL-12p40	F: 5'-CCACATTCCTACTTCTCCC-3' R: 5'-CTTGTCCGTGAAGACTCTAT-3'	500 nM	51	87	(Louw-du Toit et al. 2014)
MKP1	F: 5'-AGTACCCCACTCTACGATCAGG-3' R: 5'-TGATGGAGTCTATGAAGTCAATGG-3'	250 nM	55	250	(Rauhala et al. 2005)
CCR5	F: 5' TGGACCAAGCTATGCAGGTG 3' R: 5' CGTGTCACAAGCCCACAGAT 3'	500 nM	55	240	M. Maritz, unpublished
CD4	F: 5'-GGGGATACAGTGGAACTGACC-3' R: 5'-TCCCAAAGGCTTCTTCTTGAG-3'	500 nM	55	164	(Eszterhas et al. 2011)
CXCR4	F: 5'GAAATGGGCTCAGGGGACTAT 3' R: 5' TTCAGCCAACAGCTTCCTTGG 3	500 nM	60	417	M. Maritz, unpublished

2.8 HIV-1 infection assays

The R5 infectious molecular clone used in this study, HIV-$1_{BaL\_Renilla}$, was a gift from Dr Christina Ochsenbauer (Edmonds et al. 2010). This virus was created by insertion of a luciferase gene next to *env* in the HIV-1 NL4-3 backbone, NL-LucR.T2A-BaL.ecto (Edmonds et al. 2010).

2.8.1 Preparation of virus and determination of viral titres

Viral stocks were prepared by transfection in HEK293T cells as previously described (Pear et al. 1993), with a few modifications. HEK293T cells were seeded at 4 X 10^6 cells per 10 cm dish in full DMEM supplemented with 25 mM HEPES buffer (Lonza, Germany) for 24 h. Cells were then transfected with 12 μg HIV-$1_{BaL\text{-}Renilla}$ or DMEM as a negative no-virus control, for 48h, using XtremeGENE-9 transfection reagent (Roche Applied Science, South Africa). The media was collected and passed through a 0.22 μM filter, after which cs-FCS (Thermo Scientific, USA) was added to a final concentration of 12.5%. Aliquots of the viral stock and no-virus control were stored at -80°C.

The viral titres were determined in TZM-bl cells as previously described (Edmonds et al. 2010). Briefly, TZM-bl cells were seeded at 2.5 X 10^4 cells/well in a 96-well plate in full DMEM for 24 h. The virus was serially diluted 1:5 in full phenol red-free DMEM (Sigma Aldrich, South Africa) for 8 dilutions in a separate 96-well plate. The media was removed from the TZM-bl cells and the diluted virus, or the no-virus control, was then added in quadruplicate wells. After 72 h the cells were harvested in 70 μL/well Bright-Glo luciferase lysis buffer (Promega, USA) in the dark for 5 min. Samples were transferred into a white 96-well plate (Greiner, Germany) and fluorescence in RLU was measured on a luminometer (Modulus microplate, Promega, USA). The viral titre was calculated as log infectious units (IU)/mL using the Reed and Muench method (Reed and Muench 1938). The typical viral titre ranged from 1 X 10^4-4 X 10^5 IU/mL.

2.8.2 PBMC infection assay

Owing to the wide inter-individual variability in HIV-1 infection observed in PBMC donors, PBMCs were pre-screened for infectability prior to hormone plus infection assays. PBMCs were seeded immediately after isolation (as described in 2.4.2) at 2 X 10^5 cells/well in U-bottom 96-well plates. Aliquots of PBMCs were frozen at -80°C in full RPMI containing 5% cs-FCS until infectability was determined using the Reed and Muench method (Reed and Muench 1938).

The following day, the virus was serially diluted as for the TZM-bl assay described above. The media was removed from pelleted PBMCs and the diluted virus was then added in quadruplicate wells. Control cells (no-virus control) underwent mock infection by incubation with diluted media collected from mock-transfected HEK293T cells. After infection for 2 h at 37°C, the cells were washed three times in 1 X PBS containing 1% cs-FCS, resuspended in full RPMI containing IL-2 and incubated for a further 5 days. PBMCs were pelleted by centrifugation at 1200 rpm for 5 min then harvested for *Renilla* luciferase expression (infection) using *Renilla* luciferin according to the manufacturer's specifications (Promega, USA) with a few modifications. Briefly, PBMCs were resuspended in 70 µL/well 1 X reporter lysis buffer with *Renilla* luciferin after incubation for 10 min in the dark at room temperature. Lysed cells were transferred into a white 96-well plate (Greiner, Germany) and fluorescence in RLU was measured on a luminometer (Modulus microplate, Promega, USA). Infection was determined using the Reed and Muench method (Reed and Muench 1938).

For experiments, infectable PBMCs were thawed from frozen stock, seeded at 4 X 10^6 in 5 mL FACS tubes and stimulated with the indicated ligands for 2 or 7 days. PBMCs were infected with 10 IU/mL HIV-$1_{BaL\text{-}Renilla}$ or the equivalent volume of no-virus control for 2 h at 37°C, followed by three washes in 1 X PBS containing 1% cs-FCS. Cells were then resuspended in full RPMI containing IL-2. Five days post-infection, cells were seeded into two U-bottom 96-well plates in quadruplicate wells. Cells on the first plate were pelleted by centrifugation at 1200 rpm for 5 min then harvested for *Renilla* luciferase expression as described above. Cells on the second plate were analysed for cell viability using the MTT assay. Briefly, cells were incubated with MTT solution (5 mg/mL MTT in PBS, filter sterilized) to a final concentration of 0.5 mg/mL for 2 h in an incubator at 37°C. Cells were pelleted by centrifugation at 1200 rpm for 5 min then resuspended in 70 µL/well Solubilisation solution (0.1 N HCl in isopropanol) and transferred into a new 96-well plate. Absorbance was measured at 595 nm on a spectrophotometer (Thermo Scientific, USA). Infection was calculated as RLU of each quadruplicate divided by the average absorbance at 595 nm of the four quadruplicate wells (RLU/MTT). Relative infection was determined by setting the vehicle control (EtOH) in the presence of virus to 100% or as indicated in the figure legend.

2.8.3 TZM-bl infection assay

TZM-bl cells were seeded in quadruplicate wells at 2.5 X 10^4 cells/well in two 96-well plates and allowed to adhere overnight. Cells were then stimulated with the indicated ligands in phenol red-free SF-DMEM for 48 h at 37°C in an incubator. The media was removed and cells were incubated with 10 IU/mL HIV-$1_{BaL\text{-}Renilla}$ or the equivalent volume of no-virus control (prepared as in 2.8.1) in phenol red-free RPMI. After 72 h the cells on one plate were harvested in Bright-Glo luciferase lysis buffer

as described in 2.8.1 and cells on the other plate were harvested for cell viability using the MTT assay as described in 2.8.2. Infection as RLU/MTT was calculated as for PBMCs (2.8.2). Relative infection was determined by setting the vehicle control (EtOH) in the presence of virus to 100% or as indicated in the figure legend.

2.8.4 Media add-back onto TZM-bl cells infection assay

The effect of soluble factors secreted into growth media from cervical cell lines on HIV-1 infectivity in indicator TZM-bl cells was measured in a media add-back assay based on a previous protocol (Patel et al. 2014). TZM-bl cells were seeded at 2.5 X 10^4 cells/well in two 96-well plates for 24 h. Supernatants from knockdown and over-expression experiments in End1/E6E7 and HeLa cells were collected at the time of harvest and stored at -80°C. These supernatants were diluted 1:20 in phenol-free DMEM prior to use. Half of the diluted supernatants (100 µL) was incubated on the TZM-bl cells for 1 h at 37°C, while the other half of diluted supernatants was incubated with 10 IU/mL HIV-$1_{BaL\text{-}Renilla}$ or the equivalent volume of no-virus control in phenol red-free DMEM for 1 h at 37°C. After this incubation, the control/virus-supernatant mixture was added to TZM-bl cells in the corresponding wells. After 72 h the cells on one plate were harvested in Bright-Glo luciferase lysis buffer as described in 2.8.1 and cells on the other plate were harvested for cell viability using the MTT assay as described in 2.8.2. Infection as RLU/MTT was calculated as for PBMCs (2.8.2). Relative infection was determined by setting the vehicle control (EtOH) in the presence of virus to 100% or as indicated in the figure legend.

2.9 Immunofluorescence

2.9.1 Slide preparation

COS1 cells were seeded onto sterilized, acid-washed coverslips at 1.4 X 10^5 cells/well in 6-well plates and allowed to adhere for 24 h. Cells were then transfected with 125 ng each pcDNA3.1-hGR, pSG5-hPR-A and pMT-hPR-B using XtremeGENE-9 transfection reagent for 24 h, then stimulated with the indicated ligands for a further 24 h in SF-DMEM. Cells were then washed with ice cold 1 X PBS and fixed with ice cold methanol at -20°C for 10 min. After fixing, cells were washed again three times with cold 1 X PBS for 5 min, then blocked with 5% (w/v) BSA in PBS for 1 h at room temperature. Cells were then incubated with 1:250 anti-GR (H300, Santa Cruz Biotechnology), and 1:1000 anti-PR (NCL-LPGR-312, Leica Biosystems) antibodies diluted in 5% (w/v) BSA-PBS in a humidified chamber for 1 h at room temperature, followed by three washes in 1 X PBS for 5 min each. The

highest dilutions of antibodies that generated suitable fluorescent signals were established during pilot experiments. Cells were then incubated with 1:500 Alexa488-labelled anti-rabbit (to detect GR) and 1:1000 Cy 3-labelled anti-mouse (to detect PR) antibodies in 5% (w/v) BSA-PBS in a humidified chamber in the dark for 1h at room temperature. Cells were washed three times in 1% (w/v) BSA-PBS for 5 min each, followed by staining with Hoechst in PBS (100 μg/mL) for 5 min. Coverslips were subsequently mounted onto glass slides with 45 μL mowiol (Calbiochem, Merck, South Africa) and dried overnight in the dark at room temperature. Slides were stored in the dark at 4°C prior to fluorescence visualization.

2.9.2 Confocal microscopy

Slides of COS1 cells from 3 independent experiments prepared in 2.9.1 were imaged at the Confocal and Light Microscope Unit, Department of Human Biology, University of Cape Town, using a Zeiss Axiovert 200M LSM 510 Meta NLO Confocal Microscope. Six to eight representative fields of view per condition (Vehicle, Dex, R5020, MPA) were captured, with approximately 1-30 cells per field of view. These images were analyzed for co-localization using Carl Zeiss ZEN software (Version 2009), and Manders correlation and overlap coefficients were recorded (Manders et al. 1993). The data represent the mean and SEM of all the transfected cells from all fields of view from the 3 independent experiments.

2.9.3 Super-resolution structured illumination microscopy (SR-SIM)

Slides of COS1 cells from 3 independent experiments prepared in 2.9.1 were re-imaged at the Central Analytical Facility Fluorescence Microscopy Unit at the University of Stellenbosch using a Zeiss LSM 780 ELYRA S1 microscope. Two to four representative high-resolution fields of view per condition (Vehicle, Dex, R5020, MPA) and 8-20 z-stacks (0.1 μm) per field of view were captured. Channel alignment was carried out using fluorescent beads #1783-455 (Tool for Calibration Multip Spek, Carl Zeiss Microscopy Gmbh) in order to ensure accurate analysis of co-localization. Images were recreated using a structured-illumination algorithm in ZEN 2011 (Carl Zeiss Microscopy Gmbh). Single channel images captured using ZEN 2011 were converted to .tiff files for a representative z-stack per image and these files were then processed into 8-bit format using ImageJ. Co-localization analysis was carried out using the Just Another Co-localization Plug-in (JACoP) tool in ImageJ (Bolte and Cordelieres 2006). The data represent the mean and SEM of all the fields of view per condition, per independent experiment.

2.10 Statistical analysis

All statistical tests were performed using GraphPad Prism software (version 7) and are indicated, as well as n values indicating number of independent experiments, in the figure legends,. All data were first analysed for normal distribution using a D'Agostino and Pearson normality test for experiments with n values of 8 or more, or a Shapiro Wilk normality test for experiments with n values less than 8. Normally distributed data were analyzed using either one-way ANOVA with either Dunnett's (compared to control sample) or Tukey's (compared to every other sample) post tests for multiple comparisons or unpaired t tests for comparisons between two samples. Non-normally distributed data was analysed using a non-parametric Kruskal-Wallis test (non-parametric one-way ANOVA) with Dunn's post-test (compared to control sample) for multiple comparisons or a Wilcoxon signed-rank test for comparisons between two samples. For some molecular data such as western blot densitometry and pilot experiments (Appendix B), less stringent tests (unpaired t tests or Wilcoxon signed-rank tests) were used. All grouped data sets, that is where each condition was in triplicate or quadruplicate, were analyzed using two-way ANOVA with either Dunnett's or Tukey's post tests for multiple comparisons, since this version of GraphPad Prism does not offer a non-parametric two-way ANOVA. Graphs are plotted as mean ± SEM and the variation between independent experiments is represented by the spread of the black dots, indicating individual experiments.

Chapter 3

Both MPA used in injectable contraception and exogenous hormones mimicking different phases of the menstrual cycle increase HIV-1 infection, but via potentially different mechanisms

3.1 Background and aims

Since MPA is derived from P_4 and was designed to imitate the biological actions of P_4, MPA prevents pregnancy by mimicking the high-P_4 state of the luteal phase of the menstrual cycle. Both MPA and the luteal phase have been linked to increased susceptibility to HIV-1 infection. However, there is limited research directly comparing the effects of both menstrual cycle hormones and MPA on HIV-1 infection.

Therefore, the aims of this section were to answer the following research questions:

(i) Do exogenous hormones mimicking the menstrual cycle phases or contraception with MPA increase HIV-1 infection?

(ii) Do hormones mimicking the menstrual cycle phases or contraception with MPA affect regulation of biomarkers likely relevant to HIV-1 infection and immune function?

- Do menstrual cycle hormones and MPA differentially regulate
  - immune cell populations and their activation?
  - co-receptor expression?
  - immunomodulatory gene expression?

(iii) Are the effects on HIV-1 infection between menstrual cycle hormones and MPA mediated by the GR?

- Do hormones mimicking the menstrual cycle phases or contraception with MPA change GR levels, thereby increasing HIV-1 infection?

To answer these questions, human PBMCs were used as a model for the circulatory system and TZM-bl human as a model of infectable cervical cells. These cells were stimulated with exogenous hormone combinations of E_2 and P_4 to mimic the phases of the menstrual cycle – referred to hereafter as "luteal phase E_2/P_4" (10 nM P_4 + 400 pM E_2), or "follicular phase E_2/P_4" (1 nM P_4 + 400 pM E_2), – or with 100 nM MPA to represent near-peak serum levels of contraception with the injectable DMPA. Cells were infected with the R-tropic HIV-1 infectious molecular clone, HIV-$1_{BaL\_Renilla}$ using luciferase assays as a proxy for HIV-1 infection.

3.2 Luteal phase E_2/P_4 and MPA increase HIV-1 infection in PBMCs

In order to assess the effect of menstrual cycle hormones or MPA on systemic HIV-1 infectivity, PBMCs from female donors stimulated with exogenous E_2/P_4 mimicking the luteal phase or follicular phase or with exogenous MPA for 2 or 7 days were infected with HIV-$1_{BaL\_Renilla}$. A significant 3-fold increase in HIV-1 infection was observed comparing the absence (no-virus control) to the presence of virus (HIV-$1_{BaL\_Renilla}$) (Appendix B, Fig. B1.1). Since there were no differences in relative luciferase activity between the no-virus control for each stimulation condition at either day (Appendix B, Fig. B1.1), only the vehicle control from Day 2 is shown as a representative in Fig. 3.2.1.

Stimulation with luteal phase E_2/P_4 significantly increased HIV-1 infection at 7 days (5.3-fold, Fig 3.2.1). Stimulation with MPA significantly increased HIV-1 infection at both 2 (12.3-fold) and 7 days (7.4-fold), with a greater fold increase than for the menstrual cycle phases (at 2 days: 5.4-fold greater than luteal phase, 5-fold greater than follicular phase; at 7 days: 1.4-fold greater than luteal phase, 12-fold greater than follicular phase; Fig. 3.2.1). HIV-1 infection for the vehicle control at 7 days was significantly greater than the vehicle control at 2 days, thereby indicating productive infection was achieved (Appendix B, Fig. B1.2).

The large variability in relative infection in PBMCs was not due to changes in cell viability but appears to reflect inherent biological variation in infection between experiments (Appendix B, Table B1).

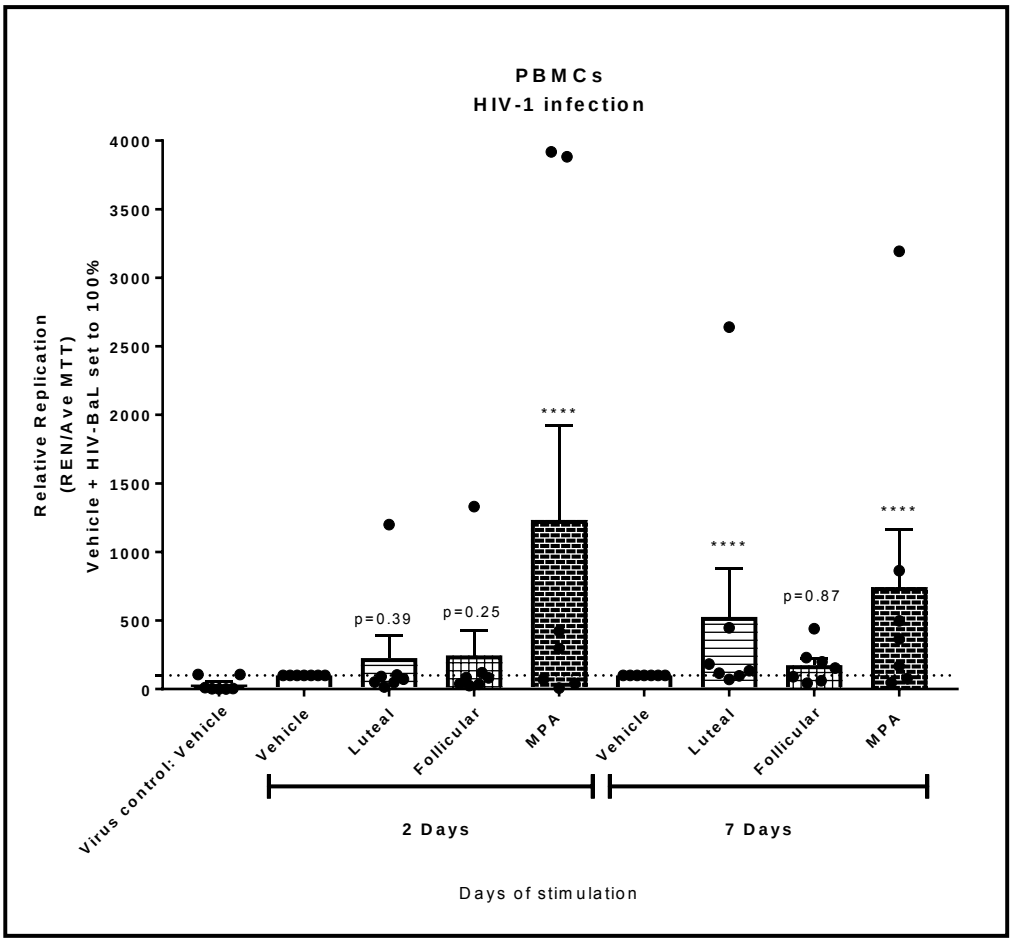


Figure 3.2.1. HIV-1 infection in PBMCs increases in response to luteal phase E_2/P_4 and MPA. PBMCs were seeded at 4 X 10^6 in FACS tubes and stimulated for 2 or 7 days with hormones representing the luteal phase (10 nM P_4 + 400 pM E_2), follicular phase (1 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM). PBMCs were subsequently infected with 10 IU/mL HIV-$1_{BaL\_Renilla}$ (HIV-BaL) or equivalent volume of no-virus control (labelled as Virus control) for 2 h, washed and re-suspended in ligand-free full RPMI for 5 days. Cells were re-seeded in round-bottom 96 well plates in quadruplicate wells per condition at 2 X 10^5 cells/well and harvested for *Renilla* luciferase (infection) and for cell viability (MTT). The results show pooled data from at least 3 independent experiments, with 2-3 PBMC donors each, for a total of 7 independent donors. Relative infection was calculated as *Renilla* luciferase (RLU) divided by average absorbance at 595nm (MTT) for the quadruplicate wells. Infection was plotted relative to each day's vehicle control in the presence of virus set to 100%. Statistical comparisons were carried out using a two-way ANOVA with Dunnett's multiple comparisons post test. Stars above bars indicate significance compared to own vehicle control in the presence of virus, with **** indicating $p<0.0001$.

3.3 Menstrual cycle phase hormones and MPA differentially regulate markers likely relevant to HIV-1 infection and immune function

Since both luteal phase E_2/P_4 and MPA significantly increased HIV-1 infection in PBMCs, with MPA increasing HIV-1 infection to a greater extent, the potential mechanisms of infection were further investigated in PBMCs and TZM-bl cells. Owing to their established or suggested roles in HIV-1 infection, the variables investigated were (i) the frequency of HIV-1 target immune cell populations in PBMCs, (ii) the expression of the CD4 receptor and co-receptors CCR5 and CXCR4, (iii) a marker for early immune activation, CD69, and (iv) the expression of the pro-inflammatory cytokine IL-6.

3.3.1 Menstrual cycle phase hormones and MPA differentially regulate the frequency of immune cell populations and their activation in PBMCs

The frequency of CD3+, CD4+ and CD8+ T-lymphocytes and CD14+ monocytes and the density i.e. expression of these receptors was assessed by flow cytometry in PBMCs stimulated for 7 days with MPA or luteal/follicular phase E_2/P_4. Additionally the frequency and expression of the activation marker CD69 was investigated within these immune cell populations. The ratio of CD4+ to CD8+ cell frequency was calculated for total cells, CD69+ and CCR5+ cells. The combined raw data (mean, SEM) for the frequency of cells expressing CD3, CD4, CD8, CD14, CD69, CCR5 and CD4/CD8 ratio, and expression of CD3/4/8/14, CD69 and CCR5 on T cells and monocytes are shown in Table 3.3.1.1 and 3.3.1.2, respectively.

Stimulation with MPA, unlike luteal or follicular phase E_2/P_4, significantly increased the frequency of CD4+ T-lymphocytes 1.1-fold and decreased the frequency of CD8+ T-lymphocytes 1.35-fold (Fig. 3.3.1.1a). However none of the hormone combinations changed the frequency of CD3+ or CD14+ cells (Fig. 3.3.1.1a, Table 3.3.1.1) nor the frequency of CD69-expressing T-cells, but MPA significantly decreased the frequency of activated CD69+CD14+ monocytes (Fig. 3.3.1.1b, Table 3.3.1.1). MPA, but not luteal/follicular phase E_2/P_4, significantly increased the CD4/CD8 ratio 1.5-fold in CD3+ cells (Fig. 3.3.1.1c) and CD69+CD3+ cells (Fig. 3.3.1.1d).

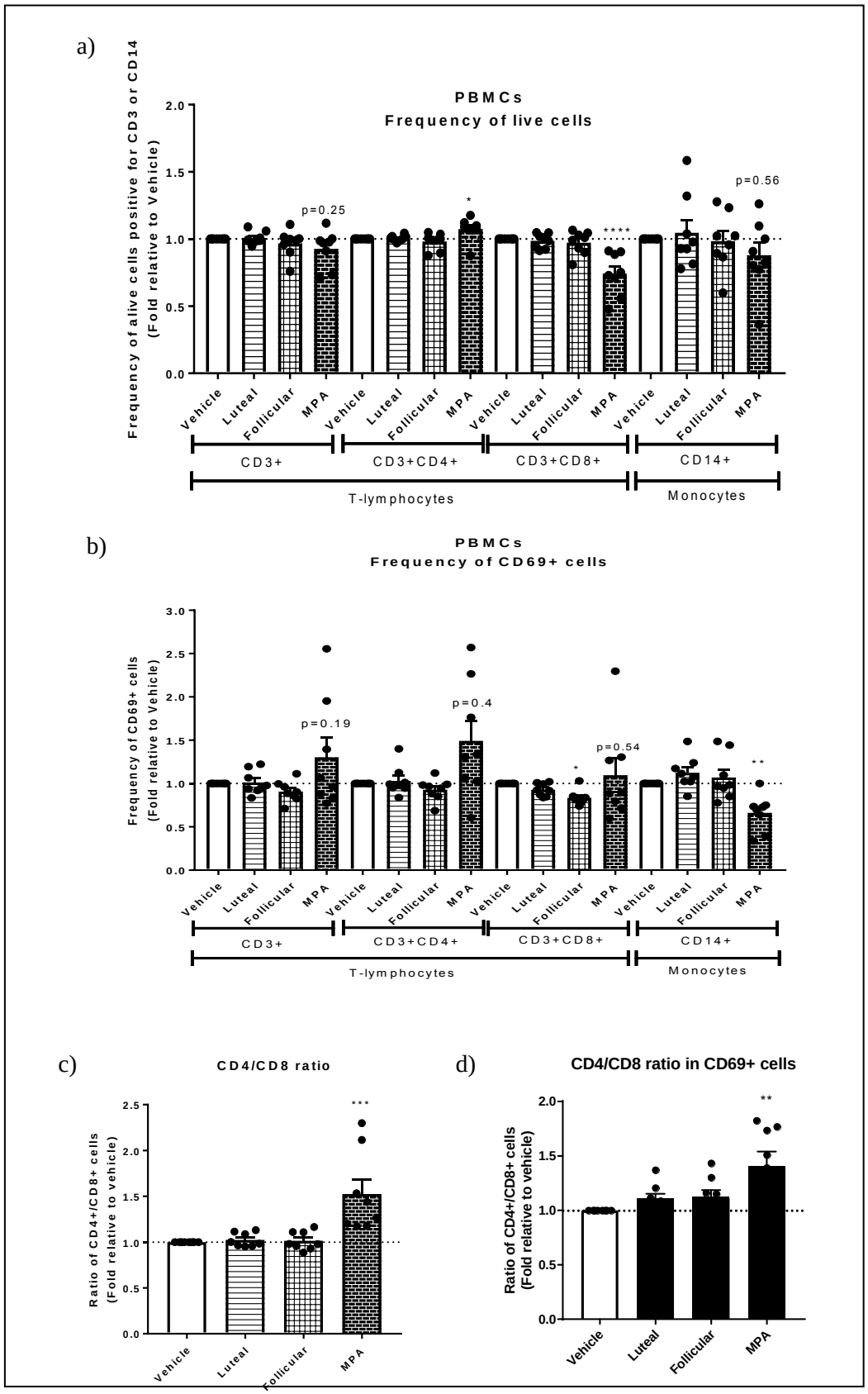


Figure 3.3.1.1. MPA, unlike luteal or follicular phase E_2/P_4, increases the frequency of CD4+ T cells, decreases the frequency of total CD8+ T cells and activated CD14+CD69+ monocytes in PBMCs, and increases the CD4/CD8 ratio in total and CD69+ cells, while follicular phase E_2/P_4 decreases the frequency of activated CD8+CD69+ T cells. PBMCs were seeded at 4 X 10^6 in FACS tubes and stimulated for 7 days with hormones representing the luteal phase (10 nM P_4 + 400 pM E_2), follicular phase (1 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM). PBMCs were

subsequently stained with conjugated antibodies specific for the relevant markers, fixed and then sorted by flow cytometry. Samples were gated as shown in Appendix C to exclude dead cells and separating cell populations using Fluorescence Minus One (MFO) controls for CD14, CCR5 and CD69. The results are pooled from 2 independent experiments, with 4 donors each, for a total of 8 independent donors. Data are plotted as frequency of cell populations (a), frequency of CD69+ cell populations (b), or relative ratio of CD4+ to CD8+ cell frequency for total (c) or CD69+ (d) cells, and are plotted relative to the vehicle control set to 1. Statistical comparisons were carried out using a parametric one-way ANOVA with Dunnett's multiple comparisons post test (a: CD3, CD8, CD14; b: CD3, CD14; c; d) or a non-parametric Kruskal-Wallis test with Dunn's multiple comparisons post test (a: CD4; b: CD4, CD8). Stars above bars indicate significance compared to own vehicle control, with ****, ***, ** and * indicating $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

Whereas the frequency looks at the percentage of cells expressing at least one of the above markers, expression levels account for the number (or density) of markers expressed between the immune cell populations. The expression of these same markers was calculated from the median fluorescent intensity score per number of cells expressing the relevant markers, generated by FlowJo software.

MPA, unlike the menstrual cycle hormones, significantly decreased the expression of CD3, CD4 and CD8, while no change was observed for CD14 (Fig. 3.3.1.2a). For the activation marker CD69, no differences were observed with luteal or follicular phase E_2/P_4, but stimulation with MPA significantly increased the expression of CD69 in CD4+ T-lymphocytes 1.14 fold (Fig. 3.3.1.2b).

Together these results suggest that increased HIV-1 infection with MPA could be due to increased frequency of CD3+CD4+ T-cells and decreased frequency of CD3+CD8+ T-cells (that is, increased CD4/CD8 ratio) and increased activation of CD4+ T cells through CD69 expression, thereby enhancing their potential for infection.

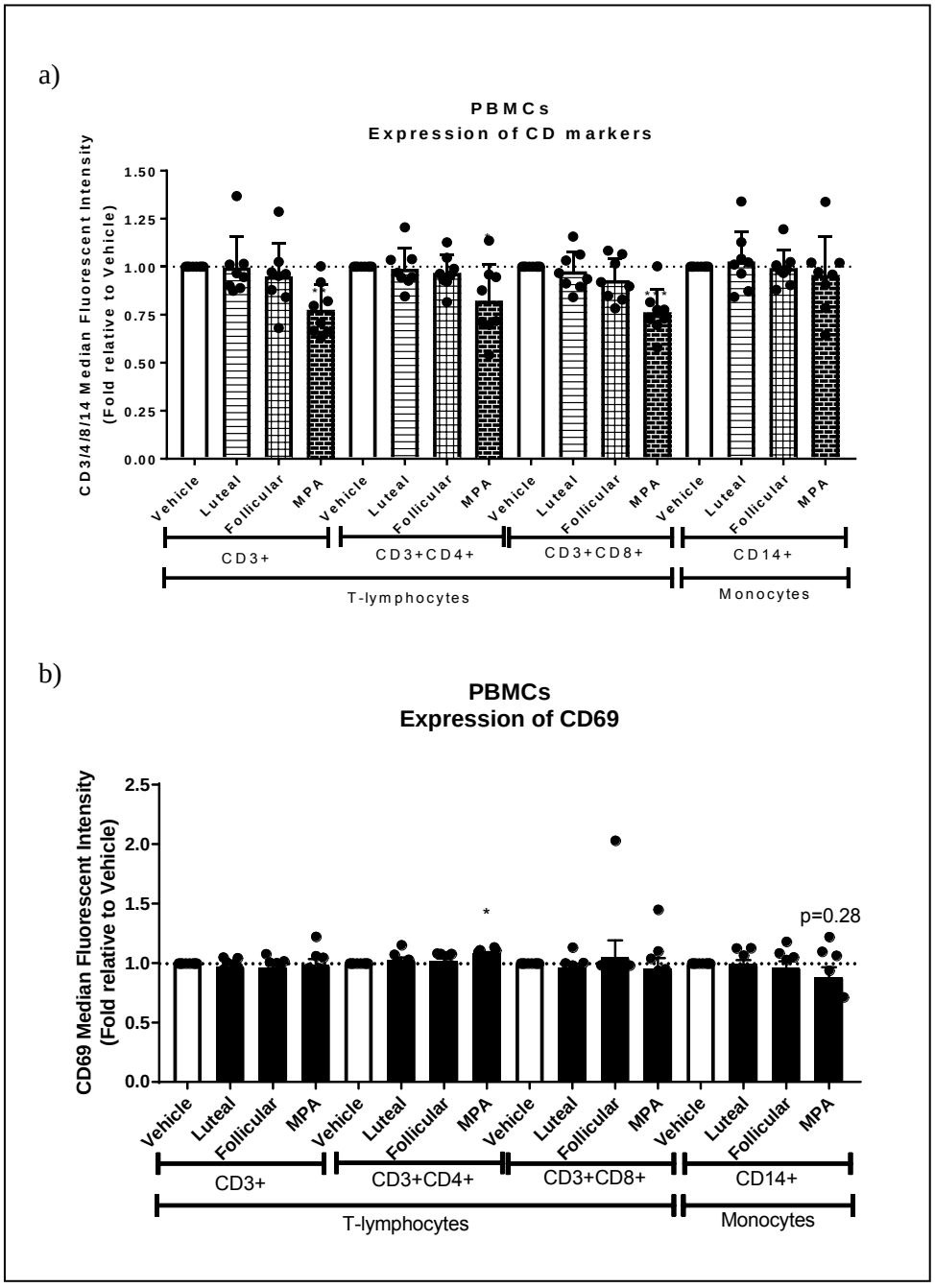


Figure 3.3.1.2. MPA, unlike luteal or follicular phase E_2/P_4, decreases CD3, CD4 and CD8 expression, but increases the expression of CD69 in activated CD4+CD69+ T-lymphocytes in PBMCs. PBMCs were seeded at 4 X 10^6 in FACS tubes and stimulated for 7 days with hormones representing the luteal phase (10 nM P_4 + 400 pM E_2), follicular phase (1 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM). PBMCs were subsequently stained, fixed and then sorted by flow cytometry. Samples were gated and analyzed as per Appendix C. The results are pooled from 2 independent experiments, with 4 donors each, for a total of 8 independent donors. CD3, 4, 8 or 14 (a) or CD69 (b) expression (MFI) was plotted relative to the vehicle control set to 1. Statistical comparisons were carried out using a parametric one-way ANOVA with Dunnett's multiple comparisons post test (a: CD4, CD8; b: CD3, CD4, CD14) or a non-parametric Kruskal-Wallis test with Dunn's multiple comparisons post test (a: CD3, CD14; b: CD8). Stars above bars indicate significance compared to own vehicle control, with ***, ** and * indicating $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

3.3.2 Menstrual cycle phase hormones and MPA differentially regulate CCR5 co-receptor expression in PBMCs and TZM-bl cells

The frequency of CD3+, CD4+ and CD8+ T-lymphocytes and CD14+ monocytes expressing the HIV-1 co-receptor CCR5, and expression levels of CCR5 on these cells were also assessed by flow cytometry in PBMCs stimulated for 7 days with luteal or follicular phase E_2/P_4 or MPA.

Stimulation with MPA, unlike luteal or follicular phase E_2/P_4, showed a 2-fold numerical increase in the frequency of CCR5-expressing CD3+ and CD4+ cells that did not reach statistical significance (Fig. 3.3.2.1a). The CD4/CD8 frequency ratio in CCR5+ cells was not significantly different between the hormone stimulations and the vehicle control (Fig. 3.3.2.1b).

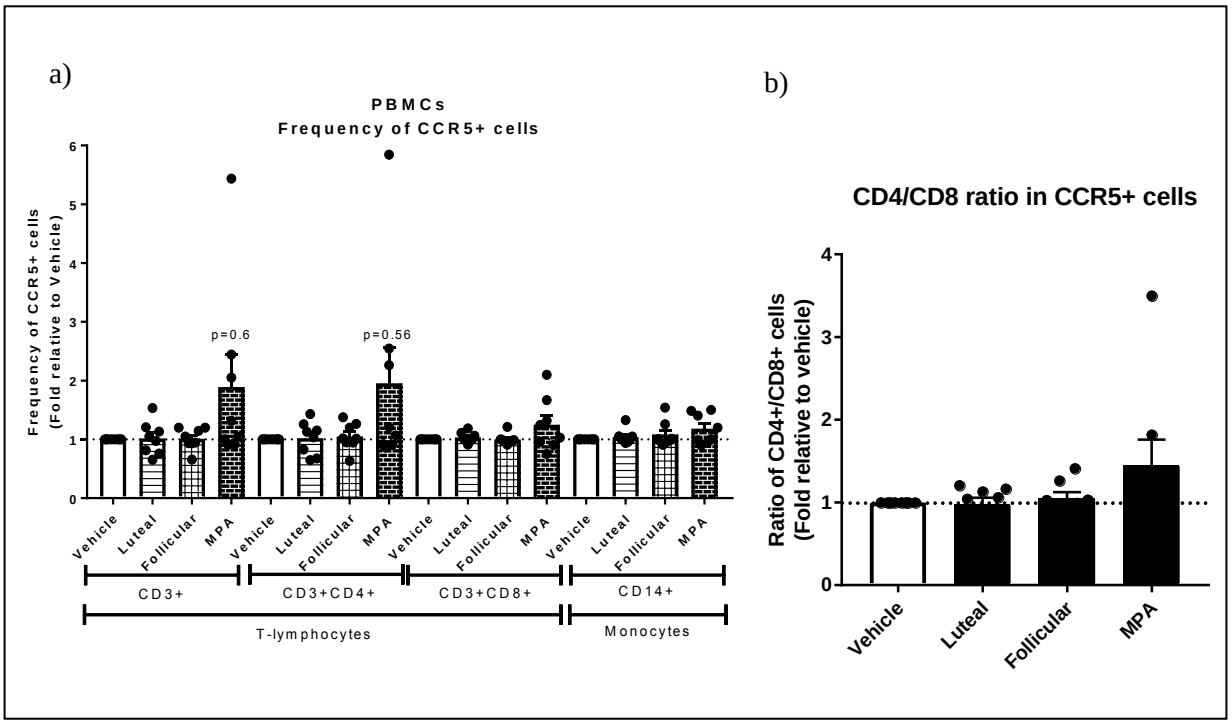


Figure 3.3.2.1. MPA, unlike luteal or follicular phase E_2/P_4, appears to increase the frequency of CCR5+ cells in PBMCs. PBMCs were seeded at 4 X 10^6 in FACS tubes and stimulated for 7 days with hormones representing the luteal phase (10 nM P_4 + 400 pM E_2), follicular phase (1 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM). PBMCs were subsequently stained, fixed and then sorted by flow cytometry. Samples were gated and analyzed as per Appendix C. The results are pooled from 2 independent experiments, with 4 donors each, for a total of 8 independent donors. Frequency of CCR5+ cell populations (a) and relative ratio of CD4+ to CD8+ cell frequency for CCR5+ cells (b) was plotted relative to the vehicle control set to 1. Statistical comparisons were carried out using a non-parametric Kruskal-Wallis test with Dunn's multiple comparisons post test. No significant differences were observed, but p values above bars indicate a comparison to each vehicle control.

Stimulation with MPA, unlike luteal or follicular phase E_2/P_4, significantly increased the expression of the HIV-1 co-receptor CCR5 in CD4+ (1.32-fold) and CD3+ T-lymphocytes (1.44-fold, Fig. 3.3.2.2). Although not significant, there appeared to be a trend for increased CCR5 expression in CD8+ T-lymphocytes (2 donors reaching 2-fold) (Fig. 3.3.2.2). CCR5 expression was not altered in CD14+ monocytes (Fig. 3.3.2.2).

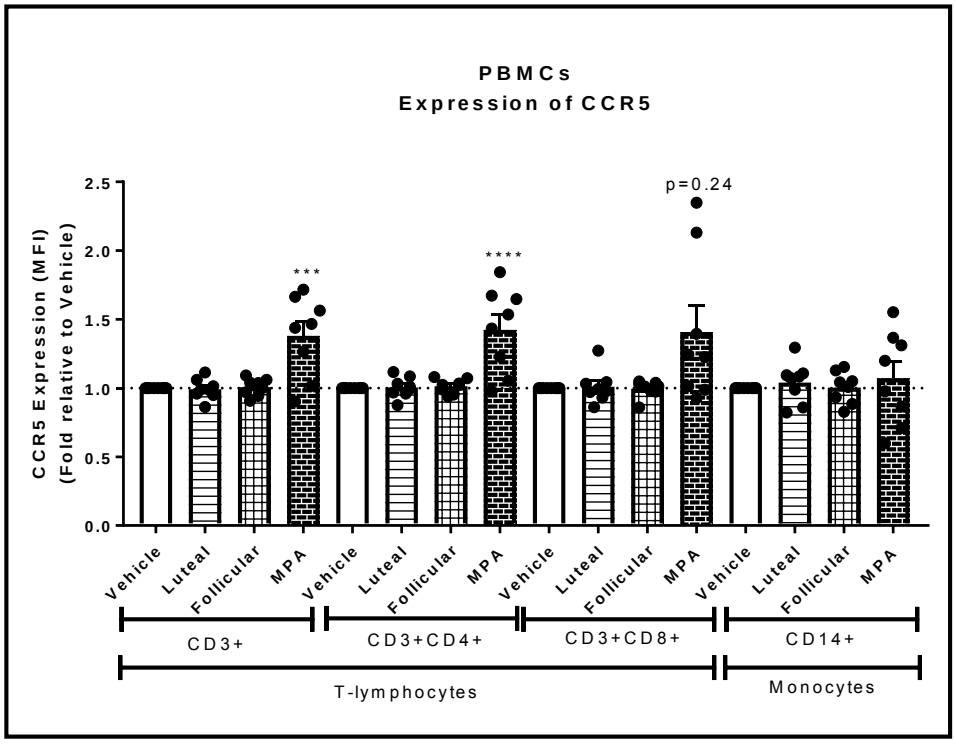


Figure 3.3.2.2. MPA, unlike luteal or follicular phase E_2/P_4, increases the expression of CCR5 in CD3+, CD4+ and CD8+ T-lymphocytes in PBMCs. PBMCs were seeded at 4 X 10^6 in FACS tubes and stimulated for 7 days with hormones representing the luteal phase (10 nM P_4 + 400 pM E_2), follicular phase (1 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM). PBMCs were subsequently stained, fixed and then sorted by flow cytometry. Samples were gated and analyzed as per Appendix C. The results are pooled from 2 independent experiments, with 4 donors each, for a total of 8 independent donors. CCR5 expression (MFI) was plotted relative to the vehicle control set to 1. Statistical comparisons were carried out using a parametric one-way ANOVA with Dunnett's multiple comparisons post test (CD3, CD4, CD14) or non-parametric Kruskal-Wallis test with Dunn's multiple comparisons post test (CD8). Stars above bars indicate significance compared to own vehicle control, with **** and *** indicating $p<0.0001$ and $p<0.001$, respectively.

Table 3.3.1.1. Frequencies of total, CD69+ and CCR5+ cells expressing the markers CD3, CD4, CD8 (T-lymphocytes) or CD14 (monocytes).

Immune cell phenotype: Frequency	Vehicle		Luteal		Follicular		MPA	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
CD3+ (% total)	79.70	3.57	79.98	3.10	76.71	3.75	73.13	3.90
CD3+CD69+	5.63	0.96	5.73	1.07	5.05	0.88	6.16	0.53
CD3+CCR5+	23.44	5.23	24.66	5.61	23.36	4.81	32.24	4.06
CD4+ (% total)	63.43	4.63	63.84	4.75	62.06	4.57	**67.43**	4.13
CD4+CD69+	6.84	0.92	6.98	0.92	6.16	0.52	9.44	1.32
CD4+CCR5+	21.03	4.47	22.68	5.27	21.71	4.32	28.98	3.55
CD8+ (% total)	27.35	4.03	27.13	4.29	26.83	4.38	**21.37**	4.37
CD8+CD69+	6.38	0.97	5.98	1.03	**5.31**	0.78	6.33	0.82
CD8+CCR5+	47.55	8.44	48.91	8.64	47.24	8.12	53.15	6.57
CD14+ (% total)	36.81	13.52	34.14	12.43	35.38	12.98	35.05	13.19
CD14+CD69+	29.98	1.73	33.11	1.32	31.71	2.79	**19.19****	1.81
CD14+CCR5+	72.06	8.93	72.91	7.75	75.05	7.4	79.60	5.14
CD4/CD8 ratio (total)	3.16	0.94	3.23	0.93	3.18	0.90	**4.84**	1.28
CD4/CD8 ratio: CD69+	1.33	0.35	1.41	0.33	1.41	0.30	**1.65**	0.24
CD4/CD8 ratio: CCR5+	0.43	0.05	0.44	0.07	0.47	0.08	0.58	0.11

Mean and SEM are shown as percentages, not normalized to fold change, and were calculated from 8 independent donors. CD69+ and CCR5+ cells are frequencies of the cells expressing CD3, CD4, CD8 or CD14. Statistical comparisons were carried out using either a parametric one-way ANOVA with Dunnett's post test (CD3+CD69+, CD3+CCR5+, CD4+, CD8+, CD8+CD69+, CD8+CCR5+, CD14+CD69+) or a non-parametric Kruskal-Wallis test with Dunn's post test (CD3+, CD4+CD69+, CD4+CCR5+, CD14+, CD14+CCR5+, CD4/CD8-total, CD4/CD8-CD69, CD4/CD8-CCR5). Stars next to values indicate significance compared to own vehicle control, with ** indicating $p<0.01$. Where the fold changes (relative to vehicle) were significantly different, as shown in Fig. 3.3.1.1 and 3.3.2.1, the values in the table are in bold. ** $p<0.01$.

Table 3.3.1.2. Expression of CD69 and CCR5 in cells expressing the markers CD3, CD4, CD8 (T-lymphocytes) or CD14 (monocytes).

Immune cell phenotype: Expression	Vehicle		Luteal		Follicular		MPA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CD3	9039.00	476.50	8915.00	461.30	8535.00	602.20	**6946.00***	422.90
CD3+CD69+	4178.00	431.60	4056.88	451.60	4028.75	444.60	4023.50	340.00
CD3+CCR5+	2609.25	142.70	2579.63	128.50	2621.75	140.40	**3551.38****	245.00
CD4	8333.00	596.90	8241.00	659.40	8098.00	690.00	**6659.00**	358.90
CD4+CD69+	2170.75	234.00	2206.63	219.00	2196.00	242.00	**2328.38**	240.20
CD4+CCR5+	2496.38	104.00	2516.50	149.60	2539.25	140.30	**3533.63****	278.50
CD8	44095.00	8157.00	43658.00	8328.00	42219.00	8429.00	**32038.00**	5220.00
CD8+CD69+	4972.75	550.60	4770.13	524.00	5032.38	590.60	4499.50	318.60
CD8+CCR5+	1193.38	146.90	1197.50	141.60	1170.75	116.80	1650.50	263.50
CD14	2354.00	274.60	2432.00	339.90	2336.00	287.40	2221.00	309.20
CD14+CD69+	4202.75	122.00	4163.75	217.70	4069.50	302.20	3725.00	403.20
CD14+CCR5+	5203.88	1223.00	5345.88	1333.00	4970.75	1107.00	4803.25	1012.00

Mean and SEM represent the median fluorescent intensities, not normalized to fold change, of either CD69 or CCR5 expressed on double-positive cells and were calculated from 8 independent donors. Statistical comparisons were carried out using either a parametric one-way ANOVA with Dunnett's post test (CD3, CD3+CCR5+, CD4, CD4+CD69+, CD4+CCR5+, CD8, CD14+CD69+) or a non-parametric Kruskal-Wallis test with Dunn's post test (CD3+CD69+, CD8+CD69+, CD8+CCR5+, CD14, CD14+CCR5+). Stars next to values indicate significance compared to own vehicle control, with ** and * indicating $p<0.01$ and $p<0.05$ respectively. Where the fold changes (relative to vehicle) were significantly different, as shown in Fig. 3.3.1.2 and 3.3.2.2, the values in the table are in bold. ** $p<0.01$, * $p<0.05$.

Using the modified cervical carcinoma cell line, TZM-bl, as a model in which to study the biological mechanisms of infection, gene expression of the host receptor/co-receptors involved in HIV-1 infection, CD4, CXCR4 and CCR5 was assessed. This was carried out using real-time qPCR in response to hormones mimicking the luteal or follicular phases of the menstrual cycle or contraception with the injectable MPA for 2 days.

TZM-bl cells stimulated with MPA but not luteal or follicular phase E_2/P_4 significantly up-regulated CCR5 mRNA levels 1.5-fold (Fig. 3.3.2.3a) and significantly increased CD4 mRNA levels 1.6-fold (Fig. 3.3.2.3b). Interestingly, MPA but not luteal or follicular phase E_2/P_4 appeared to down-regulate expression of CXCR4 (Fig. 3.3.2.3c), the co-receptor utilized during infection with X-tropic viruses, although this was not significant.

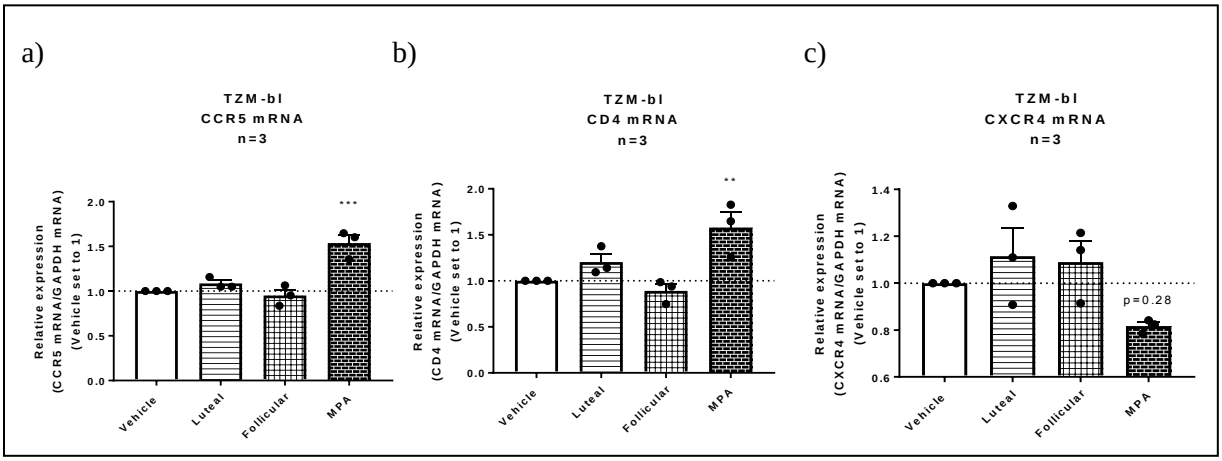


Figure 3.3.2.3. MPA, unlike luteal or follicular phase E_2/P_4, up-regulates the total mRNA expression of CCR5 and CD4 but down-regulates the expression of CXCR4 in TZM-bl cells. TZM-bl cells were seeded at 1 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were then stimulated with hormones representing the luteal phase (10 nM P_4 + 400 pM E_2), follicular phase (1 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM) in SF-DMEM for 48 h. Cells were washed and RNA was extracted. cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of CCR5 (a), CD4 (b) and CXCR4 (c) were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to the vehicle control set to 1. The results are pooled from 3 independent experiments. Statistical comparisons were carried out using a parametric one-way ANOVA with Dunnett's multiple comparisons post test. Stars above bars indicate significance compared to own vehicle control, with *** and ** indicating $p<0.001$ and $p<0.01$ respectively.

Together, these results suggest that contraception with MPA, unlike the P_4-dominant luteal phase of the menstrual cycle, may increase HIV-1 infection through up-regulating the frequency of HIV-1 target CD4+ T cells, and down-regulating the frequency of cytotoxic CD8+ T cells. The consequent increase in the CD4/CD8 ratio implies that enhanced infection can occur through increased proportions of cells being infected with HIV-1 relative to cells that can fight HIV-1 infection. Furthermore, MPA, unlike menstrual cycle hormones, may increase HIV-1 infection by increasing expression of CD69 on activated CD4+ T-lymphocytes in PBMCs, and increasing expression of the HIV-1 co-receptor CCR5 in both PBMCs and TZM-bl cells.

3.3.3 Menstrual cycle phase hormones and MPA differentially regulate pro-inflammatory IL-6 gene expression in PBMCs and TZM-bl cells

Although it is not established whether DMPA use is associated with increased or decreased levels of inflammatory markers, most studies in cell lines suggest that MPA is immunosuppressive (see section 1.4.3). In order to explore this further using the pro-inflammatory cytokine IL-6 as a marker for inflammation, IL-6 mRNA levels in response to MPA and menstrual cycle phase hormones were investigated in PBMCs and TZM-b cells.

In PBMCs, stimulation with MPA but not luteal or follicular phase E_2/P_4 for 2 days significantly decreased IL-6 mRNA levels 2.6-fold (Fig. 3.3.3.1a). A similar decrease of 1.8-fold was observed at 7 days, although this was not significant (Fig. 3.3.3.1a). Like PBMCs, TZM-bl cells stimulated with MPA but not luteal or follicular phase E_2/P_4 for 2 days also significantly decreased IL-6 mRNA levels 3.5-fold (Fig. 3.3.3.1b). In both PBMCs and TZM-bl cells, the repression of IL-6 by MPA was also significantly different to luteal phase E_2/P_4 (Fig. 3.3.3.1).

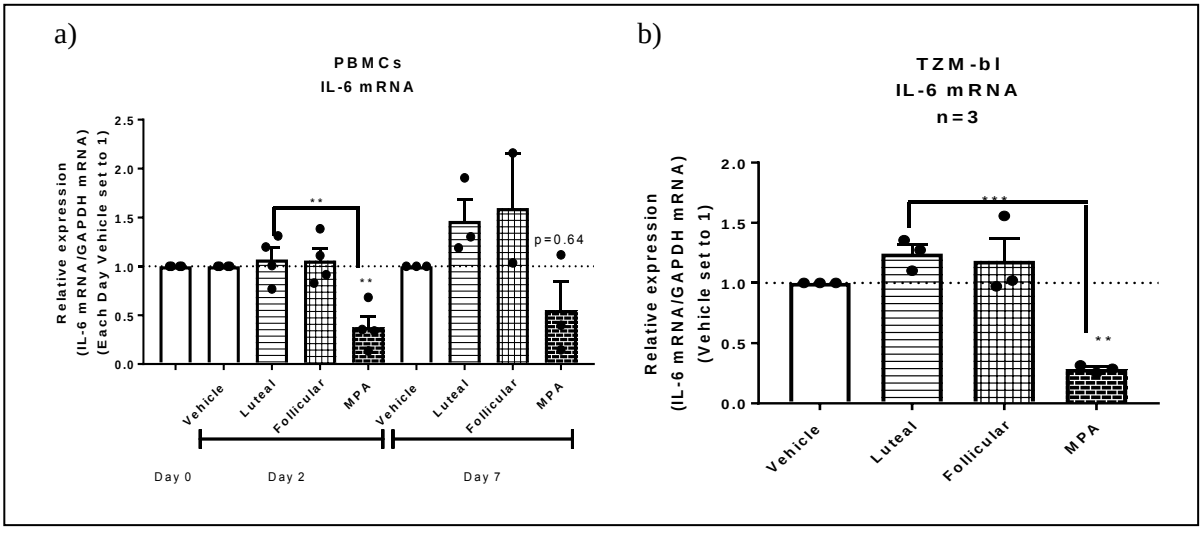


Figure 3.3.3.1. MPA, unlike luteal or follicular phase E_2/P_4, represses the expression of IL-6 in PBMCs and TZM-bl cells. PBMCs (a) were seeded at 4 X 10^6 in FACS tubes and stimulated for 2 or 7 days in full RPMI, while TZM-bl cells (b) were seeded at 1 X 10^5 cells/well in 12 well plates and stimulated for 2 days in SF-DMEM. Hormones represent the luteal phase (10 nM P_4 + 400 pM E_2), follicular phase (1 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM). Upon harvesting, PBMCs (a) were pelleted by centrifugation. PBMCs and TZM-bl cells were washed, RNA was extracted and cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of IL-6 were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to the vehicle control set to 1. The results are pooled from 3 (TZM-bl and PBMCs Day 7) or 4 (PBMCs Day 0-2) independent experiments. Statistical comparisons were carried out using a parametric one-way ANOVA with Tukey's multiple comparisons post test. Stars above bars indicate significance compared to each vehicle control unless otherwise indicated by lines, with *** and ** indicating $p<0.001$ and $p<0.01$ respectively.

Together these results suggest that contraception with MPA, unlike the P_4-dominant luteal phase of the menstrual cycle, may promote an anti-inflammatory response in both PBMCs and TZM-bl cells.

3.4 The GR is required for the increased HIV-1 infection in response to MPA, but not menstrual cycle phase hormones

The GR is a major regulator of the immune system and inflammatory processes. It has been established that both MPA and P_4 bind to the GR, with MPA having a much higher relative binding affinity than P_4 (see section 1.4.1). In order to investigate whether GR is required for enhanced HIV-1 infection observed for both luteal phase E_2/P_4 and MPA, GR protein levels and the effects of GR antagonism on HIV-1 infection in PBMCs were assessed.

3.4.1 Increased HIV-1 infection in response to stimulation with MPA but not luteal or follicular phase E_2/P_4 is mediated via the GR

The role of the active GR in changing HIV-1 infection in response to luteal or follicular phase E_2/P_4 or MPA was assessed by co-stimulating PBMCs with the GR/PR antagonist, RU486. Since PBMCs only express the GR but not the PR [Appendix A, Fig. A1.1, and (Tomasicchio et al. 2013)], the effects of RU486 in these experiments are likely to represent the antagonism of GR only.

Similar to earlier data (Fig. 3.2.1), stimulation with luteal phase E_2/P_4 (1.5-fold) and MPA (2.7-fold) for 7 days significantly increased HIV-1 infection in PBMCs, while the apparent increase (1.4-fold) observed with follicular phase E_2/P_4 was not significant (Fig. 3.4.1.1).

In the absence of exogenous hormones, co-stimulation with RU486 significantly increased HIV-1 infection 5.6-fold (Fig. 3.4.1.1a), suggesting that RU486 is a partial agonist for infection. Furthermore, the increased HIV-1 infection observed with MPA, but not luteal phase E_2/P_4, was significantly repressed 2-fold when co-stimulated with RU486 (Fig. 3.4.1.1b). This effect was also observed when HIV-1 infection was plotted from only the PBMC donors showing increased infection with MPA ("MPA responders", Appendix B, Fig. B1.3). Together, these data suggest that MPA-induced HIV-1 infection requires the active GR, but that unliganded GR protects against infection in the absence of exogenous hormones.

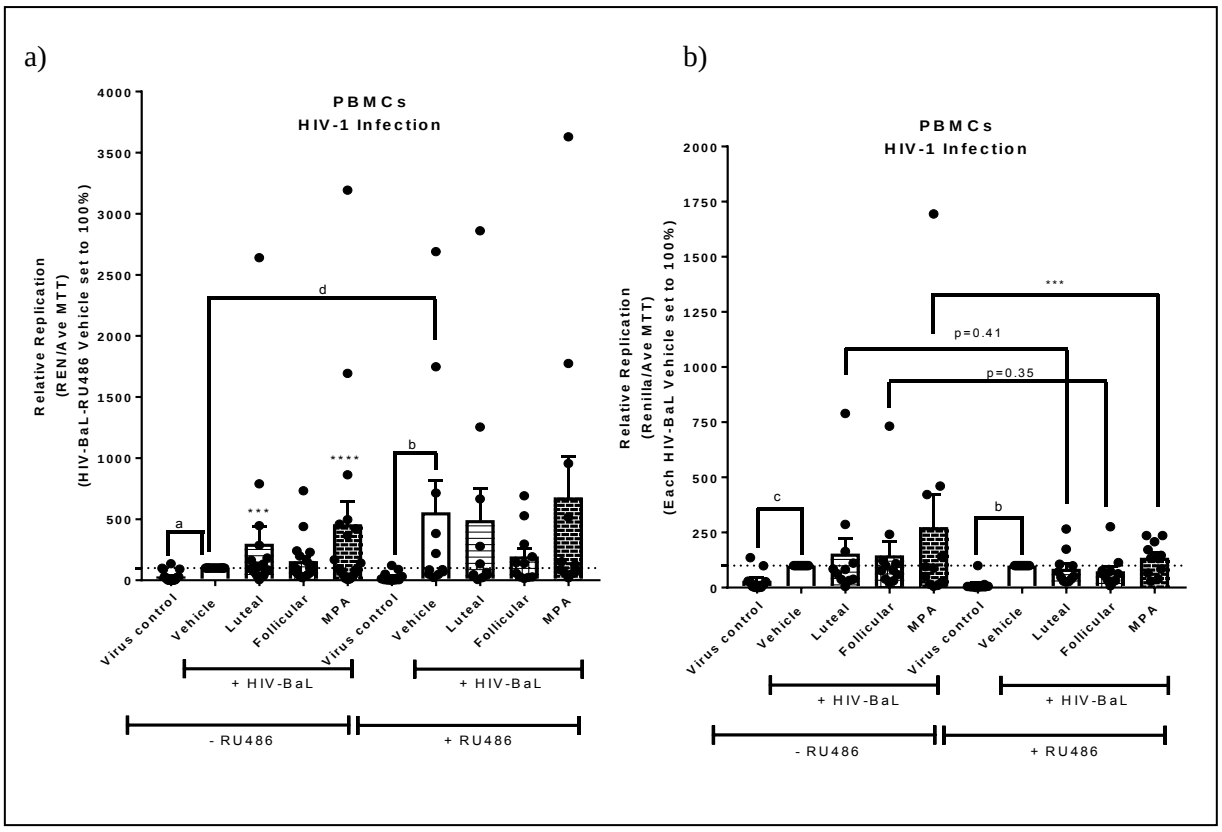


Figure 3.4.1.1. The GR antagonist RU486 represses the MPA- but not luteal phase E_2/P_4-induced increase in HIV-1 infection in PBMCs. PBMCs were seeded at 4 X 10^6 in FACS tubes and stimulated for 7 days with hormones representing the luteal phase (10 nM P_4 + 400 pM E_2), follicular phase (1 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM). PBMCs were subsequently infected with 10 IU/ml HIV-$1_{BaL\_Renilla}$ (HIV-BaL) or equivalent volume of no-virus control (labelled as Virus control) for 2 h, washed and re-suspended in ligand-free full RPMI for 5 days. Cells were re-seeded in round-bottom 96 well plates in quadruplicate wells per condition at 2 X 10^5 cells/well and harvested for *Renilla* luciferase (infection) and for cell viability. The results show pooled data from at least 4 independent experiments, with 2-3 PBMC donors each, for a total of 11 independent donors for both – and + RU486. Additionally, the HIV-1 infection data for 7 days' stimulation from Fig. 3.2.1 was included in the –RU486 data set in (a) (n=18). Relative infection was calculated as *Renilla* luciferase (RLU) divided by average absorbance at 595nm (MTT) for the quadruplicate wells. Infection was plotted relative to the vehicle control in the presence of virus but the absence of RU486 (-RU486) set to 100% in (a) and both vehicle controls (±RU486) in the presence of virus set to 100% in (b). Statistical comparisons were carried out using a two-way ANOVA with Dunnett's (a) or Tukey's (b) multiple comparisons post test, indicated by stars (*), or a Wilcoxon test, indicated by letters (a-d). Stars above bars indicate significance compared to the vehicle control in the presence of virus but absence of RU486, unless otherwise indicated by lines. Letters above bars indicate significance for the comparison indicated by lines, with ****/a, ***/b, **/c and */d indicating $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

3.4.2 GR protein levels are decreased in response to MPA but not menstrual cycle phase hormones in PBMCs

It has previously been demonstrated that the agonist ligand-activated GR undergoes ligand-dependent turnover (Hoeck et al. 1989, Bellingham et al. 1992, Oakley and Cidlowski 1993). Since previous research from the present author's laboratory has shown that MPA acts like a glucocorticoid (Koubovec et al. 2004, Avenant et al. 2010b, Tomasicchio et al. 2013, Govender et al. 2014, Hapgood et al. 2014b), it would be expected that, as a proof of GR involvement, MPA would promote a decrease in GR protein levels. Therefore the levels of GR protein in PBMCs were assessed in response to stimulation with luteal or follicular phase E_2/P_4 or MPA.

Total protein was harvested from matched samples during PBMC infection assays (Fig. 3.2.1) and was probed for GR protein levels by western blotting (Fig. 3.4.2.1a, b). The representative western blot indicates that GR protein levels were lower when stimulated with MPA but not luteal or follicular phase E_2/P_4. However when the western blots from all donors were pooled together and analyzed by densitometry, the large variability between experiments suggests the differences in GR protein levels are not significant (Fig. 3.4.2.1b).

In order to confirm that the effects with MPA were via the GR only, it was assessed whether PR protein was possibly up-regulated in PBMCs. Following reports that PR protein has been detected in PBMCs (Asin et al. 2008, Cabrera-Munoz et al. 2012) and the knowledge that PR expression is regulated by E_2 (Kastner et al. 1990), it was attempted to promote PR protein expression in PBMCs by stimulation with the exogenous luteal phase E_2/P_4 for 7 days. However, as shown by the representative western blot, this combination of E_2 and P_4 did not up-regulate the endogenous PR (Fig. 3.4.2.1c).

The above results suggest that in response to MPA, HIV-1 infection increases in PBMCs, in a manner involving the active GR, since GR protein levels appeared to be decreased with MPA stimulation. In contrast, luteal phase E_2/P_4 increases HIV-1 infection in PBMCs, but this appears to not be mediated by the GR, and GR protein levels were unaffected. The results further imply that MPA, but not menstrual cycle hormones, promotes an anti-inflammatory response by repressing expression of IL-6. In combination with the other data showing that MPA increases the CD4/CD8 ratio in total and CD69+ cells, CCR5 expression and the frequency of activated CD4+CD69+ T cells, the results from this chapter imply that MPA and the luteal phase increase HIV-1 infection via different mechanisms.

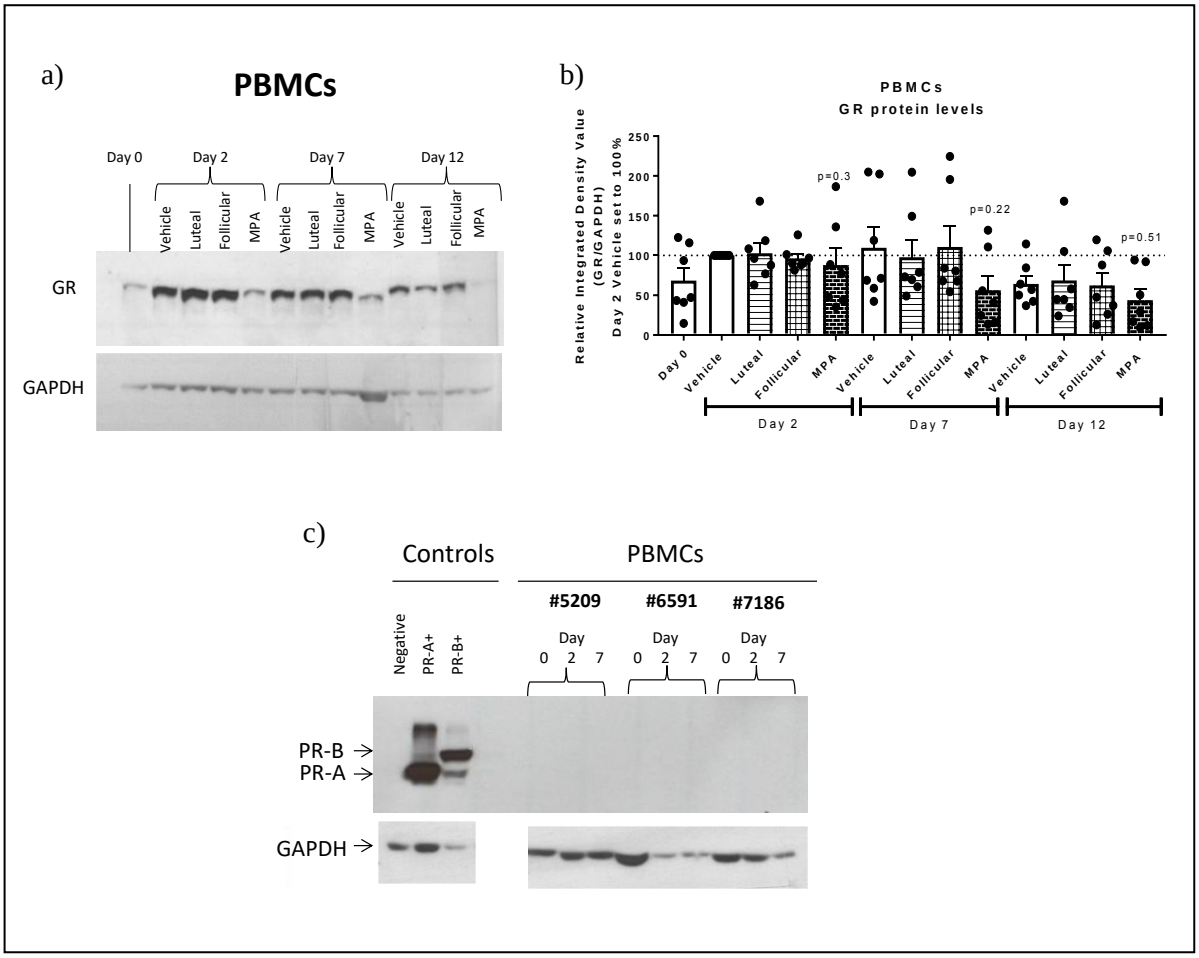


Figure 3.4.2.1. GR protein levels appear to decrease in PBMCs stimulated with MPA but not luteal or follicular phase E_2/P_4. (a, b) PBMCs were seeded at 4 X 10^6 in FACS tubes and stimulated with hormone combinations representing the luteal phase (10 nM P_4 + 400 pM E_2), follicular phase (1 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM). From matched samples at Day 0, 2, 7 and 12, 8 million PBMCs were pelleted and harvested for total protein using TAPS buffer. Total protein lysates were analyzed for GR protein levels by western blotting using GAPDH as a loading control. Blots were scanned and quantified for relative GR levels by calculating integrated density values. (a) Representative western blot from donor #9937. Apparent very differences in GR band sizes may be due to pH effects of the TAPS buffer from protein isolation or limited differential proteolysis. If this effect is due to different degrees of GR protein degradation, use of proteasomal inhibitors such as MG-132 may reverse the effect in future work. (b) Densitometry indicating GR protein relative to GAPDH protein, pooled from 7 independent donors. GR levels are plotted relative to the vehicle control at Day 2 set to 100 %. (c) PBMCs (8 million) from 3 independent donors (#5209, #6591, #7186) were stimulated with hormone combinations representing the luteal phase (10 nM P_4 + 400 pM E_2) for 0, 2 or 7 days then pelleted and harvested for total protein using TAPS buffer. Lysates were analyzed for PR protein levels by western blotting using GAPDH as a loading control. (b) Statistical comparisons were carried out using a non-parametric Kruskal-Wallis test with Dunn's multiple comparisons post test. No significant differences were observed, but p values above bars indicate a comparison to each day's vehicle control.

Chapter 4

MPA-induced and basal HIV-1 infection and immunomodulatory gene expression change when the relative levels of GR and PR are altered

4.1 Background and aims

It has previously been shown that of the steroid receptors, only GR is detectable in PBMCs (Appendix A, Fig. A1.1) while GR and AR are detectable in TZM-bl cells (Appendix A, Fig. A2.1). However, tissues at the site of infection in the FRT express other steroid receptors; importantly the PR. Expression of PR is under the control of the fluctuating levels of E_2 during the menstrual cycle. Since both GR and PR are activated by MPA, this raises the question whether these receptors can be activated at the same time in the FRT of DMPA users, and whether HIV-1 infection in the FRT is modulated by the levels of these receptors.

Expression of both GR and PR protein in ectocervical explant tissue from premenopausal women in the luteal and follicular phase was confirmed by western blotting (Appendix B, Fig. B2.1, Appendix D, Table D1). In the samples with high detectable levels of total protein, as shown by GAPDH levels, there appeared to be some differences in GR/PR levels between the luteal and follicular phases (Appendix B, Fig. B2.1, Appendix D, Table D1). However, a pattern was difficult to deduce. This variation in protein levels could reflect biological inter-individual differences in protein levels, differences in relative composition of cell types, different durations of sample storage (Appendix D, Table D1) and/or differences in protein degradation during sample processing. Nevertheless, other research from the present author's laboratory (Ray 2015) observed higher levels of PR in ectocervical explants from the follicular phase compared to the luteal phase (Appendix A, Fig. A3.1), suggesting that the relative levels of GR/PR change during the menstrual cycle.

Furthermore, in cervical explants from postmenopausal women, in which endogenous E_2/P_4 levels are both much lower than in premenopausal women (Appendix D, Table D1), both GR and PR protein were detectable by western blotting in both endo- and ectocervix (Appendix B, Fig. B2.2), Despite high inter-individual variation in protein expression, greater PR levels were detectable in the endocervix than ectocervix samples (Appendix B, Fig. B2.2), suggesting that the relative GR/PR levels change depending on the cervical compartment, at least in postmenopausal samples.

Together these results imply that the relative GR/PR levels in cervical tissue *ex vivo* vary depending on the menstrual cycle and menopausal status. However the consequences of changing GR/PR levels

on immune function and HIV-1 susceptibility have not been studied. Therefore a molecular manipulation of GR/PR levels by siRNA knockdown and over-expression strategies was established in human cell lines in order to investigate the consequences of changing relative GR/PR levels on MPA-induced immunomodulatory gene expression and HIV-1 infection.

The aims of this section were therefore to answer the following research questions:

(i) Does changing the relative levels of GR and PR modify MPA-induced HIV-1 infection?
- Does the increase in HIV-1 infection with luteal phase E_2/P_4 and MPA change with relative GR/PR levels?
- Does HIV-1 infection in the absence of MPA and luteal phase E_2/P_4 change with relative GR/PR levels?

(ii) Does changing the relative levels of GR and PR modify MPA-induced and/or basal expression of genes relevant to HIV-1 infection and immune function, specifically the following?
- HIV-1 receptor and co-receptor expression
- Immunomodulatory gene expression

(iii) Is a difference in HIV-1 infection between relative levels of GR and PR due to a possible change in expression of soluble mediators?

In order to answer these questions, several cervical cell lines were used to model cells in the female reproductive tract, the site of heterosexual HIV-1 infection. These included End1/E6E7 immortalized endocervical epithelial cells; HeLa cervical carcinoma cells and TZM-bl cells, which are modified HeLa cells that are infectable with HIV-1. While TZM-bl cells endogenously express GR and AR (Appendix A, Fig. A2.1) and End1/E6E7 cells express GR, AR and ERα (Appendix A, Fig. A2.2), HeLa cells only express GR (Appendix A, Fig. A2.1). The expression of GR in these three cell lines renders them suitable for studying GR-mediated HIV-1 infection and immunomodulatory gene expression. The relative levels of GR/PR were changed by using siRNA to decrease expression of the endogenous GR (siGR), as compared to non-silencing control (NSC) siRNA, with or without the simultaneous over-expression of PR-B. This leads to four conditions: NSC-PR-B, siGR-PR-B, NSC+PR-B and siGR+PR-B, also referred to throughout this chapter as “high GR/no PR”, “low GR/no PR”, “high GR/high PR” and “low GR/high PR”, respectively. Pilot experiments to confirm decreased GR expression, PR-B over-expression and GR- or PR-mediated gene expression were performed in these cell lines (Appendix B, Fig. B3.1, 3.2, 4.1, 4.2, 4.3).

Over-expression of GR/PR in U2OS cells, which express very low levels of endogenous GR, was further explored as a strategy in which to study the effects of changing the relative levels of GR/PR on

endogenous immunomodulatory gene expression. However, despite predictable Dex-induced, GR-mediated responses on the transactivation gene GILZ, robust and reproducible repression of IL-6 by Dex was not observed (not shown, reported in Appendix B5). Furthermore the breast cancer cell lines, MCF-7 and T47D, in which GR and PR are endogenously expressed (Appendix B, Fig. B6.1), were investigated in parallel for their suitability to study changing GR/PR. However, despite functional GR/PR activity on the TAT-GRE-LUC reporter gene in MCF-7 cells (Appendix B, Fig. B6.2a, b), reproducible GR-mediated transcriptional responses on endogenous genes were not observed (Appendix B, Fig. B6.3a, b). GR-mediated responses in T47D cells were not achieved on the TAT-GRE-LUC reporter gene (Appendix B, Fig. B6.2d) nor endogenous genes (Appendix B, Fig. B6.3c, d) and attempts to over-express GR in T47D cells were unsuccessful (Appendix B, Fig. B6.4). In contrast, End1/E6E7 and HeLa cells have been shown to exhibit transactivation and transrepression of endogenous genes such as GILZ and IL-6, mediated by the endogenous GR [(Govender 2014) and Appendix B, Fig. B3.1-2]. In addition, PR-B could be successfully over-expressed in these cells, making End1/E6E7 and HeLa cells suitable model cell lines in which to investigate GR/PR-mediated gene expression (reported in Chapter 4), while U2OS/COS1 and MCF-7 cells were used in assays other than gene expression studies (Chapter 5).

4.2 MPA-induced HIV-1 infection changes when the relative levels of GR/PR are altered in TZM-bl cells

Since increased HIV-1 infection with luteal phase E_2/P_4 and MPA was shown in PBMCs, HIV-1 infection was assessed in response to luteal phase E_2/P_4 and MPA in TZM-bl cells in which the relative levels of GR/PR were changed by siRNA and over-expression strategies.

4.2.1 The increase in HIV-1 infection with MPA, but not luteal phase E_2/P_4, depends on GR but not PR-B levels

Stimulation with MPA significantly increased HIV-1 infection 1.5-fold in TZM-bl cells (Fig. 4.2.1.1a). While HIV-1 infection appeared to increase 1.3-fold in response to luteal phase E_2/P_4, this was not significant (Fig. 4.2.1.1a). The large variability in relative infection in TZM-bl cells was not due to changes in cell viability but appears to reflect inherent biological variation in infection between experiments (Appendix B, Table B1).

The increase in HIV-1 infection with MPA (NSC) was lost when GR expression was decreased using siRNA targeting the GR (siGR) (Fig. 4.2.1.1a). This effect occurred both in the presence and absence

of over-expressed PR-B. Although high GR/high PR (NSC+PR-B) appeared to further increase MPA-induced HIV-1 infection (1.7-fold) compared to high GR/no PR (NSC-PR-B), this effect was not significant (p=0.4456). Decreased GR levels by about 40% (Fig. 4.2.1.1c) and PR-B over-expression by 1.5-2-fold (Fig. 4.2.1.1d) were confirmed by western blotting on parallel samples (Fig. 4.2.1.1b). Taken together, these results show that MPA increases HIV-1 infection in a GR-mediated, PR-B-independent manner.

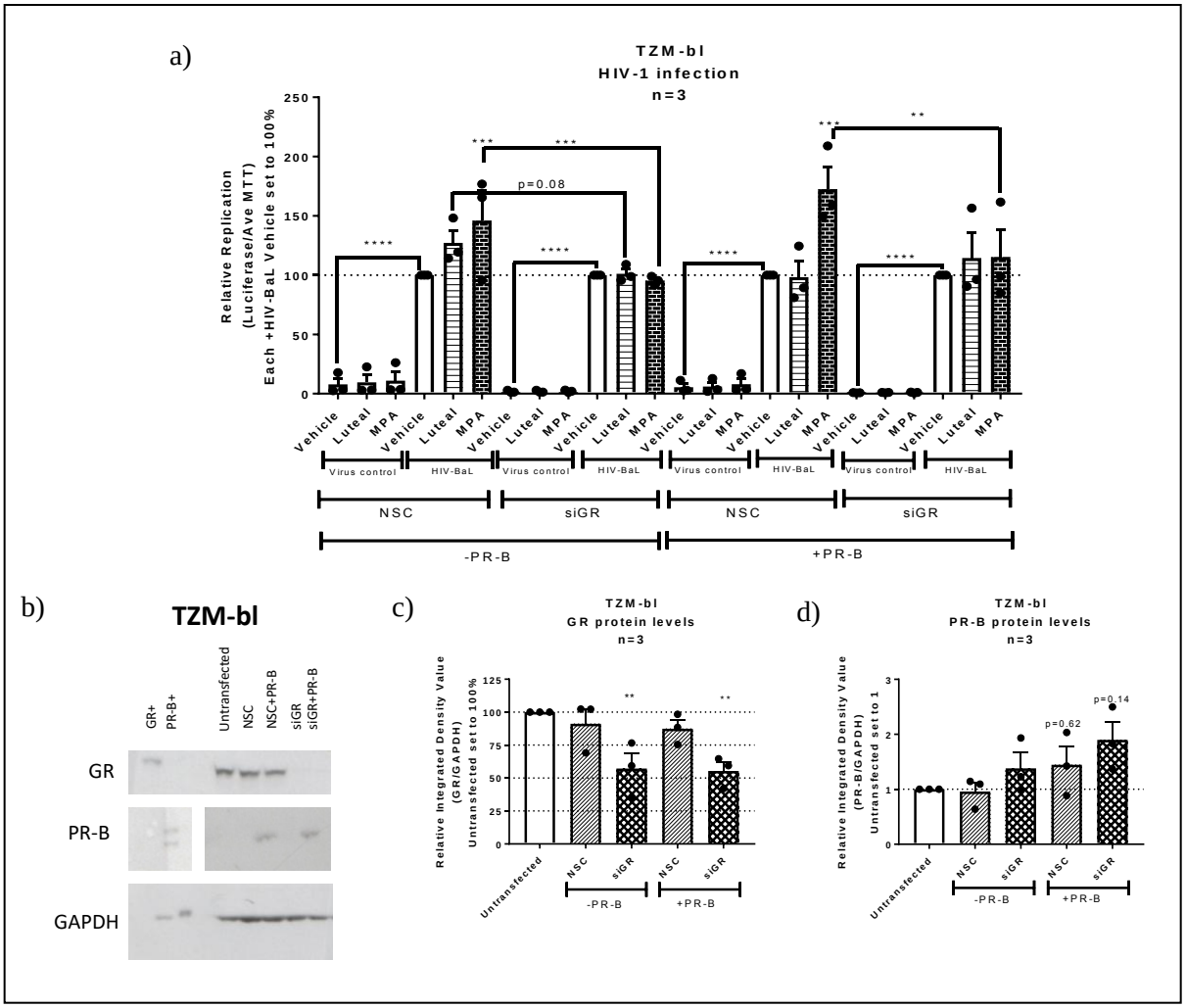


Figure 4.2.1.1. MPA-induced HIV-1 infection is mediated by the GR in TZM-bl cells. TZM-bl cells were seeded at 1 X 10^5 cells/well in 12 well plates and left to adhere overnight. Cells were subsequently transfected with 10 nM non-silencing control (NSC) or siRNA targeting the human GR (siGR) for 24 h, then transiently transfected with 500 ng/well empty vector pcDNA3 or pMT-hPR-B for 24 h. (a) Cells were re-seeded at 2.5 X 10^4 cells/well into 96-well plates in quadruplicate wells for another 24 h, followed by stimulation for 24 h with hormone combinations representing the luteal phase (10 nM P_4 + 400 pM E_2) or peak levels of the injectable contraceptive MPA (100 nM). Media was removed and replaced with phenol red-free DMEM containing 10 IU/mL HIV-$1_{BaL\_Renilla}$ (HIV-BaL) or equivalent volume of no-virus control (labelled as Virus control). Cells were harvested 72 h later for luciferase (infection) and for cell viability. The results are pooled from 3 independent experiments where each point was in quadruplicate. Relative infection was calculated as luciferase (RLU) divided by average absorbance at 595nm (MTT) for the quadruplicate wells.

Infection was plotted relative to the vehicle control for NSC-PR-B in the presence of virus set to 100%. (b) Cells seeded and transfected in parallel were harvested in SDS sample buffer and lysates were analyzed for GR and PR-B levels by western blotting using GAPDH as a loading control. A representative blot is shown. Blots were probed first for GR then stripped and re-probed for PR-B. (c, d) Western blots were scanned and quantified for relative GR (c) or PR-B (d) levels by calculating integrated density values. Data are pooled from 3 independent experiments. GR levels are plotted relative to the untransfected control set to 100% (c), while PR-B levels are plotted relative to the untransfected control set to 1 (d). Statistical comparisons were carried out using a two-way ANOVA with Tukey's (a) or Dunnett's (c, d) multiple comparisons post test. Stars above bars indicate significance compared to each vehicle in the presence of virus (a) or to the untransfected control (c, d), unless otherwise indicated by lines, with *** and ** indicating $p<0.001$ and $p<0.01$ respectively.

4.2.2 HIV-1 infection in the absence of exogenous hormones changes when the relative levels of GR/PR are altered

Since antagonism of the GR in the absence of exogenous hormones increased HIV-1 infection in PBMCs (Fig. 3.4.1.1), it was hypothesized that decreasing GR levels using siRNA in TZM-bl cells would also increase HIV-1 infection. Therefore the effect of directly changing the relative GR/PR levels on HIV-1 infection in the absence of exogenous MPA or luteal phase E_2/P_4 in TZM-bl cells was assessed.

HIV-1 infection was significantly higher when GR levels were decreased (siGR) compared to basal GR levels (NSC), both in the presence (3.1-fold, siGR+PR-B) and absence (2.9-fold, siGR-PR-B) of PR-B (Fig. 4.2.2.1a). With high GR/high PR (NSC+PR-B), HIV-1 infection was significantly increased 1.7-fold, compared to high GR/low PR (Fig. 4.2.2.1a). However the highest HIV-1 infection was observed for low GR/high PR (3.1-fold, siGR+PR-B, Fig. 4.2.2.1a), although this was not an additive effect. This same pattern was observed in the previous figure (Fig. 4.2.1.1) when vehicle controls were plotted relative to NSC-PR-B (not shown).

Decreased GR levels by about 50% (Fig. 4.2.2.1c) and PR-B over-expression by about 10-20-fold (Fig. 4.2.2.1d) were confirmed by western blotting on parallel samples (Fig. 4.2.2.1b). The low GR/high PR combination appeared to express lower PR-B protein levels on the western blot than the high GR/high PR combination, despite equal amounts of PR-B expression vector transfected (Fig. 4.2.2.1b). However, when pooled samples were analyzed densitometrically, this difference was not significant (Fig. 4.2.2.1d). Nevertheless, this suggests that the siRNA targeting the GR may also silence the expression of PR-B or another factor required for PR-B expression.

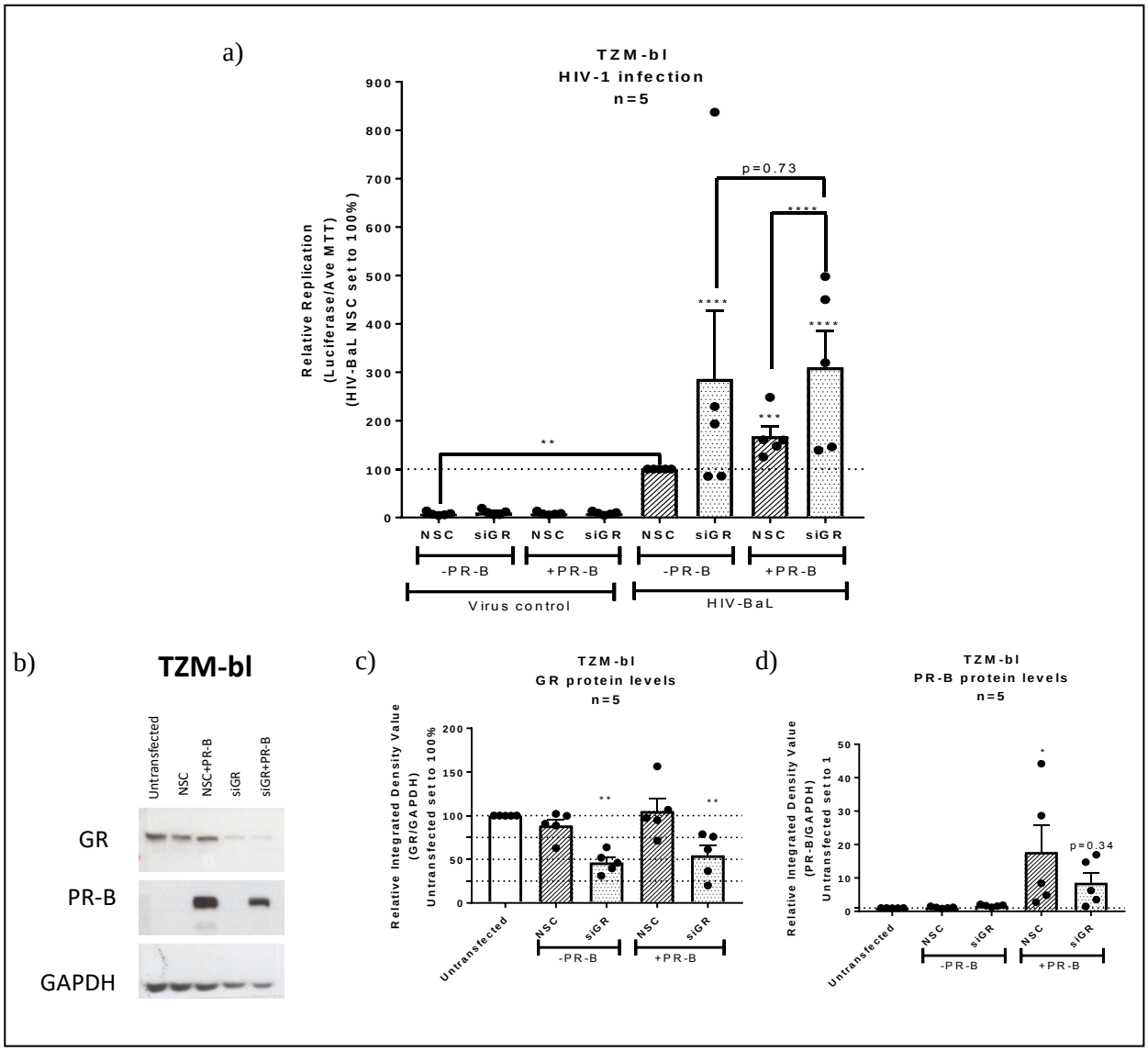


Figure 4.2.2.1 Basal HIV-1 infection in the absence of exogenous hormones in TZM-bl cells increases with low GR/high PR. TZM-bl cells were seeded at 1 X 10^5 cells/well in 12 well plates and left to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 24 h, then transiently transfected with 500 ng/well empty vector pcDNA3 or pMT-hPR-B for 24 h. (a) Cells were re-seeded at 2.5 X 10^4 cells/well into 96-well plates in quadruplicate wells for another 24 h, then infected with 10 IU/mL HIV-$1_{BaL\_Renilla}$ (HIV-BaL) or equivalent volume of no-virus control (labelled as Virus control) by addition to the wells. Cells were harvested 72 h later for luciferase (infection) and for cell viability. The results are pooled from 5 independent experiments where each point was in quadruplicate. Relative infection was calculated as luciferase (RLU) divided by average absorbance at 595nm (MTT) for the quadruplicate wells. Infection was plotted relative to NSC-PR-B in the presence of virus set to 100%. (b) Cells seeded and transfected in parallel were harvested in SDS sample buffer and lysates were analyzed for GR and PR-B levels by western blotting using GAPDH as a loading control. (c, d). A representative blot is shown. Western blots were scanned and quantified for relative GR (c) or PR-B (d) levels by calculating integrated density values. Data are pooled from 5 independent experiments.GR levels are plotted relative to the untransfected control set to 100% (c), while PR-B levels are plotted relative to the untransfected control set to 1 (d). Statistical comparisons were carried out using a two-way ANOVA with Tukey's (a) or Dunnett's (c, d) multiple comparisons post test. Stars above bars indicate significance compared to NSC-PR-B in the presence of virus (a) or to the untransfected control (c, d), unless otherwise indicated by lines, with ****, ***, ** and * indicating $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

Together these results suggest that in the absence of MPA, unliganded basal levels of GR seem to protect against HIV-1 infection, while PR seems to increase HIV-1 infection; and in the presence of MPA, the MPA-induced HIV-1 infection is mediated by the GR only, while PR has no significant effect.

4.3 Altering the relative levels of GR/PR modifies the MPA-induced expression of genes relevant to HIV-1 infection and immune function in TZM-bl cells

The previous section showed that HIV-1 infection in TZM-bl cells increased in the presence of MPA, in a manner dependent on GR levels, and furthermore that HIV-1 infection in the absence of exogenous hormones increased with low GR/no PR, high GR/high PR and low GR/high PR compared to high GR/no PR. In order to investigate possible mechanisms of infection, the effect of changing relative GR/PR levels on expression of genes relevant to HIV-1 infection and immune function was assessed.

4.3.1 Basal HIV-1 CD4 receptor, but not CCR5 or CXCR4 co-receptor, expression changes when GR/PR levels are altered in TZM-bl cells in the absence of exogenous hormones

TZM-bl cells stably express high levels of the CD4 receptor and the HIV-1 co-receptors CXCR4 and CCR5 (Platt et al. 1998). The previous chapter showed that MPA increased the expression of CCR5 in CD3+ and CD3+CD4+ T-cells in PBMCs (Fig. 3.3.2.2) and increased CCR5 mRNA levels in TZM-bl cells (Fig. 3.3.2.3a). Furthermore, CCR5 is the co-receptor used by the R5-tropic infectious molecular clone used in our experiments, HIV-1 $_{BaL\_Renilla}$.

Interestingly, CCR5 mRNA levels were not modified by altering the relative levels of GR/PR in TZM-bl cells, in the absence of exogenous hormones (Fig. 4.3.1.1a). In contrast, CD4 mRNA levels were significantly increased 1.35-fold with low GR/no PR (siGR-PR-B), but unaffected by PR-B over-expression (Fig. 4.3.1.1b). CXCR4 mRNA levels were not significantly changed compared to high GR/no PR (Fig. 4.3.1.1c). Decreased GR levels by about 40% (Fig. 4.2.1.1c) and PR-B over-expression by 1.5-2-fold (Fig. 4.2.1.1d) were confirmed by western blotting on parallel samples (Fig. 4.2.1.1b).

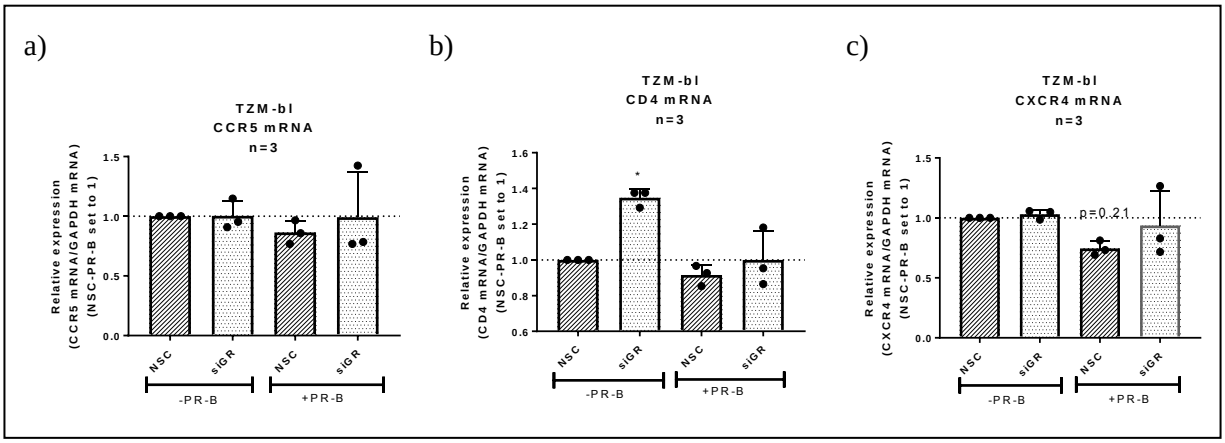


Figure 4.3.1.1. Basal CD4 but not CCR5 or CXCR4 expression is increased with low GR/no PR. TZM-bl cells were seeded at 1 X 10^5 cells/well in 12 well plates and left to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 24 h, then transiently transfected with 500 ng/well empty vector pcDNA3 or pMT-hPR-B for 24 h. Media was changed to SF-DMEM for a further 24 h, then cells were washed. RNA was extracted and cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of CCR5 (a), CD4 (b) and CXCR4 (c) were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to NSC-PR-B set to 1. Decreased GR expression and PR-B over-expression were confirmed by western blotting in parallel samples (Fig. 4.2.1.1b-d). The results are pooled from 3 independent experiments. Statistical comparisons were carried out using a two-way ANOVA with Dunnett's multiple comparisons post test. Stars above bars indicate significance compared to NSC-PR-B, with. ** indicating $p<0.01$.

These results suggest that changing GR levels does not influence basal CCR5 or CXCR4 mRNA levels in TZM-bl cells, but there is an increase in the basal levels of the CD4 receptor when GR expression is decreased. Furthermore these results show that over-expression of PR-B did not change expression of CCR5, CD4 or CXCR4.

4.3.2 MPA-induced immunomodulatory gene expression changes when GR/PR levels are altered in End1/E6E7 and HeLa cells

Given the knowledge that GR regulates immunomodulatory genes and that MPA activates both the GR and the PR, the effect of altering the relative GR/PR levels on immunomodulatory gene expression in response to GR- (Dex), PR- (R5020) or GR/PR-specific (MPA) ligands was next investigated. This was carried out using the more physiologically relevant endocervical epithelial cell line, End1/E6E7, and the cervical carcinoma cell line, HeLa, both expressing endogenous GR and in which PR-B protein could be over-expressed (Appendix B, Fig. 3.1c, 3.2c, 4.1b, 4.2b). In order to interpret these results, the data were plotted relative to each vehicle control, where comparisons

between vehicle and ligands within the same transfection condition and between ligands from different transfection conditions (NSC vs siGR) were made.

In End1/E6E7 cells, the model transactivation gene, GILZ (Fig. 4.3.2.1a), was significantly induced 4-fold by Dex in a typical GR-mediated response (NSC) that was reduced to 2-fold when GR expression was decreased (siGR), in both the absence and presence of PR-B, although this reduction was not significant (Fig. 4.3.2.1a). MPA also appeared to increase the transactivation of GILZ, but to a lower extent than Dex, and this also appeared to occur in a GR-dependent manner (Fig. 4.3.2.1a). The numerical fold reduction for MPA between NSC (high GR) and siGR (low GR) conditions appeared to be greater in the presence (0.5-fold) than the absence (0.3-fold) of PR-B (Fig. 4.3.2.1a). MPA-induced GILZ up-regulation appeared to increase with high GR/high PR (1.9-fold) compared to high GR/no PR (1.5-fold), although this was not significant (Fig. 4.3.2.1a). These results suggest that the presence of PR-B may alter the GR-mediated regulation of GILZ in response to MPA but not Dex, although not conclusively.

With high GR/no PR, the model transrepression gene, IL-6 (Fig. 4.3.2.1b), was significantly repressed by Dex (4.9-fold), in a GR-dependent manner, since decreasing GR expression lifted this repression (low GR/no PR, Fig. 4.3.2.1b). Dex appeared to repress IL-6 with high GR/high PR (2.8-fold), suggesting that Dex repression may be PR-B-independent (Fig. 4.3.2.1b). In the absence of PR-B, MPA appeared to repress IL-6 in a GR-dependent manner (Fig. 4.3.2.1b). In the presence of PR-B, however, MPA no longer repressed IL-6 either with high GR/high PR (NSC+PR-B) or low GR/high PR (siGR+PR-B, Fig. 4.3.2.1b). This suggests that the presence of PR-B changes MPA-induced IL-6 expression to no longer be anti-inflammatory, unlike the result for Dex.

In response to R5020, both GILZ and IL-6 mRNA fluctuated around basal levels (Fig. 4.3.2.1a, b), suggesting that no PR-specific response was observed on these genes.

Decreased GR levels by about 50% (Fig. 4.3.2.1d) and PR-B over-expression by about 2-fold (Fig. 4.3.2.1e) were confirmed by western blotting on parallel samples (Fig. 4.3.2.1c). Similar to TZM-bl cells, PR-B protein levels were lower in the presence of siGR than NSC siRNA oligonucleotides, although this difference was not statistically significant (Fig. 4.3.2.1e).

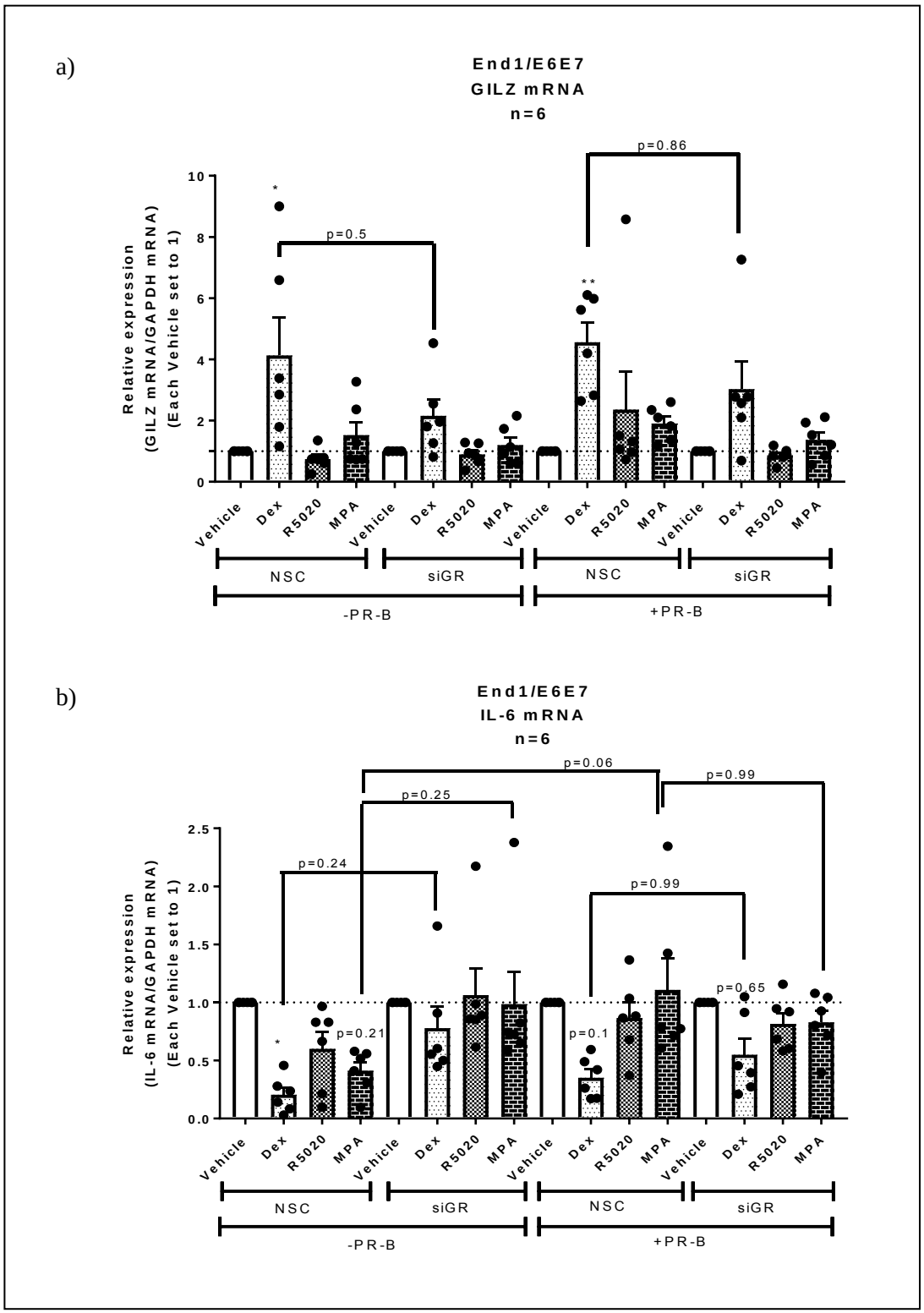


Figure 4.3.2.1. The presence of PR-B alters the GR-mediated transcriptional responses to MPA on GILZ and IL-6 in End1/E6E7 cells. Continued on next page.

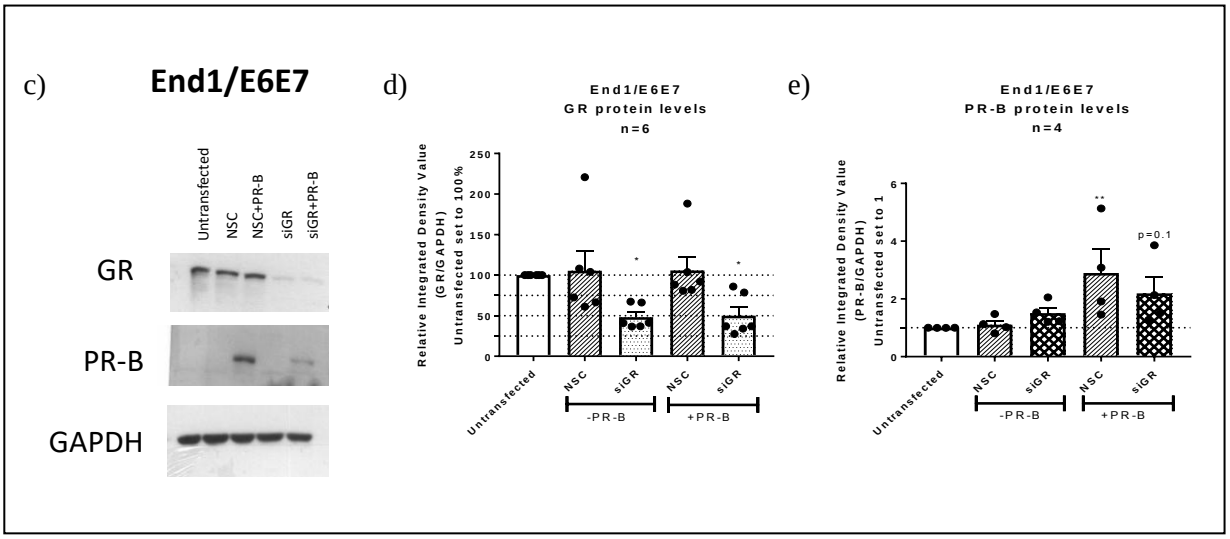


Figure 4.3.2.1. The presence of PR-B alters the GR-mediated transcriptional responses to MPA on GILZ and IL-6 in End1/E6E7 cells. Cells were seeded at 1.5 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 24 h, then transiently transfected with 250 ng/well empty vector pcDNA3 or pMT-hPR-B for 24 h. Cells were stimulated with 100 nM final concentration of the indicated ligands in SF-KSFM for a further 24 h, then cells were washed. RNA was extracted and cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of GILZ (a) and IL-6 (b) were normalized to GAPDH mRNA levels. Relative expression was determined either by normalizing to each vehicle control set to 1. The results are pooled from 6 independent experiments. (c) Cells seeded and transfected in parallel were harvested in SDS sample buffer and lysates were analyzed for GR and PR-B levels by western blotting using GAPDH as a loading control. A representative blot is shown. Blots were probed first for GR then stripped and re-probed for PR-B. Western blots were scanned and quantified for relative GR (d) or PR-B (e) levels by calculating integrated density values. Data are pooled from 4 (PR-B) or 6 (GR) independent experiments, since PR-B protein was not detectable in every experiment. GR levels are plotted relative to the untransfected control set to 100% (d), while PR-B levels are plotted relative to the untransfected control set to 1 (e). Statistical comparisons were carried out using a two-way ANOVA with Tukey's (a, b) or Dunnett's (d, e) post test. Stars above bars indicate significance compared to each vehicle control (a, b) or to the untransfected control (d, e), unless otherwise indicated by lines, with ***, ** and * indicating $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

The same experiment was carried out in HeLa cells, in which greater amounts of PR-B expression vector DNA could be transfected for PR-B over-expression without noticeable effects on cell proliferation and/or viability, unlike End1/E6E7 cells (Appendix B4).

Similar to End1/E6E7 cells, GILZ mRNA was significantly induced 6.7-fold by Dex in the presence of endogenous GR (NSC) and this was reduced to 2.9-fold when GR expression was decreased (siGR), both in the absence (significantly) and presence (near-significantly) of PR-B (Fig. 4.3.2.2a). Similarly, MPA also induced GILZ expression, to a lesser extent than Dex, and this induction was greater with high GR/high PR (4.2-fold, NSC+PR-B, significant) than high GR/no PR (2.5-fold, NSC-PR-B, not significant, Fig. 4.3.2.2a). The numerical fold reduction for MPA between NSC (high GR) and siGR (low GR) conditions was greater in the presence (2.3-fold) than the absence (0.5-fold) of PR-B, although these changes did not reach statistical significance (Fig. 4.3.2.2a). Similar to End1/E6E7 cells, these results in HeLa cells suggest that Dex regulation of GILZ is PR-B-independent, but PR-B alters the response to MPA.

Also similar to results for End1/E6E7 cells with high GR/no PR, in HeLa cells IL-6 mRNA was significantly repressed by Dex (2.8-fold), in a GR-dependent manner, since this repression was lifted back to basal levels when GR expression was decreased (low GR/no PR, Fig. 4.3.2.2b). In the presence of PR-B, Dex near-significantly repressed IL-6 (2.3-fold), in a GR-dependent manner (Fig. 4.3.2.2b). MPA appeared to repress IL-6 with high GR/no PR and high GR/high PR (Fig 4.3.2.2b). However, while this apparent repression was lifted to basal levels with low GR/no PR, MPA continued to repress IL-6 by 1.5-fold with low GR/high PR (siGR+PR-B), although not significantly (Fig. 4.3.2.2b). This suggests that the presence of PR-B promotes MPA repression of IL-6 independent of GR levels in HeLa cells, unlike in End1/E6E7 cells.

Decreased GR levels by about 25% (Fig. 4.3.2.2d) and PR-B over-expression by about 7-fold (Fig. 4.3.2.2e) were confirmed by western blotting on parallel samples (Fig. 4.3.2.2c). Similar to results for End1/E6E7 and TZM-bl cells, in HeLa cells PR-B protein levels were lower in the presence of siGR than NSC siRNA oligonucleotides, although this was not statistically significant (Fig. 4.3.2.2c).

Two additional interesting observations were made when the mRNA levels were plotted relative to the NSC-PR-B vehicle control (Appendix B4). Firstly, in End1/E6E7 cells with high GR/high PR, the Dex-induced increase of GILZ was not as high as expected, or as high as observed with high GR/no PR, although this effect was not significantly different (Appendix B, Fig. B4.4a). A similar trend was observed for the transactivation gene MKP1 in these cells (not shown). This dampening of the GR-mediated Dex response on GILZ was not observed for MPA in End1/E6E7 cells (Appendix B, Fig. B4.4a), nor for Dex or MPA in HeLa cells (Appendix B, Fig. B4.5a), suggesting that the presence of PR-B can change GR-mediated transcriptional regulation in a ligand- and cell-specific manner.

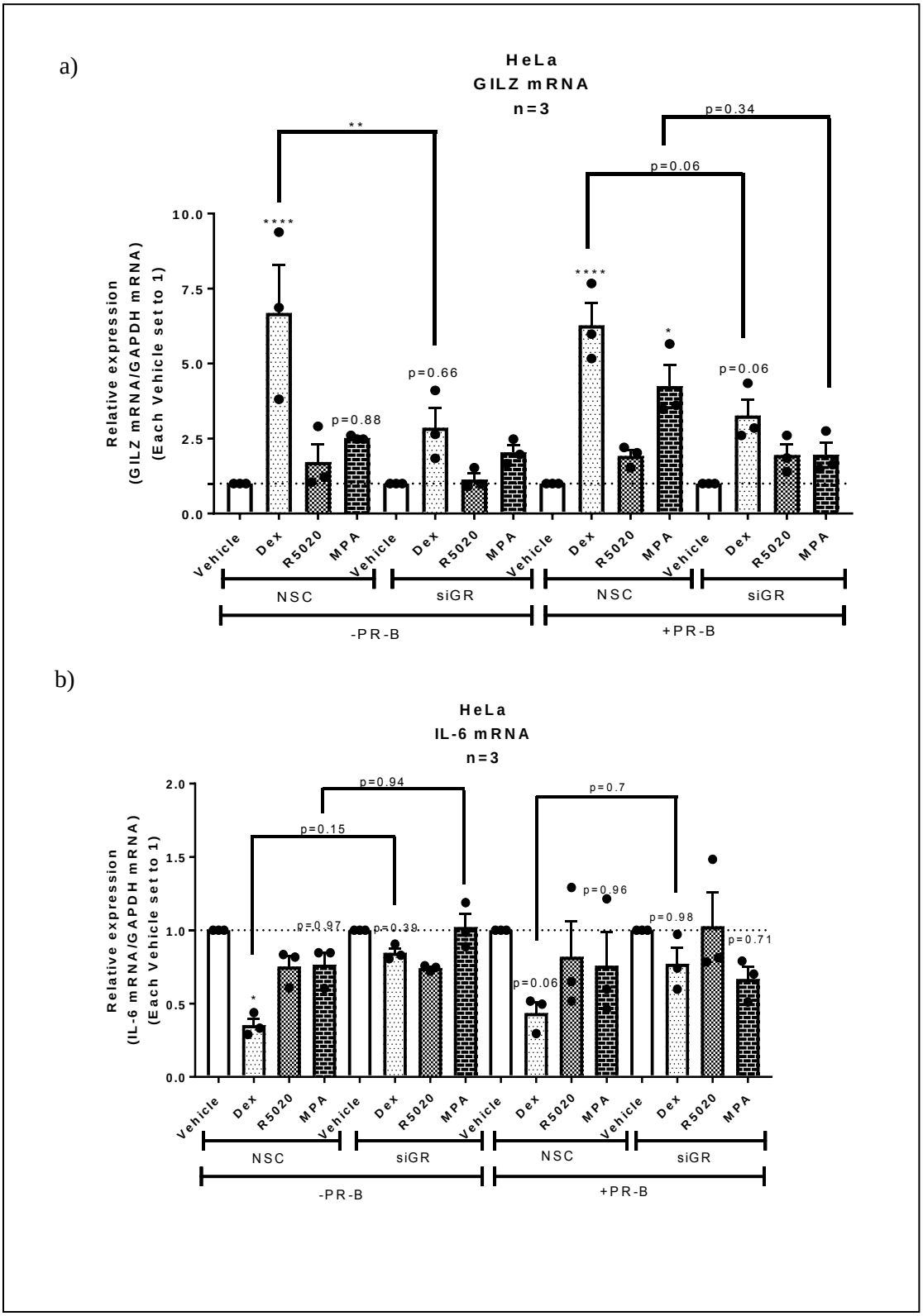


Figure 4.3.2.2. The presence of PR-B alters the GR-mediated transcriptional responses to MPA on GILZ and IL-6 in HeLa cells. Continued on next page.

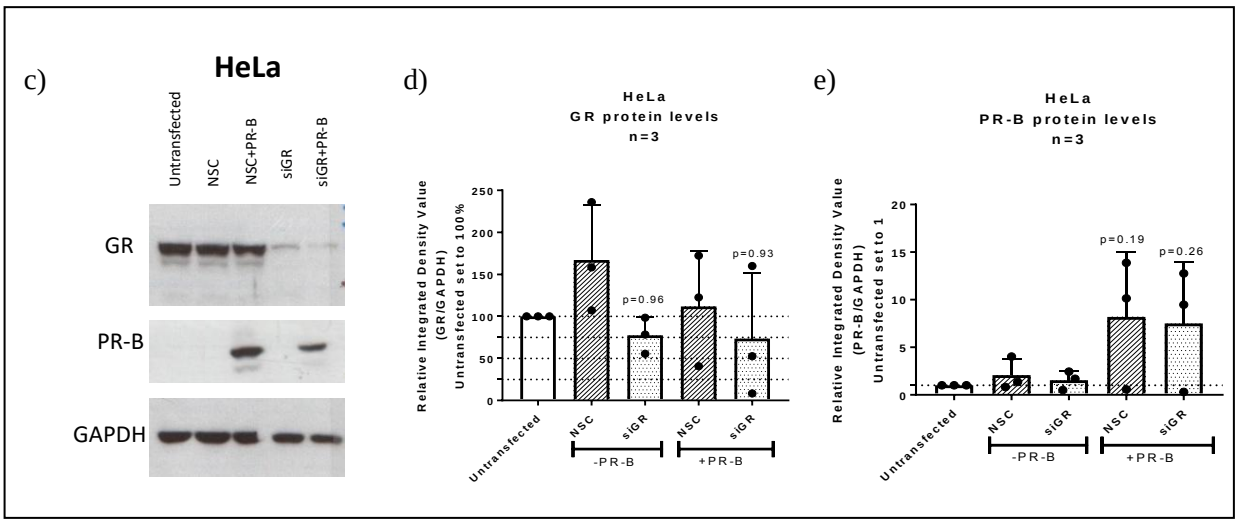


Figure 4.3.2.2. The presence of PR-B alters the GR-mediated transcriptional responses to MPA on GILZ and IL-6 in HeLa cells. Cells were seeded at 1 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 24 h, then transiently transfected with 500 ng/well empty vector pcDNA3 or pMT-hPR-B for 24 h. Cells were stimulated with 100 nM final concentration of the indicated ligands in SF-DMEM for a further 24 h, then cells were washed. RNA was extracted and cDNA was synthesized from 500 ng RNA then used in real-time qPCR. Relative mRNA levels of GILZ (a) and IL-6 (b) were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to each vehicle control set to 1 (a, b). The results are pooled from 3 independent experiments. (c) Cells seeded and transfected in parallel were harvested in SDS sample buffer and lysates were analyzed for GR and PR-B levels by western blotting using GAPDH as a loading control. A representative blot is shown. Blots were probed first for GR then stripped and re-probed for PR-B. Western blots were scanned and quantified for relative GR (d) or PR-B (e) levels by calculating integrated density values.GR levels are plotted relative to the untransfected control set to 100% (d), while PR-B levels are plotted relative to the untransfected control set to 1 (e). Statistical comparisons were carried out using a two-way ANOVA with Tukey's (a, b) or Dunnett's (d, e) multiple comparisons post test. Stars above bars indicate significance compared to each vehicle control (a, b) or to the untransfected control (d, e), unless otherwise indicated by lines, with ****, ***, ** and * indicating $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

Secondly, in End1/E6E7 cells in the presence of over-expressed PR-B (high GR/high PR), the basal levels of GILZ decreased (Appendix B, Fig. B4.4a) while IL-6 increased (Appendix B, Fig. B4.4b) compared to high GR/no PR. Increased basal IL-6 mRNA levels were observed with decreased GR protein levels (Appendix B, Fig. B4.4b), which has been observed previously in these cells (Verhoog et al. 2011). Furthermore with low GR/high PR, basal IL-6 mRNA levels were further increased, although not significantly (Appendix B, Fig. B4.4b). In HeLa cells, basal IL-6 levels significantly increased about 4-fold with high GR/high PR and low GR/high PR compared to high GR/no PR (Appendix B, Fig. B4.5b). Together these results indicate that the relative GR/PR levels can also regulate immune function gene expression in a ligand-independent manner.

Following the above results indicating that responses to MPA appear to change when the relative levels of GR/PR were altered, the effect of only MPA, without the GR/PR-specific ligands, on immunomodulatory gene regulation was investigated. The same RNA samples were re-analyzed together with additional independent experiments for MPA-induced GILZ and IL-6 gene expression in End1/E6E7 (2, to make n=8) and HeLa (1, to make n=4) cells (Fig 4.3.2.3). GR and PR-B protein levels were confirmed previously (Fig. 4.3.2.1c, Fig. 4.3.2.2c).

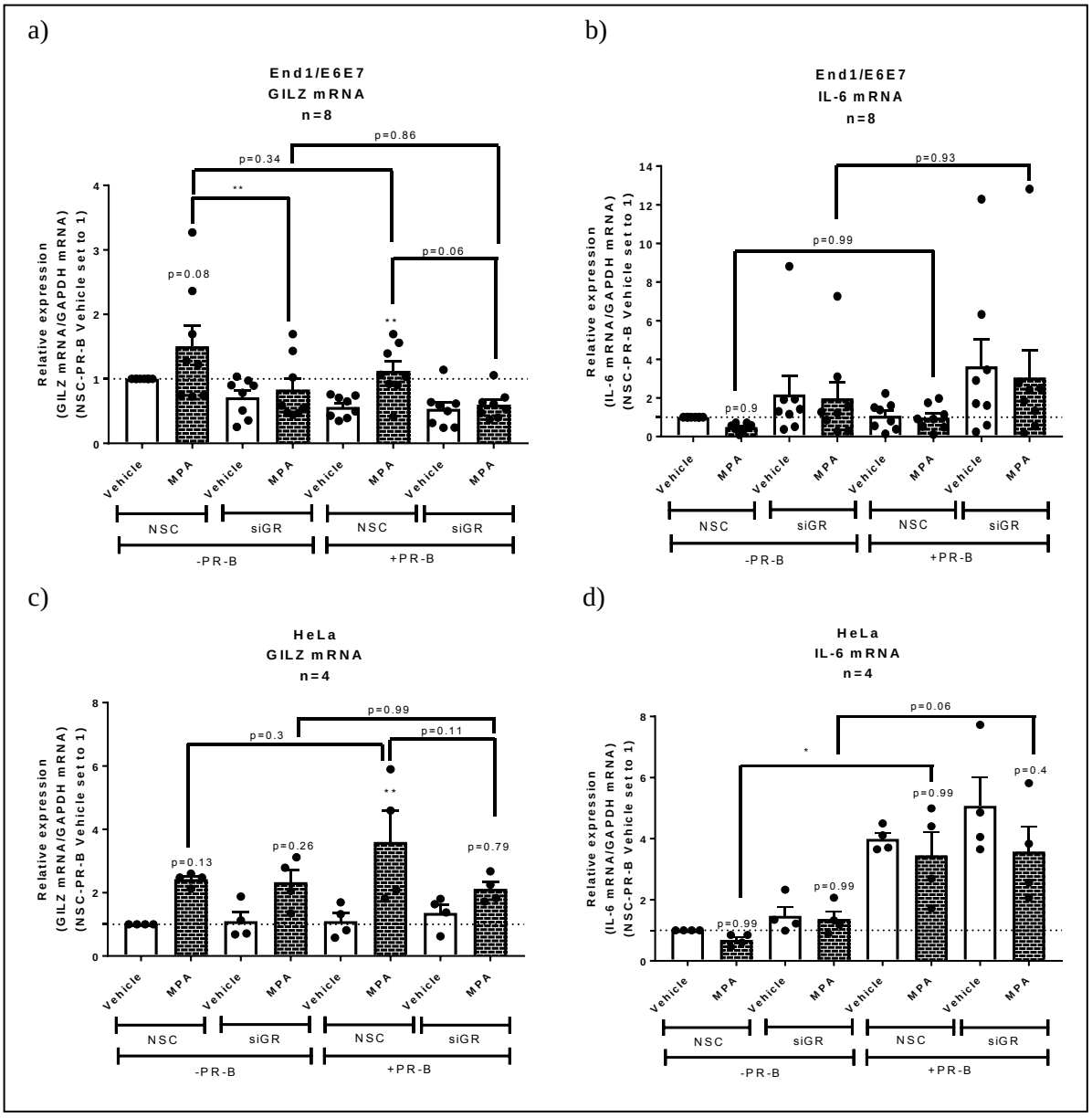


Figure 4.3.2.3. MPA-induced, GR-mediated regulation IL-6 in End1/E6E7 and HeLa cells changes in the presence of PR-B. Cells were seeded at 1.5 X 10^5 cells/well (End1/E6E7, a, b) or 1 X 10^5 cells/well (HeLa, c, d) in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 24 h, then transiently transfected with 250 ng/well (End1/E6E7, a, b) or 500 ng/well (HeLa, c, d) empty vector pcDNA3 or pMT-hPR-B for 24 h. Cells were stimulated with 100 nM final concentration of the indicated ligands in SF-KSFM (End1/E6E7, a, b) or SF-DMEM (HeLa, c, d) for a further

24 h, then cells were washed. RNA was extracted and cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of GILZ (a, c) and IL-6 (b, d) were normalized to GAPDH mRNA levels. Relative expression was determined either by normalizing to NSC-PR-B set to 1. The results are pooled from 8 (End1/E6E7, a, b) or 4 (HeLa, c, d) independent experiments. Statistical comparisons were carried out using a two-way ANOVA with Tukey's multiple comparisons post test. Stars above bars indicate significance compared to each vehicle control unless otherwise indicated by lines, with ** and * indicating $p<0.001$ and $p<0.05$ respectively. Decreased GR expression and PR-B over-expression were confirmed previously by western blotting in parallel samples (Fig. 4.3.2.1c-e; Fig. 4.3.2.2c-e).

Two main comparisons were made upon interpretation of these results: GR-mediated responses (comparing NSC vs siGR) and the effect of PR-B over-expression on GR-mediated responses (-PR-B vs +PR-B).

Analysis of GR-mediated responses showed that MPA induction of GILZ in End1/E6E7 cells required the GR, independent of PR-B levels (Fig. 4.3.2.3a). MPA induction of GILZ in HeLa cells did not change when GR levels were decreased (Fig. 4.3.2.3c). In the absence of PR-B, repression of IL-6 in End1/E6E7 (Fig. 4.3.2.3b) and HeLa (Fig. 4.3.2.3d) cells showed a trend of a GR-mediated response.

Analysis of the presence of PR-B on GR-mediated responses showed that repression of IL-6 by MPA (high GR/high PR) was not elevated when GR levels were decreased (low GR/high PR, siGR+PR-B) in either End1/E6E7 (Fig. 4.3.2.3b) or HeLa (Fig. 4.3.2.3d) cells. This may suggest that PR-B blocks GR-mediated repression of IL-6 in response to MPA. Interestingly, in HeLa cells, a GR-mediated effect on GILZ was only observed in the presence of PR-B, although not significant (Fig. 4.3.2.3c).

When comparing high GR/no PR (NSC-PR-B) to high GR/high PR (NSC+PR-B), or comparing low GR/no PR (siGR-PR-B) to low GR/high PR (siGR+PR-B), there were no significant differences in GILZ expression in End1/E6E7 cells (Fig. 4.3.2.3a). Similarly, the presence of PR-B had no significant effects on IL-6 expression in End1/E6E7 cells (Fig. 4.3.2.3b) or GILZ in HeLa cells (Fig. 4.3.2.3c). However, IL-6 expression significantly increased in the presence of PR-B in HeLa cells, both when comparing high GR/high PR to high GR/no PR, and comparing low GR/high PR to low GR/no PR (Fig. 4.3.2.3d).

Taken together, these results suggest that the effects of changing GR/PR on MPA-induced responses are gene- and cell type-specific. Furthermore this implies that the balance of pro-inflammatory versus anti-inflammatory responses to MPA depend on the relative levels of GR/PR. These findings are summarized in Table 6.3 in the Discussion.

4.3.3 Basal immunomodulatory gene expression changes when GR/PR levels are altered in End1/E6E7 and HeLa cells

In the absence of exogenous ligands, basal changes in immunomodulatory gene expression were also observed when the relative levels of GR/PR were altered (Fig. 4.3.2.3, Appendix B, Fig. B4.4 and B4.5). In order to further characterize basal changes in gene expression, the same RNA samples were re-analyzed for GILZ and IL-6 mRNA levels in End1/E6E7 and HeLa cells, and for a selection of additional immunomodulatory genes in HeLa cells. GR and PR-B protein levels were confirmed previously (Fig. 4.3.2.1c, Fig. 4.3.2.2c).

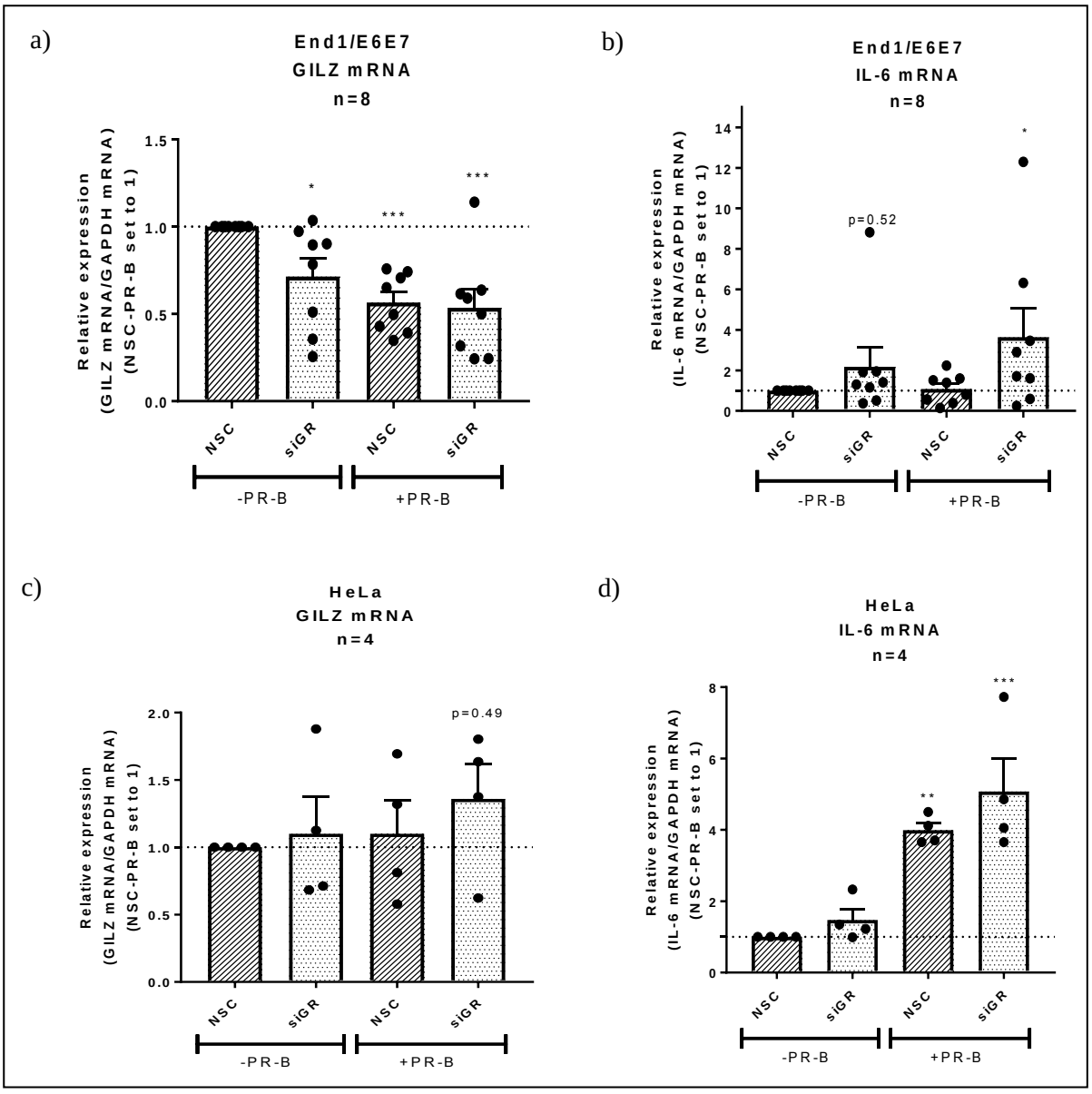


Figure 4.3.3.1. The basal expression of IL-6 in End1/E6E7 and HeLa cells, and GILZ in End1/E6E7 cells, changes when the relative levels of GR/PR are altered. Cells were seeded at 1.5 X 10^5 cells/well (End1/E6E7, a, b) or 1 X 10^5 cells/well (HeLa, c, d) in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 24 h, then transiently transfected

with 250 ng/well (End1/E6E7, a, b) or 500 ng/well (HeLa, c, d) empty vector pcDNA3 or pMT-hPR-B for 24 h. Cells were stimulated with 100 nM final concentration of the indicated ligands in SF-KSFM (End1/E6E7, a, b) or SF-DMEM (HeLa, c, d) for a further 24 h, then cells were washed. RNA was extracted and cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of GILZ (a, c) and IL-6 (b, d) were normalized to GAPDH mRNA levels. Relative expression was determined either by normalizing to NSC-PR-B set to 1. The results are pooled from 8 (End1/E6E7, a, b) or 4 (HeLa, c, d) independent experiments. Statistical comparisons were carried out using a two-way ANOVA with Dunnett's multiple comparisons post test. Stars above bars indicate significance compared to NSC-PR-B, with ***, ** and * indicating $p<0.001$, $p<0.01$ and $p<0.05$ respectively. Decreased GR expression and PR-B over-expression were confirmed previously by western blotting in parallel samples (Fig. 4.3.2.1c-e; Fig. 4.3.2.2c-e).

In End1/E6E7 cells, basal GILZ mRNA levels were significantly reduced with high GR/high PR (1.8-fold) and with low GR/high PR (1.9-fold), compared to high GR/no PR (Fig. 4.3.3.1a). Basal IL-6 mRNA levels significantly increased with low GR/high PR (3.6-fold, Fig. 4.3.3.1b).

In HeLa cells, contrary to End1/E6E7 cells, basal GILZ mRNA levels appeared to increase 1.4-fold with low GR/high PR compared to high GR/no PR (Fig. 4.3.3.1c). Basal IL-6 mRNA levels, however, were significantly increased with high GR/high PR (4-fold) and, like for End1/E6E7 cells, with low GR/high PR (5-fold, Fig. 4.3.3.1d). Taken together, these results suggest that the basal expression of GILZ and IL-6 are regulated in a PR-B-dependent, GR-dependent manner in End1/E6E7 cells and in a PR-B-dependent, GR-independent manner in HeLa cells.

To further assess gene-specific effects, additional immunomodulatory genes, including the pro-inflammatory chemokines RANTES and IL-8, the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine IL-12, were assessed by qPCR for changing expression in response to altered GR/PR levels in HeLa cells.

RANTES mRNA levels were near-significantly increased 12-fold with low GR/high PR compared to high GR/no PR (Fig. 4.3.3.2a). No statistically significant changes were observed for IL-8 (Fig 4.3.3.2b), IL-12p35 (Fig. 4.3.3.2c), IL-10 (Fig. 4.3.3.2d) or IL-12p40 (Fig. 4.3.3.2e) mRNA levels.

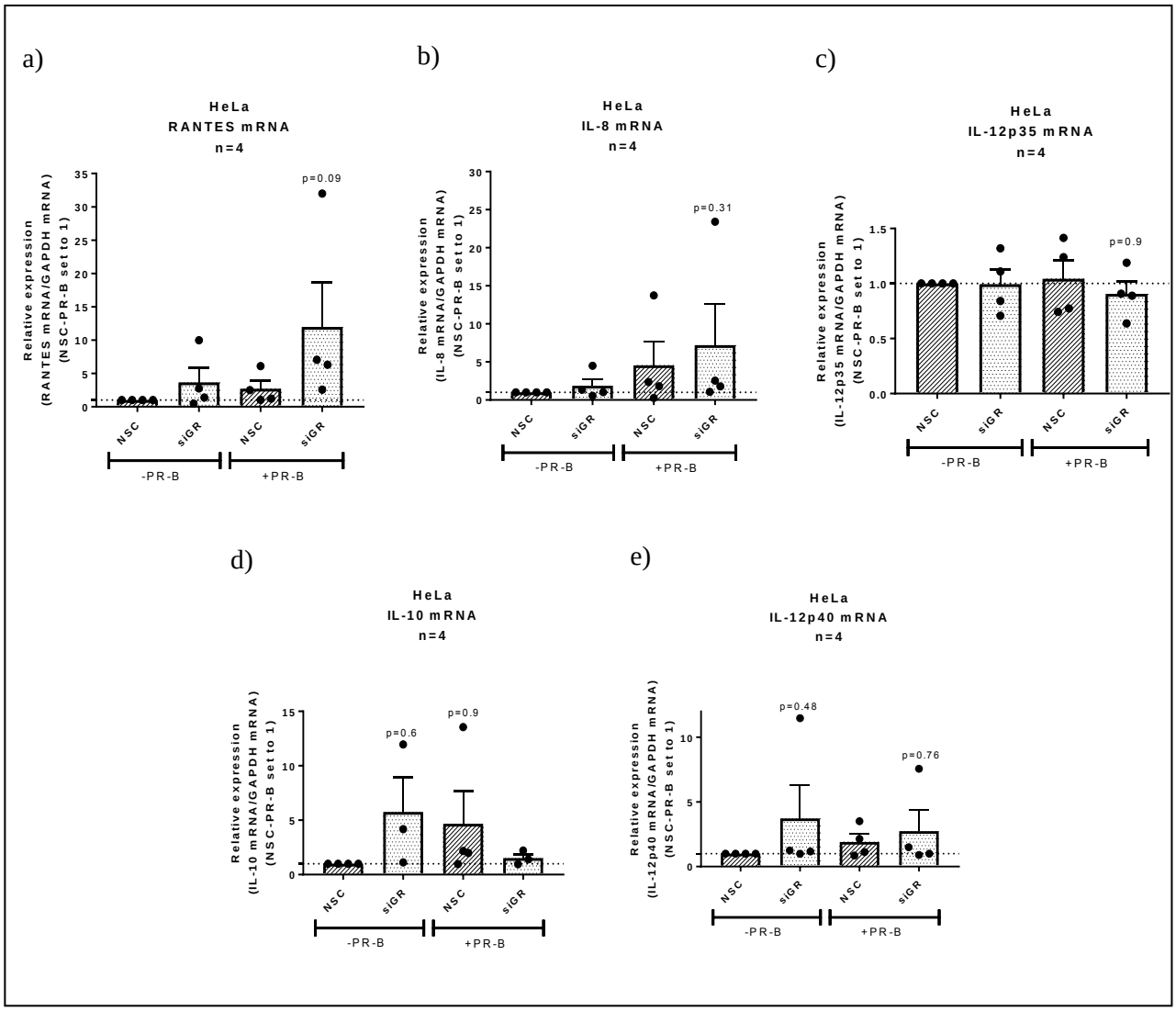


Figure 4.3.3.2. The basal expression of some but not other immunomodulatory genes appears to change when the relative levels of GR/PR are altered in HeLa cells. Cells were seeded at 1 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 24 h, then transiently transfected with 500 ng/well empty vector pcDNA3 or pMT-hPR-B for 24 h. Cells were incubated in SF-DMEM for a further 24 h, then washed. RNA was extracted and cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of RANTES (a), IL-8 (b), IL-12p35 (c), IL-10 (d) and IL-12p40 (e) were normalized to GAPDH mRNA levels. Relative expression was determined either by normalizing to NSC-PR-B set to 1 (b, d, f). The results are pooled from 4 independent experiments. Statistical comparisons were carried out using a two-way ANOVA with Dunnett's multiple comparisons post test. No significant differences were observed, but p values above bars indicate a comparison to NSC-PR-B. Decreased GR expression and PR-B over-expression were confirmed previously by western blotting in parallel samples (Fig. 4.3.2.2c-e).

These results suggest that the effects of changing the relative levels of GR/PR are different for different genes. Changing GR/PR in HeLa cells to low GR/high PR could increase expression of the immunomodulatory genes RANTES and possibly IL-8, in the absence of exogenous ligands, similar to IL-6 expression in both End1/E6E7 and HeLa cells. This increase in pro-inflammatory gene expression could subsequently alter the inflammatory response depending on the relative levels of GR and PR, in the FRT of women using either no hormonal contraceptives or with very low levels of MPA. These findings are summarized in Table 6.4 in the Discussion.

4.4 Differences in HIV-1 infection with changing GR/PR levels is mediated by soluble factors, but likely not IL-6

Previous results in this chapter showed that in the absence of exogenous ligands, changing the relative levels of GR/PR, particularly with low GR/high PR, resulted in a change in basal mRNA expression of some key cytokines and chemokines (Fig. 4.3.3.1, Fig. 4.3.3.2). Secreted soluble cytokines and/or chemokines are likely to play a role in modulating HIV-1 susceptibility in the FRT. In order to assess the effect of these soluble mediators on HIV-1 infection, growth media (supernatants) containing secreted modulators were collected from End1/E6E7 and HeLa cells in which GR/PR levels had been altered. Decreased GR expression and PR-B protein over-expression in End1/E6E7 (Fig. 4.3.2.1c-e) and HeLa (Fig. 4.3.2.2c-e) cells was confirmed by western blotting in previous samples. These supernatants were subsequently cultured together with HIV-$1_{BaL\_Renilla}$ (HIV-BaL) onto TZM-bl cells in a conditioned media add-back assay.

HIV-1 infection was significantly increased in TZM-bl cells cultured with End1/E6E7 supernatants in which the GR expression was decreased, both in the absence (1.7-fold, siGR-PR-B) and presence (1.5-fold, siGR+PR-B) of PR-B (Fig. 4.4.1a). In TZM-bl cells cultured with HeLa supernatants, however, HIV-1 infection only appeared to increase after exposure to supernatants from low GR/high PR (siGR+PR-B) HeLa cells (1.9-fold, Fig. 4.4.1b).

These results suggest that decreasing GR expression in End1/E6E7 cells, with or without the simultaneous over-expression of PR-B, causes, either directly or indirectly, changes the secretion of soluble mediators into the surrounding growth media that increase HIV-1 infection in TZM-bl cells. In HeLa cells, however, only low GR/high PR influences the secretion of soluble mediators that increase HIV-1 infection in TZM-bl cells, suggesting that in this system susceptibility to HIV-1 only increases with low GR/high PR. This would suggest a role for the GR in protecting against HIV-1 infection and for PR-B to increase susceptibility to HIV-1.

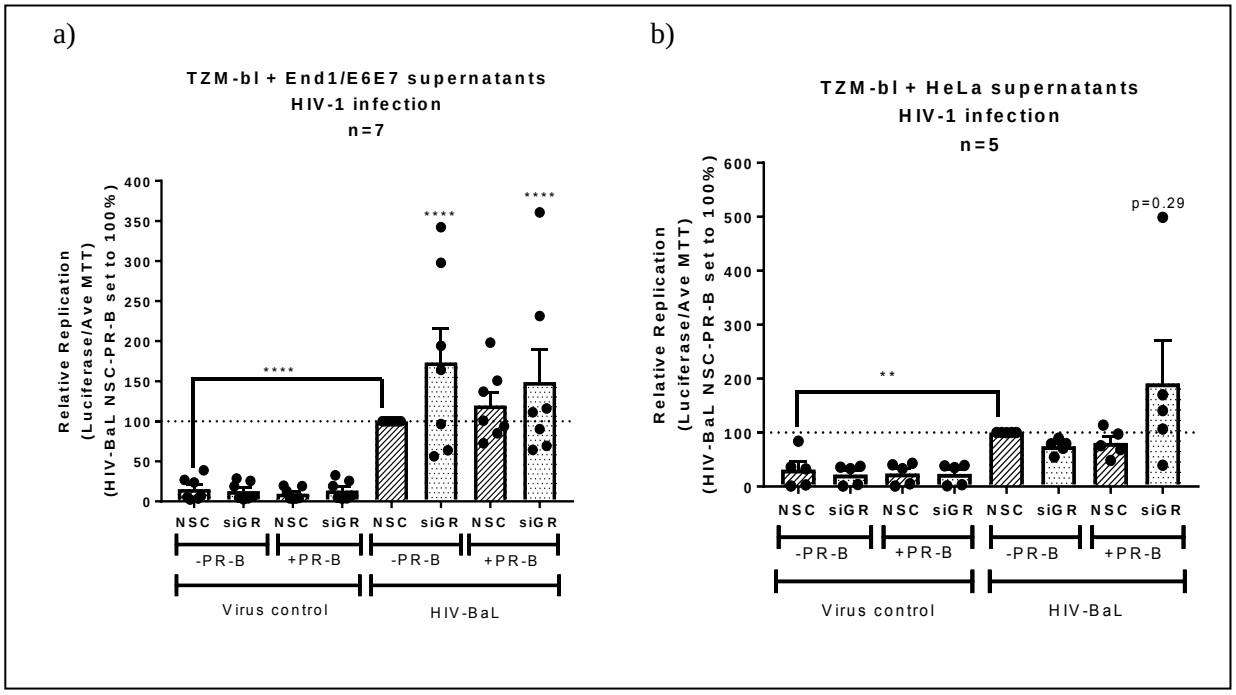


Figure 4.4.1. Low GR/high PR in the absence of exogenous hormones causes changes in soluble mediators from End1/E6E7 and HeLa cells that increase HIV-1 infection when cultured with TZM-bl cells. Growth media supernatants from End1/E6E7 (a) and HeLa (b) cells in which the relative levels of GR/PR were directly changed by decreased GR expression and PR-B over-expression were collected during previous experiments and stored at -80°C (Fig. 4.3.2.1, 4.3.2.2). TZM-bl cells were seeded in quadruplicate wells at 2.5 X 10^4 cells/well in 96-well plates and left to adhere overnight. Supernatants were thawed, diluted 1:20 with phenol red-free DMEM and half was incubated with 10 IU/mL HIV-$1_{BaL\_Renilla}$ (HIV-BaL) or equivalent volume of no-virus control (labelled as Virus control) for 1 h at 37°C, while the other half was added to the TZM-bl cells for 1 h at 37°C. The supernatant-virus/control mix was subsequently added onto the TZM-bl cells which were incubated for 72 h after which cells were harvested for luciferase (infection) and for cell viability (MTT). The results are pooled from 7 (End1/E6E7) or 5 (HeLa) independent experiments. Relative infection was calculated as luciferase (RLU) divided by average absorbance at 595nm (MTT) for the quadruplicate wells. Infection was plotted relative to NSC-PR-B in the presence of virus set to 100%. Statistical comparisons were carried out using a two-way ANOVA with Tukey's multiple comparisons post test (a) or an unpaired t test (b). Stars above bars indicate significance compared to NSC-PR-B in the presence of virus unless otherwise indicated by lines, with ****, ***, ** and * indicating $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

A possible candidate secreted factor that may play a role in HIV-1 infection following add-back onto TZM-bl cells is the cytokine IL-6, although it is not established that the change in IL6 levels affects HIV-1 infection. Basal IL-6 mRNA levels were shown to be up-regulated with low GR/high PR in End1/E6E7 and HeLa cells (Fig. 4.3.3.1b, d). Following this observation, IL-6 protein levels were determined by ELISA in the growth media collected from these cells.

IL-6 protein levels ranged from 100-400 pg/mL and were significantly increased 3.4-fold in supernatants collected from HeLa cells in which low GR/high PR were expressed, relative to high GR/no PR (NSC-PR-B, Fig. 4.4.2a). This mirrors the pattern observed for IL-6 mRNA in HeLa cells (Fig. 4.3.3.1d). In End1/E6E7 cells, however, IL-6 protein levels were 10 pg/mL or lower and appeared to be decreased with low GR/no PR and low GR/high PR (Fig. 4.4.2b). This could be because less IL-6 protein is expressed in End1/E6E7 than HeLa cells. Alternatively, there could be sample degradation in End1/E6E7 supernatants due to storage in SF-KSFM, which may be less cryoprotective than SF-DMEM in which HeLa supernatants were stored. However, this was not directly tested.

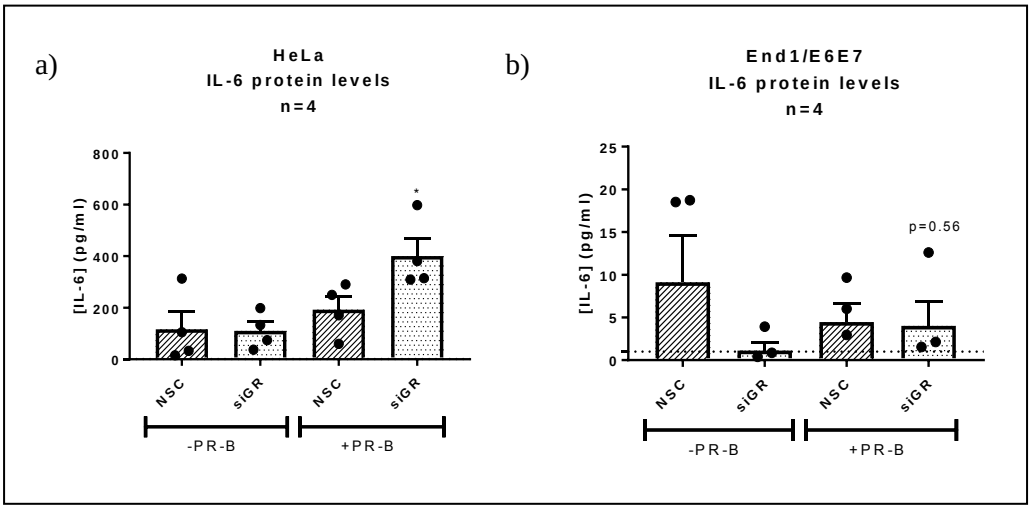


Figure 4.4.2. Secreted IL-6 protein increases when GR is knocked down and PR-B is over-expressed in HeLa but not End1/E6E7 cells. Growth media supernatants from End1/E6E7 (a) and HeLa (b) cells in which the relative levels of GR/PR were directly changed by decreased GR expression and PR-B over-expression were collected during previous experiments and stored at -80°C (Fig. 4.3.2.1, 4.5). Supernatants were diluted 1:20 and analysed for absolute IL-6 protein expression using the Human IL-6 High Sensitivity ELISA. Statistical comparisons were carried out using a two-way ANOVA with Dunnett's multiple comparisons post test. Stars above bars indicate significance compared to NSC-PR-B, with * indicating $p<0.05$.

Low GR/high PR was previously shown to up-regulate IL-6 mRNA (Fig. 4.3.3.1d) and IL-6 protein (Fig. 4.4.2a) in HeLa cells and cause changes in soluble factors from both End1/E6E7 and HeLa cells that increased HIV-1 infection in TZM-bl cells (Fig. 4.4.1a, b). These observations led to the consideration that IL-6 may be the soluble factor that increases HIV-1 infection. In order to test this hypothesis, TZM-bl cells were stimulated with increasing doses of exogenous recombinant IL-6 protein at the same time as infection with HIV-$1_{BaL\_Renilla}$ (HIV-BaL) using the same protocol as for the supernatant add-back assays. Since IL-6 concentrations in HeLa cells ranged from 100-400 pg/mL, TZM-bl cells were incubated with increasing doses of exogenous IL-6 ranging from 0-200 pg/mL. As shown in Figure 4.4.3, however, increasing doses of exogenous IL-6 used in the same manner as the supernatants did not increase HIV-1 infection in TZM-bl cells. This would suggest that IL-6 alone, within these concentrations, does not increase HIV-1 infection in this mode system. Thus it is likely that it is a combination of secreted factors, perhaps including RANTES and/or IL-8 (Fig. 4.3.3.2) that increase HIV-1 infection in TZM-bl cells exposed to End1/E6E7 or HeLa supernatants.

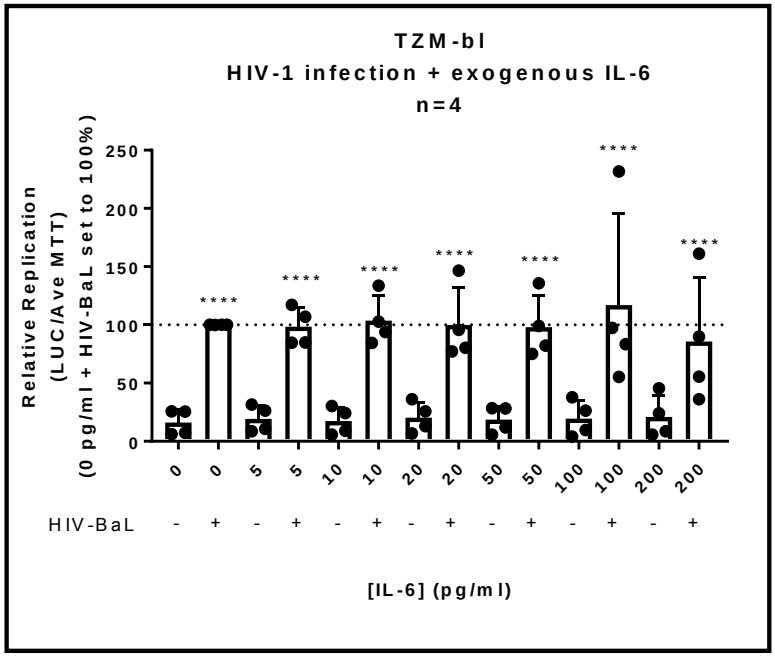


Figure 4.4.3. Exogenous IL-6 does not dose-dependently increase HIV-1 infection in TZM-bl cells. TZM-bl cells were seeded in quadruplicate wells at 2.5 X 10^4 cells/well in 96-well plates and left to adhere overnight. Recombinant IL-6 was diluted to the indicated concentration in phenol red-free DMEM and half was incubated with 10 IU/mL HIV-$1_{BaL\_Renilla}$ (HIV-BaL) or equivalent volume of no-virus control for 1 h at 37°C, while the other half was added to the TZM-bl cells for 1 h at 37°C. The IL-6-virus/control mix was subsequently added onto the TZM-bl cells which were incubated for 72 h after which cells were harvested for luciferase (infection) and for cell viability (MTT). The results are pooled from 4 independent experiments where each point was in quadruplicate. Relative infection was calculated as luciferase (RLU) divided by average absorbance at 595nm (MTT) for the quadruplicate wells. Infection was plotted relative to 0 pg/mL IL-6 in the presence of virus set to 100%. Statistical comparisons were carried out using a two-way ANOVA with Tukey's multiple comparisons post-test. Stars above bars indicate significance comparing each concentration to its own control in the absence of virus, with **** indicating $p<0.0001$.

Taken together, the results from this chapter show that MPA-induced HIV infection requires the GR in TZM-bl cells, and the unliganded GR plays an important role in protecting against HIV-1 infection in TZM-bl cells. In general, the presence of PR-B appears to increase susceptibility to HIV-1 infection in TZM-bl cells and alters the GR-regulated MPA-induced and ligand-independent expression of some key immunomodulatory genes such as IL-6, in End1/E6E7 and/or HeLa cells. Secreted soluble mediators change in response to changing relative GR/PR levels in End1/E6E7 and TZM-bl cells. A combination of these soluble mediators, but likely not IL-6 alone, appear to mediate increased HIV-1 infection in TZM-bl cells.

Chapter 5

Modulation of GR activity by PR potentially occurs via GR-PR association and co-localization

5.1 Background and aims

The previous research chapter established that the relative levels of GR/PR influence HIV-1 infection and expression of genes relevant to immune function in the absence of exogenous ligand, with PR generally increasing infection and GR generally protecting against infection. In addition, some experiments revealed that the presence of PR-B may decrease the transcriptional activity of the liganded GR. For example, the presence of PR-B inhibited the GR-mediated immunosuppressive effect of MPA on IL-6 in both End1/E6E7 (Fig. 4.3.2.1b) and HeLa cells (Fig. 4.3.2.2b). Furthermore there are hints that GR may modulate PR activity. For example, in the presence of PR-B, basal IL-6 mRNA in HeLa cells increased when GR levels were decreased (Fig. 4.3.2.3d, Fig. 4.3.3.1d) which might suggests that PR regulation of IL-6 might only occur with low GR.

In light of this apparent co-regulation of HIV-1 infection and immunomodulatory gene expression by GR and PR-B, the question arose as to whether GR and PR regulate the same gene/s at the same time and whether the GR and PR reciprocally modulate each other's activity. Within the scope of this study, the effect of PR-B on GR activity and the crude physical proximity of GR and PR were chosen as a starting point from which to investigate these broader issues. In addition it was investigated whether the presence of the less transcriptionally active PR isoform, PR-A, influenced the effects of PR-B on GR-mediated reporter gene regulation.

The aims of this section were therefore to answer the following specific research questions:

a) Does the PR-A/B inhibit the GR's activity?
b) Do GR and PR-B associate together?
c) Are transiently expressed GR and PR-B co-expressed within the same cells?
d) Do GR and P-BR co-localize in response to different ligands?

In order to answer these questions, over-expressed (in COS1 or U2OS cells) or endogenously expressed (in MCF-7 cells) GR and/or PR were assessed for ligand-dependent regulation of the GR/PR-responsive TAT-GRE-LUC reporter gene, ligand-dependent co-immunoprecipitation of GR and PR, and ligand-dependent cellular co-localization of the GR and PR by confocal and super-resolution microscopy. Dex and R5020 were used as GR- and PR-specific ligands, respectively, while MPA was used as a GR/PR agonist.

5.2 PR-A and PR-B individually repress GR-mediated activity on the TAT-GRE-LUC reporter gene in COS1 and U2OS cells over-expressing GR and/or PR

PR-A has been shown to act as a transdominant repressor of transcriptional activity of PR-B in CV-1 monkey kidney fibroblasts (Vegeto et al. 1993). This was confirmed in U2OS cells over-expressing PR-A and/or PR-B (Appendix B Fig. B5). Furthermore PR-A was demonstrated to inhibit the transcriptional activity of the GR *in vitro* (Vegeto et al. 1993). Interestingly some results from this study – such as PR-B altering GR-mediated anti-inflammatory regulation of IL-6 (Fig. 4.3.2.1b, Fig. 4.3.2.2b) - point towards PR-B as a potential inhibitor of GR activity.

In order to further test the effect of PR-B on GR-mediated activity, monkey fibroblast COS1 cells over-expressing GR and PR-A and/or PR-B were assessed for their ligand-dependent activation of the TAT-GRE-LUC reporter gene.

In the absence of over-expressed GR, the TAT-GRE-LUC reporter gene was significantly induced 3.7-fold by Dex, most likely mediated by the low levels of endogenous GR in COS1 cells (Fig. 5.2.1a). In the presence of over-expressed GR this Dex induction increased considerably to a significant 171-fold (Fig. 5.2.1a). In the presence of PR-A or PR-B alone, this Dex-induced reporter gene activity was significantly reduced to 117-fold or 94-fold, respectively, but when both PR-A and PR-B were over-expressed together, the repression of the Dex-induced response was lifted back to 193-fold induction (Fig. 5.2.1a).

For simplification, only the Dex-induced statistical differences are shown in Figure 5.2.1a, but other significant differences were also observed with R5020 and MPA. MPA significantly induced the TAT-GRE-LUC reporter gene in the presence of GR alone (48-fold), GR with PR-B (110-fold) and GR with both PR-A and PR-B (83-fold), but not GR with PR-A (21.5-fold, $p=0.589$, Fig. 5.2.1a). Similarly, R5020 significantly induced TAT-GRE-LUC in the presence of GR with PR-B (210-fold) and GR with both PR-A and PR-B (67-fold, Fig. 5.2.1a). As expected, the R5020-induced response was significantly greater in the presence of PR-B (210-fold) than PR-A (6.2-fold, Fig. 5.2.1a), since PR-B is more transcriptionally active than PR-A. Furthermore, consistent with the repressive function of PR-A on PR-B, R5020-induced activation of TAT-GRE-LUC was significantly lower when both PR-A and PR-B were present (67-fold) compared to PR-B only (210-fold, Fig. 5.2.1a). Over-expression of GR and PR was confirmed by western blotting (Fig. 5.2.1b) and densitometric analysis (Fig. 5.2.1c-e).

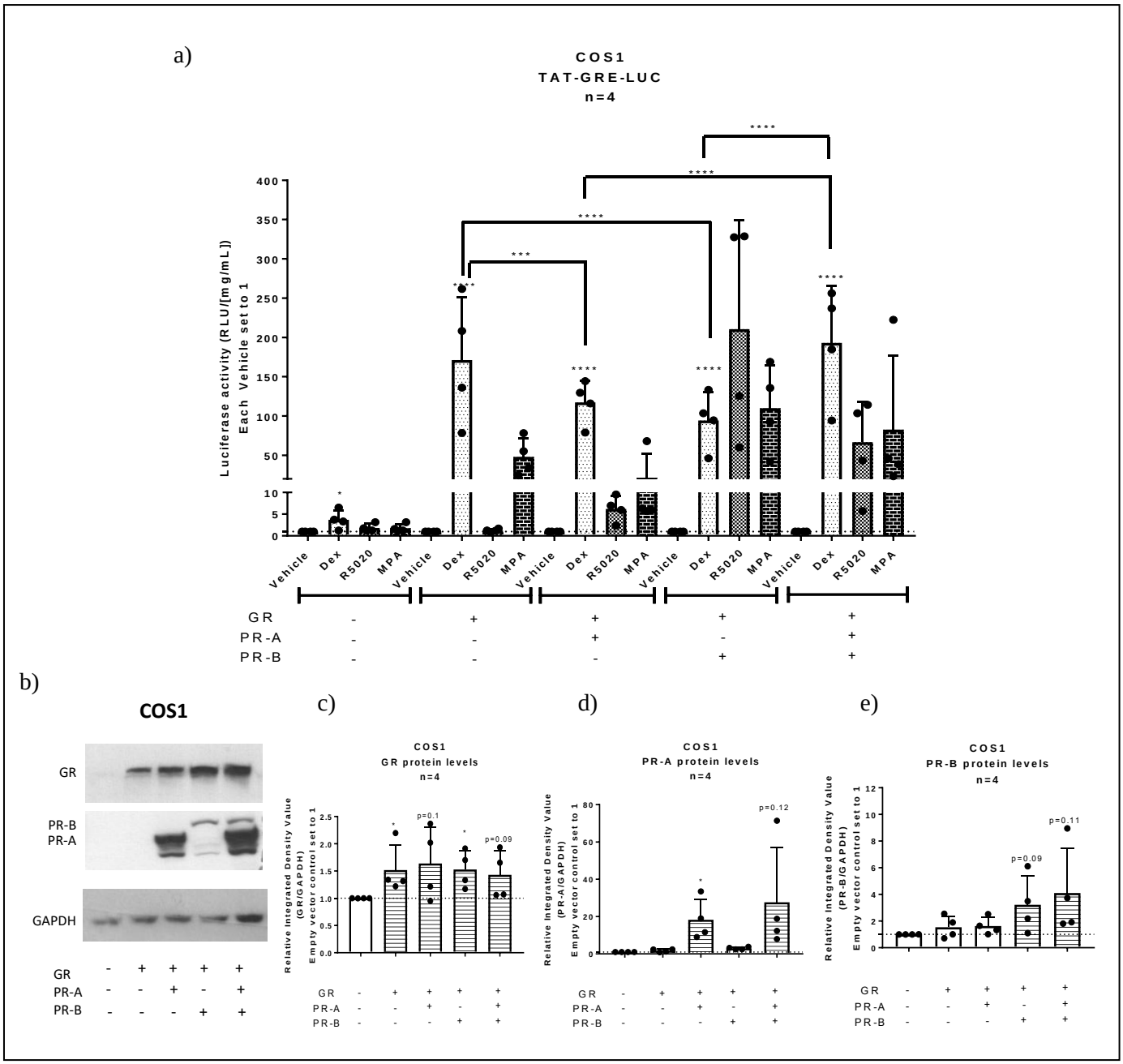


Figure 5.2.1. PR-A and PR-B individually, but not together, repress Dex-induced activation of the TAT-GRE-LUC reporter gene by GR in COS1 cells. (a) Cells were seeded at 7 X 10^4/well in 24-well plates and allowed to adhere overnight. Cells were subsequently transfected with 125 ng pcDNA3-hGR and/or pSG5-hPR-A and/or pMT-hPR-B and/or pcDNA3.1 and 47 ng pTAT-GRE-E1b-LUC for 24 h. Cells were stimulated in triplicate wells with 100 nM final concentration of the indicated ligands in SF-DMEM for 24 h, then washed and harvested in Reporter Lysis Buffer. Luciferase activity and total protein were measured. Luciferase activity was normalized to average protein concentration in mg/mL. Fold change was determined relative to each vehicle control set to 1. Data are pooled from 4 independent experiments where each point was in triplicate. (b) The remaining cell lysates were harvested in SDS sample buffer and analyzed for GR and PR-A/B levels by western blotting using GAPDH as a loading control. Blots were probed first for PR then stripped and re-probed for GR. A representative western blot is shown (b). Western blots were scanned and quantified for relative GR (c), PR-A (d) or PR-B (e) levels by calculating integrated density values. Protein levels are plotted relative to the pcDNA3 empty vector control set to 1. Statistical comparisons were carried out using a two-way ANOVA with Tukey's multiple comparisons post test (a) or a non-parametric Mann-Whitney t-test (c, -GR vs +GR) or a parametric unpaired t-test (c- e). Stars above bars indicate significant differences compared to

each vehicle control (a) or empty vector (-GR/PR-A/PR-B) control (c-e) unless otherwise indicated by lines, where ****, *** and * indicate $p<0.0001$, $p<0.001$ and $p<0.05$ respectively.

In order to test whether these observations were reproducible in a different cell line, human U2OS cells over-expressing GR and PR-A and/or PR-B were assessed for their ligand-dependent activation of the TAT-GRE-LUC reporter gene using the same protocol as for COS1 cells.

In U2OS cells, the TAT-GRE-LUC reporter gene was not induced by Dex in the absence of over-expressed GR and PR (Fig. 5.2.2a). However, in the presence of over-expressed GR, Dex significantly increased reporter gene expression by 117-fold (Fig. 5.2.2a). Similar to COS1 cells, in the presence of PR-A or PR-B alone, Dex-induced reporter gene activity was significantly reduced to 91-fold or 58-fold, respectively, but when both PR-A and PR-B were over-expressed together, the repression of the Dex-induced response was lifted back to 119-fold induction (Fig. 5.2.2a). This observation in both COS1 and U2OS cells is consistent with the apparent dampening of endogenous GR-mediated transcriptional regulation by Dex of endogenous GILZ in the presence of over-expressed PR-B in End1/E6E7 cells (Appendix B, Fig. B4.4a).

As for COS1 cells, only the Dex-induced statistical differences are shown in Figure 5.2.2a for U2OS cells, but R5020- and MPA-induced significant differences were also observed. The TAT-GRE-LUC reporter gene was significantly induced by MPA with GR alone (47-fold), GR with PR-B (75-fold) and GR with both PR-A and PR-B (28-fold), but not GR with PR-A (8-fold, $p=0.6577$, Fig. 5.2.2a). TAT-GRE-LUC was also significantly induced by R5020 in the presence of GR with PR-B (158-fold) and GR with both PR-A and PR-B (49-fold).

In addition, as for COS1 cells, the R5020-induced response was significantly greater in the presence of PR-B (158-fold) than PR-A (5.8-fold, Fig. 5.2.2a) for U2OS cells, although the difference was greater for COS1 cells. Also like COS1 cells, R5020-induced activation of TAT-GRE-LUC was significantly lower when both PR-A and PR-B were present (49-fold) compared to PR-B only (158-fold, Fig. 5.2.2a). Over-expression of GR and PR was confirmed by western blotting (Fig. 5.2.2b) and densitometric analysis (Fig. 5.2.2c-e). The differences in absolute fold changes between COS1 and U2OS cells may be attributed to different levels of endogenous GR (Appendix B, Fig. B6.1), different transfection efficiencies and/or other biological differences arising between cell lines from different species. Taken together, these data suggest that PR-B inhibits the GR-mediated activity on a reporter gene in both cell lines.

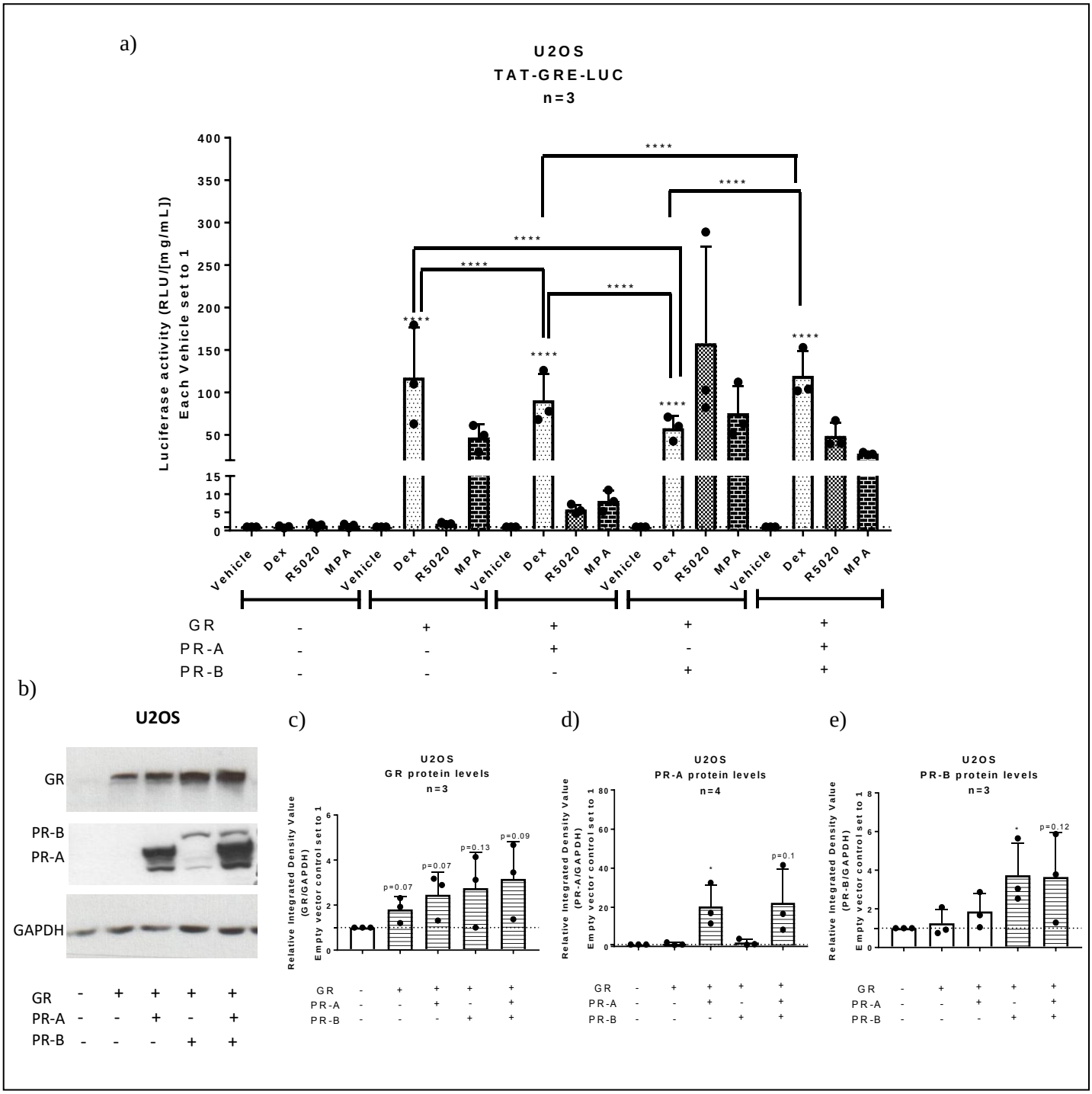


Figure 5.2.2. PR-A and PR-B individually, but not together, repress Dex-mediated activation of the TAT-GRE-LUC reporter gene by GR in U2OS cells. (a) Cells were seeded at 7 X 10^4/well in 24-well plates and allowed to adhere overnight. Cells were subsequently transfected, stimulated and harvested as described for COS1 cells (Fig. 5.2.1). Luciferase activity was normalized to average protein concentration in mg/mL. Fold change was determined relative to each vehicle control set to 1. Data are pooled from 3 independent experiments where each point was in triplicate. (b) The remaining cell lysates were harvested in SDS sample buffer and analyzed for GR and PR-A/B levels by western blotting using GAPDH as a loading control. Blots were probed first for PR then stripped and re-probed for GR. A representative western blot is shown (b). Western blots were scanned and quantified for relative GR (c), PR-A (d) or PR-B (e) levels by calculating integrated density values. Protein levels are plotted relative to the pcDNA3 empty vector control set to 1. Statistical comparisons were carried out using a two-way ANOVA with Tukey's multiple comparisons post

test (a) or a parametric unpaired t-test (c-e). Stars above bars indicate significant differences compared to each vehicle control (a) or empty vector (-GR/PR-A/PR-B) control (c-e) unless otherwise indicated by lines, where **** and * indicate $p<0.0001$ and $p<0.05$ respectively.

5.3 GR and PR associate in the same protein complex

In order to test the hypothesis that the presence of PR modulates the activity of GR by the receptors associating with each other, the ability of GR and PR to co-immunoprecipitate was investigated in COS1 cells over-expressing GR and PR-B. These cells were chosen as they express very low levels of endogenous GR and are readily transfected in order to over-express GR and PR.

PR-B was co-immunoprecipitated with anti-GR antibody, both in the absence and presence of MPA, although there was greater co-immunoprecipitation with MPA in the indicated western blot (Fig. 5.3.1a) and in total for three out of five experiments (Fig. 5.3.1b). When experiments were pooled together (n=5) however, the 2.4-fold greater PR-B co-immunoprecipitation in response to MPA compared to the vehicle control was not statistically significant (Fig. 5.3.1b). Therefore it cannot be concluded that PR-B co-immunoprecipitation with GR is a ligand-dependent interaction. In all experiments, PR protein was not detected in the input samples (Fig. 5.3.1a, b), which may be due to low levels of PR below the limit of detection by western blot. As expected, GR was immunoprecipitated with anti-GR antibody in the presence compared to the absence of over-expressed GR (Fig. 5.3.1a, c). A faint GR band is visible in the control lane (-GR), which most likely indicates the low levels of endogenous GR in COS1 cells (Fig. 5.3.1a). GR protein was only detected in the input samples in which GR was over-expressed (Fig. 5.3.1a, c).

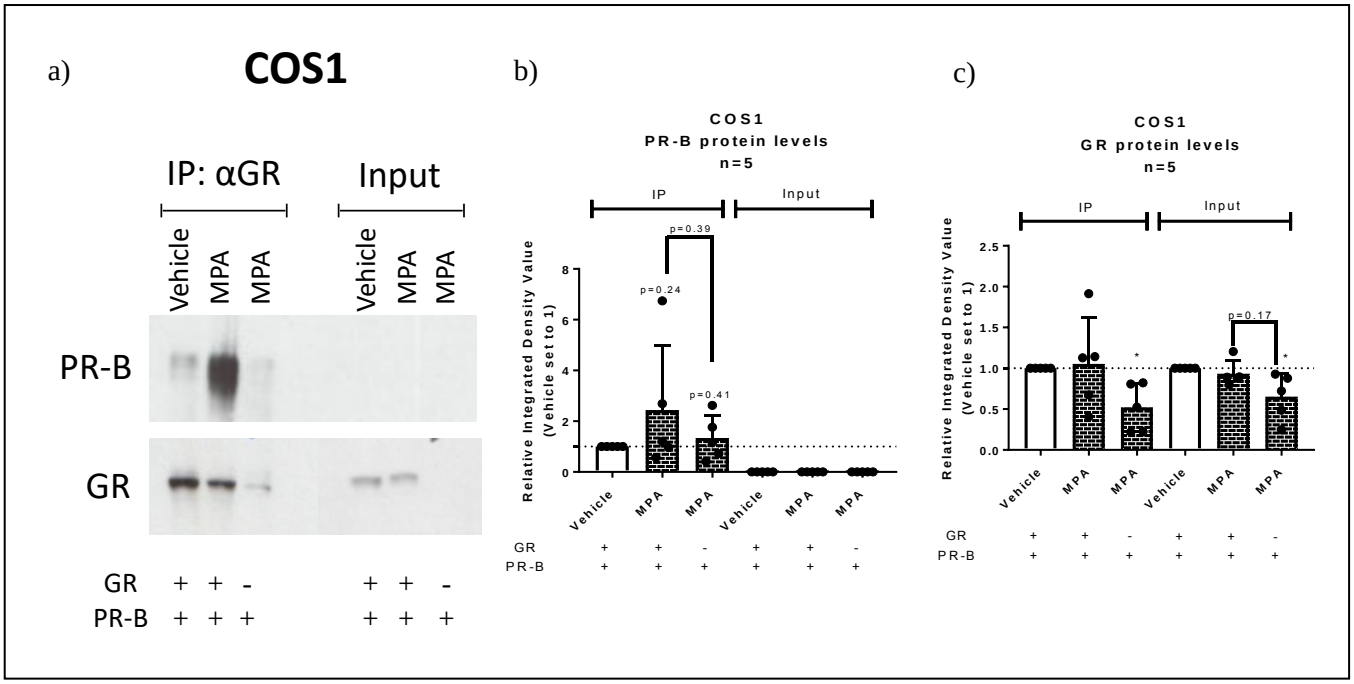


Figure 5.3.1. PR-B is co-immunoprecipitated with anti-GR antibody in COS1 cells over-expressing GR and PR. COS-1 cells were seeded at 1.5 X 10^6/dish in 10 cm dishes and allowed to adhere overnight. Cells were then transiently transfected with 3 µg pMT-hPR-B and 3 µg pcDNA3.1 or pcDNA3-hGR for 24 h and stimulated with 1 µM MPA, using EtOH as the vehicle control, for 1 h. Cells were lysed and an aliquot was analysed by western blotting for input levels of GR and PR-B. The remaining cell lysate was immunoprecipitated with anti-GR antibody and magnetic protein A/G beads. Co-immunoprecipitated proteins were analysed by western blotting on separate blots for GR and PR-B to ensure no cross-reaction between anti-GR or –PR antibodies. A western blot showing the biggest differences is shown (a). GR (95 kDa) and PR-B (114 kDa) were identified by size relative to the protein molecular weight marker. Western blots were scanned and quantified for relative PR-B (b) and GR (c) levels by calculating integrated density values. Protein levels are plotted relative to the vehicle control for both IP and input. The data are pooled from 5 independent experiments. Statistical comparisons were carried out using parametric unpaired t-tests (b, c) or a non-parametric Wilcoxon t-test (c, input Vehicle vs MPA). Stars above bars indicate significant differences compared to each vehicle control (b, c) unless otherwise indicated by lines, where ** and * indicate $p<0.01$ and $p<0.05$ respectively.

In order to eliminate any confounding factors introduced by transient transfection, the potential association of GR and PR was also investigated by co-IP in MCF-7 cells. These cells were chosen as they express detectable levels of both endogenous GR and PR.

In MCF-7 cells, PR was co-immunoprecipitated with anti-GR antibody, both in the absence and presence of MPA (Fig. 5.3.2a, b). No significant difference between the vehicle control and MPA stimulation was observed, suggesting that the GR-PR association does not depend on MPA (Fig. 5.3.2b). As expected, GR was immunoprecipitated independent of stimulation with ligand (Fig. 5.3.2a, c). The lack of signal in the IgG control lane (sample incubated with anti-rabbit secondary

antibody) confirmed that PR and GR pull-down were specific (Fig. 5.3.2b, c). Equal GR and PR levels in the input samples were also observed (Fig. 5.4b, c), although PR-A protein levels appeared to be greater than PR-B (Fig. 5.3.2a). While both PR-A and PR-B were distinguishable in the input lanes, due to over-exposure of the western blot, the isoform that predominantly co-immunoprecipitates with GR cannot be conclusively identified (Fig. 5.3.2a), but is labelled as PR-B for densitometric analysis (Fig. 5.3.2b).

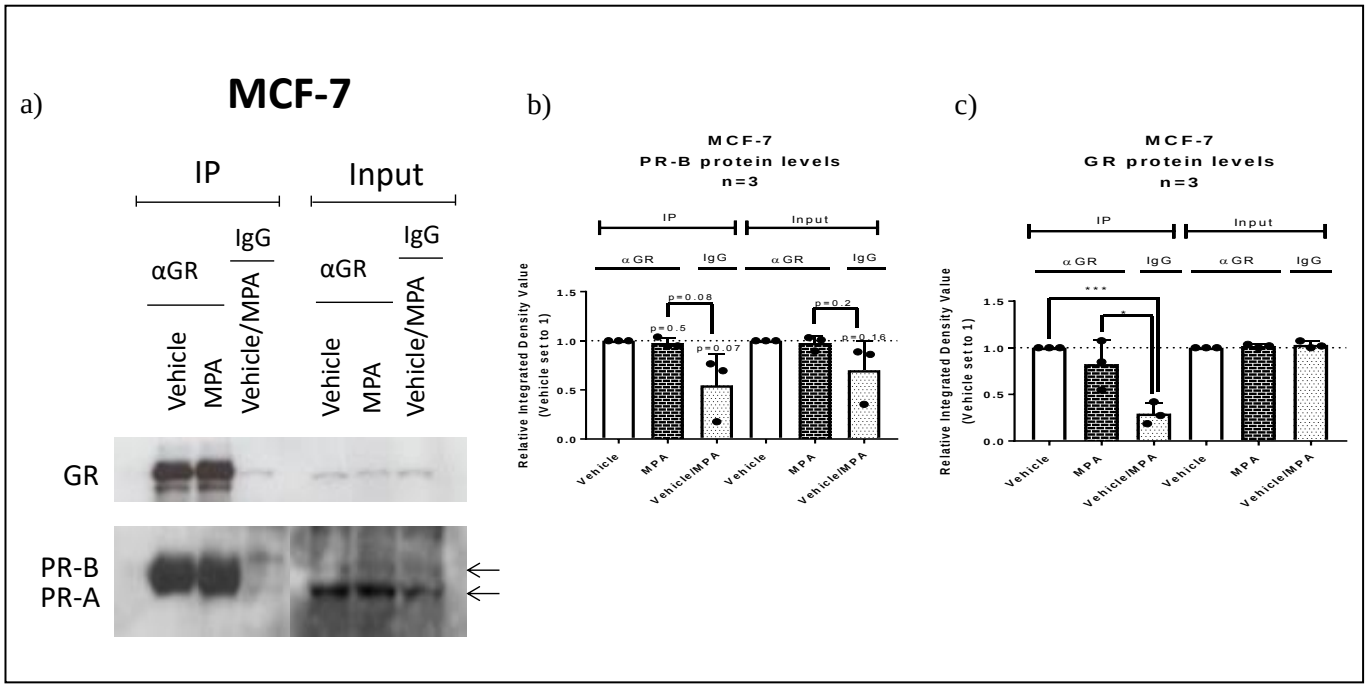


Figure 5.3.2. PR is co-immunoprecipitated with anti-GR antibody in both the absence and presence of MPA in MCF-7 cells endogenously expressing both GR and PR. MCF-7 cells were seeded at 1.5 X 10^6/dish in 10 cm dishes and allowed to adhere for 48 h. Cells were then stimulated with 1 μM MPA, using EtOH as the vehicle control, for 1 h. Cells were lysed and an aliquot was analysed by western blotting for input levels of GR and PR. The remaining cell lysate was immunoprecipitated with anti-GR antibody (Vehicle, MPA) and magnetic protein A/G beads. As a control, a mixture of equal volumes of Vehicle and MPA-stimulated cell lysates was incubated with anti-rabbit secondary antibody (IgG). Co-immunoprecipitated proteins were analysed by western blotting on separate blots for GR and PR-B to ensure no cross-reaction between anti-GR or –PR antibodies. A western blot showing the biggest differences is shown (a). GR (95 kDa), PR-A (95 kDa) and PR-B (114 kDa) were identified by size relative to the protein molecular weight marker. Western blots were scanned and quantified for relative PR-B (b) and GR (c) levels by calculating integrated density values. Protein levels are plotted relative to the vehicle control for both IP and input. The data are pooled from 3 independent experiments. Statistical comparisons were carried out using parametric unpaired t tests. Stars above bars indicate significant differences compared to each vehicle control (b, c) unless otherwise indicated by lines, where *** and * indicate $p<0.001$ and $p<0.05$ respectively.

In order to determine whether GR could also co-immunoprecipitate with PR, the reciprocal pull-down assay was carried out in MCF-7 cells. While PR was immunoprecipitated with anti-PR antibody, and GR protein was detectable in the input samples, there was insufficient GR protein co-immunoprecipitated with anti-PR for detection by western blotting (data not shown). This avenue warrants further investigation, since GR-PR association should be confirmed by a reciprocal co-IP assay. Nevertheless, taken together, these results suggest that GR and PR associate, within the same protein complex in a ligand-independent manner, both in COS1 cells over-expressing GR/PR and in MCF-7 cells expressing endogenous GR/PR.

5.4 GR and PR are co-expressed within the same cells

Crucial to the interpretation of reporter gene results in COS1 cells over-expressing GR and PR is the confirmation that GR and PR are co-expressed within the same cells. This would ensure the overall result is not a reflection of populations of cells expressing only GR or only PR. In order to test that GR and PR are expressed together in the same cells, COS1 cells were transfected and stimulated with Dex, R5020 or MPA using the same protocol used in the reporter assay (Fig. 5.2.1) and analyzed visually for transfected cells by immunofluorescence using confocal microscopy.

Overall only 17% of cells were successfully transfected. However, of those cells that were transfected, 80% expressed both GR and PR together (Fig. 5.4a). A small percentage of cells expressed only PR (12%) or only GR (8%, Fig. 5.4a). When further divided according to ligand stimulation or vehicle control, 70-90% of transfected cells expressed both GR and PR together (Fig. 5.4.1b).

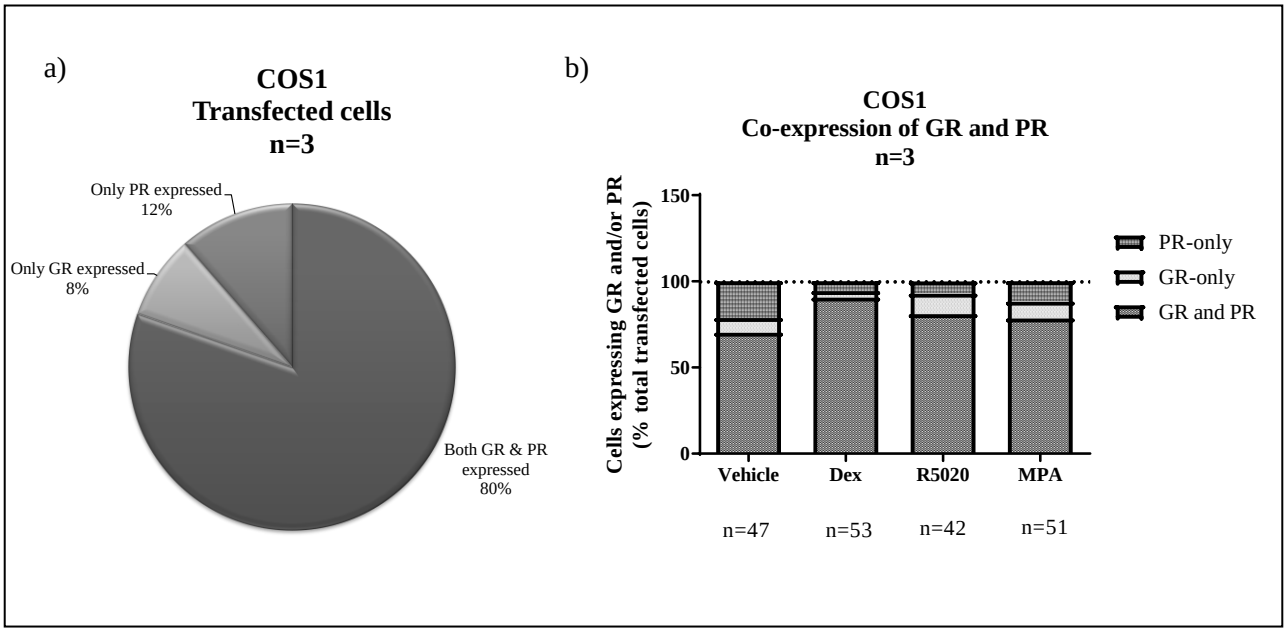


Figure 5.4. GR and PR are co-expressed within the same cells, in COS1 cells over-expressing GR and PR. COS1 cells were seeded at 1 X 10^5 cells/well in 6 well plates containing sterilized coverslips and allowed to adhere overnight. Cells were transiently transfected with 125 ng each pcDNA3-hGR, pMT-hPR-B and pSG5-hPR-A for 24 h, then stimulated with 100 nM final concentration of Dex, R5020 or MPA in SF-DMEM for 24 h, with 0.1% (v/v) EtOH as the vehicle control. Coverslips were washed, stained with anti-GR and anti-PR primary antibodies, fluorophore-conjugated secondary antibodies and Hoechst for nuclear staining. Coverslips were then mounted onto glass slides prior to immunofluorescence visualization using a confocal microscope. Images captured were counted manually for the number of transfected cells expressing GR and/or PR. The data shown are pooled from 3 independent experiments, containing 4-7 fields of view captured per slide and 1-30 cells per field of view. (a) percentages of combined transfected cells expressing GR only, PR only or both GR and PR. (b) percentages of transfected cells classified by ligand expressing GR only, PR only or both GR and PR. The total number of transfected cells analysed is indicated by the n value under each ligand.

These results confirm that GR and PR are co-expressed within the same cells, in COS1 cells transiently transfected with GR and PR and stimulated with GR- and/or PR-specific ligands.

5.5 PR is mostly nuclear while GR is located in both the cytoplasm and nucleus

The cellular distribution (i.e. nuclear or cytoplasmic localization) of GR and PR in response to Dex, R5020 or MPA in the COS1 cells over-expressing both GR and PR was also assessed by immunofluorescence. The localization was determined by visual examination of the fluorescent images and estimated to be nuclear when the fluorescent signal of the PR (red) or GR (green) channel overlapped with the blue signal of the nuclear stain (Hoechst, Fig. 5.6.1).

While PR is expected to be localized in the nucleus in the absence and presence of PR ligands, GR is expected to translocate from the cytoplasm into the nucleus upon activation by GR ligands. Consistent with this, PR was entirely nuclear in response to R5020 and MPA (Fig. 5.5a). For Dex and the vehicle control, about 25% of cells showed PR localization in both the cytoplasm and nucleus, with PR being entirely nuclear in the remaining cells (Fig. 5.5a). No PR was localized to the cytoplasm only in any of the conditions (Fig. 5.5a).

In the absence of ligands, GR was localized to the cytoplasm only in 35% of cells and to both the cytoplasm and nucleus in 62% of cells (Fig. 5.5b), suggesting that not all GR resides in the cytoplasm in the absence of ligands. Upon stimulation with any of the ligands, no GR was localized to the cytoplasm only (Fig. 5.5b). With Dex stimulation, GR was localized to the nucleus only in 28% of cells, and localized to the cytoplasm and nucleus in the remaining 72% of cells (Fig. 5.5b). In response to MPA, GR was entirely nuclear in 78% of cells, and localized to both the cytoplasm and nucleus in 28% of cells (Fig. 5.5b). Surprisingly, GR was entirely nuclear in 74% of cells stimulated with R5020, and both cytoplasmic and nuclear in the remaining 22% of cells (Fig. 5.5b). This suggests that GR can translocate into the nucleus in response to R5020, supposedly a PR-specific ligand (Fig. 5.5b).

Taken together, these results show that the PR is mostly localized to the nucleus ligand-independently, while the GR is both cytoplasmic and nuclear in the absence of ligands but is mostly nuclear in response to GR- and PR-specific ligands. This suggests that in COS1 cells expressing both GR and PR, there is overlapping GR/PR localization in the nucleus in both the presence and absence of ligands.

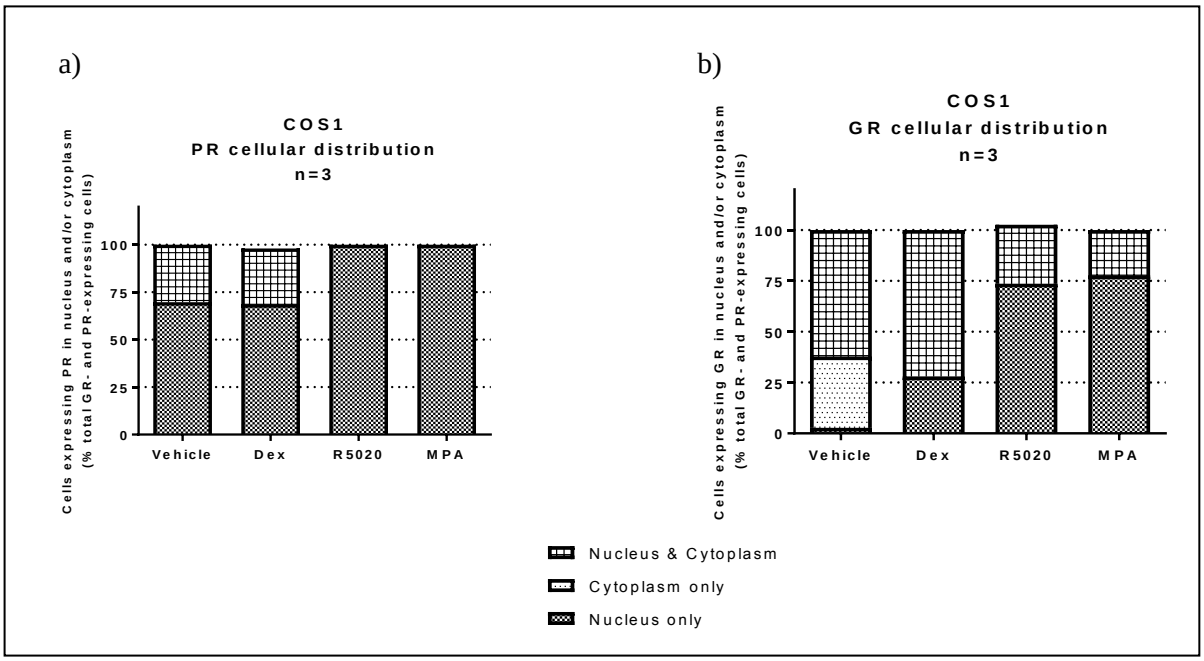


Figure 5.5. Increased nuclear versus cytoplasmic localization of GR and PR is observed in response to R5020 and MPA compared to the vehicle control in COS1 cells over-expressing both GR and PR. COS1 cells were prepared on glass slides as for Fig. 5.4 and visualized using a confocal microscope. Images captured were counted manually for the number of cells containing GR and PR localized to the nucleus and/or cytoplasm. Images shown are representative of 3 independent experiments, containing 4-7 fields of view captured per slide and 1-30 cells per field of view. Data shown are percentage of PR (a) or GR (b) expressed in the cytoplasm only, nucleus only or both cytoplasm and nucleus. The total number of transfected cells analysed is indicated by the n value under each ligand in Fig. 5.4b.

5.6 GR and PR co-localize in the nucleus in response to GR- and/or PR-specific ligands

Following the observation that GR and PR are expressed within the same COS1 cells and they both localize to the nucleus in the absence and presence of ligands, nuclear co-localization of GR and PR was quantified by immunofluorescence in the absence and presence of ligands. Whether or not GR and PR co-localize was determined here by whether signals within one fluorescent channel co-localize with signals in the other fluorescent channel. The extent of co-localization was quantified using a variety of parameters: the Manders co-localization coefficients, M1 and M2, which represent the proportion of GR signals overlapping with PR signals (M1) and the proportion of PR signals overlapping with GR signals (M2); and the Manders-derived overlap coefficient (O) which represents the degree of overlap between the two channels (Manders et al. 1993, Bolte and Cordelieres 2006). With M1/M2, co-localization is inferred with values between 0.5 and 1, for both channels; whereas no

co-localization is indicated when values are between 0 and 0.5. For the overlap coefficient O, co-localization is inferred by values between 0.6 and 1, and no co-localization indicated by values between 0 to 0.6 (Bolte and Cordelieres 2006). However, it should be noted that co-localization, as determined by this method, can only be used to suggest close spatial co-occurrence or a possible GR-PR association, given that co-localization cannot prove that the two receptors inhabit the same space, due to resolution limitations.

The white regions of the representative merged overlay images (Fig. 5.6.1) suggest that the detectable signals for GR and PR overlap in the nucleus in response to the ligands Dex, R5020 and MPA, but not the vehicle control, where GR was mostly cytoplasmic and PR was mostly found in the nucleus. Quantification of co-localization for all pooled experiments confirmed that GR and PR do not appear to co-localize in the absence of ligands (Table 5.6.1).

In the presence of the GR-specific agonist Dex, 83% of GR significantly co-localized with PR (M1) and 76% of PR significantly co-localized with GR (M2, Table 5.6.1), suggesting GR and PR co-localize in response to Dex. In the presence of the PR-specific agonist R5020, 84% of GR significantly co-localized with PR (M1) but only 61% of PR co-localized with GR (not significant, M2, Table 5.6.1). For the GR/PR agonist MPA, 74% of GR near-significantly co-localized with PR (M1) and 77% of PR significantly co-localized with GR (M2, Table 5.6.1), suggesting that GR and PR co-localize in response to MPA, similar to R5020. Consistent with the above M1/M2 values, the overlap coefficients (O) indicate significant GR/PR nuclear co-localization occurs in response to Dex, R5020 and MPA (Table 5.6.1).

Table 5.6.1. Quantification of co-localization of GR and PR in COS1 cells in response to Dex, R5020 and MPA using confocal microscopy.

		GR co-localized with PR (M1)		PR co-localized with GR (M2)		Overlap (O)	
	n	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Vehicle	22	0.483	0.074	0.39	0.078	0.666	0.044
Dex	46	0.83^{*}	0.041	0.76^{**}	0.05	0.866^{****}	0.023
R5020	29	0.84^{***}	0.061	0.61	0.072	0.821^{***}	0.017
MPA	31	$0.741^{p=0.0561}$	0.063	0.765^{***}	0.062	0.857^{****}	0.016

Data are shown for 3 independent experiments. Number of GR/PR-expressing cells evaluated is indicated by n value for each condition. Statistical comparisons were carried out comparing ligands to the vehicle control using a non-parametric Kruskal-Wallis ANOVA with Dunn's post-test (M1, M2) or a parametric one-way ANOVA with Dunnett's post-test (O). Stars next to the mean values indicate significance compared to the vehicle control, with ****, ***, ** and * indicating $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

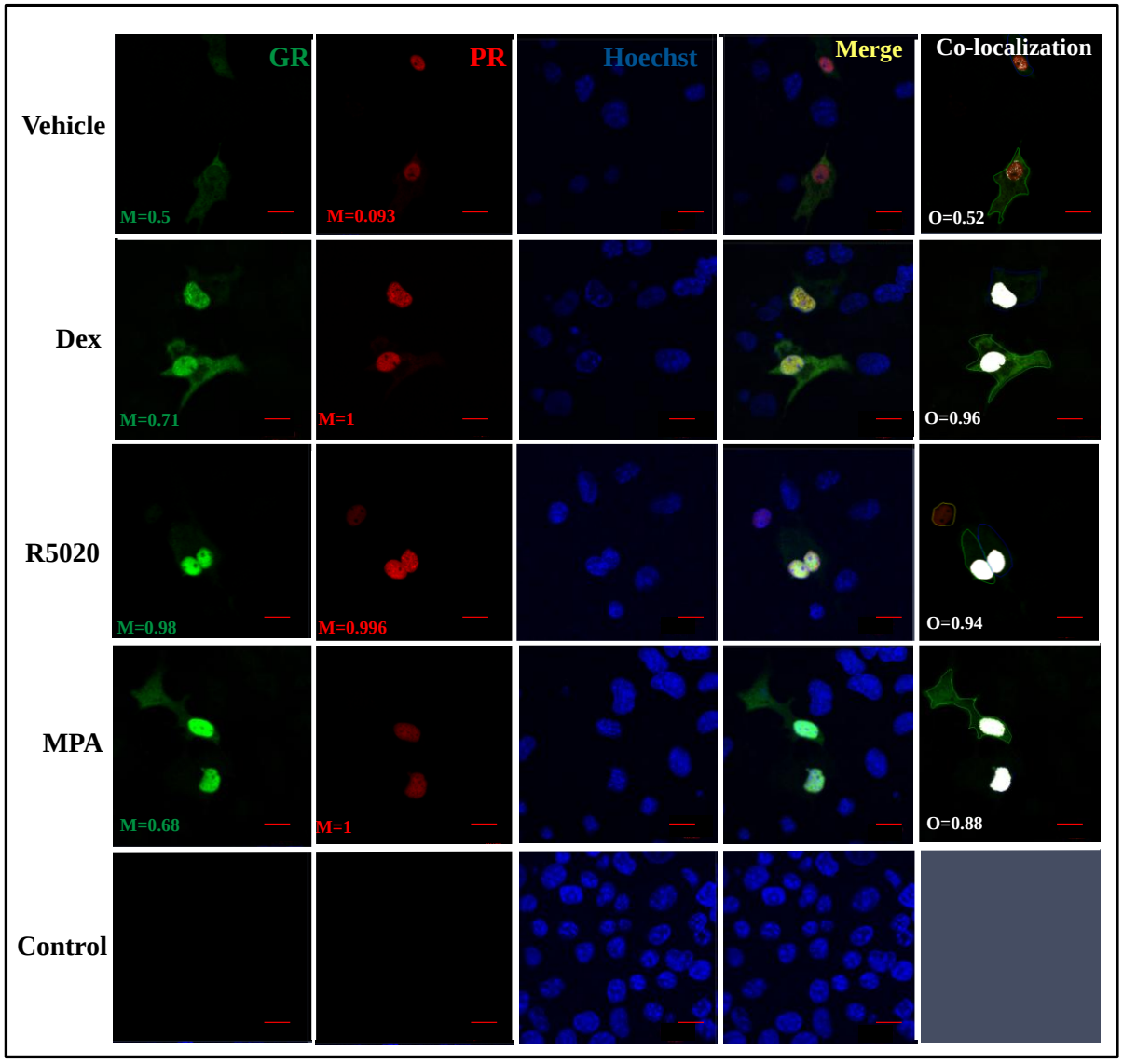
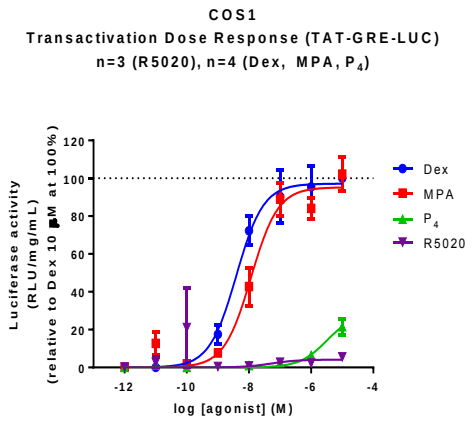


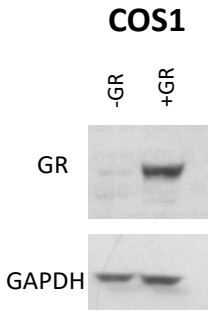
Figure 5.6.1. GR and PR co-localize in the nucleus in response to Dex, R5020 and MPA in COS1 cells over-expressing both GR and PR. COS1 cells were prepared on glass slides as for Fig. 5.4 and visualized using a confocal microscope. Images shown are representative of 3 independent experiments, containing 4-7 fields of view captured per slide and 1-30 cells per field of view. The green channel indicates detection of GR, the red channel indicates detection of PR and the blue channel indicates the nuclei by Hoechst staining. The merge panel indicates overlay of the red, green and blue channels. Quantification of co-localization was carried out on the merged images using ZEN software, generating the co-localization panel. Yellow regions in the merge panel and white regions in the co-localization panel indicate overlap of the red and green channels. The control panel represents the secondary antibody-only control slide. Co-localization was quantified using Manders coefficients (Table 5.6.1). M: Manders co-localization coefficient; indicates the mean coefficient across the double positive transfected cells showing the degree of red (PR) in the green (GR) channel and the degree of green (GR) in the red (PR) channel. O: Overlap coefficient; indicates the mean overlap coefficient across the double positive transfected cells showing the degree of overlap of the two channels. The scale bar (red) indicates 20 μm.

The observation that GR translocated into the nucleus in response to R5020, led to the concern that R5020 was perhaps activating the GR. In order to test this idea, the activation of over-expressed GR on the reporter gene TAT-GRE-LUC in response to increasing doses (1 pM-1 μM final concentration) of Dex, MPA, P_4 and R5020 was assessed (Fig. 5.6.2). GR over-expression was confirmed by western blotting (Fig. 5.6.2b).

a)



b)



c)

	Dex	MPA	P_4	R5020
Efficacy (% ± SEM)	97.15 ± 3.863	95.22 ± 4.278	28.42 ± 4.026	4.141 ± 6.06
Potency/ EC_{50} (M)	3.937 e-009	1.170 e-008	3.189 e-006	N/A

Figure 5.6.2. The PR agonist R5020, like P_4, does not transactivate the TAT-GRE-LUC reporter gene in COS1 cells over-expressing GR. COS1 cells were seeded in 10 cm dishes at 1 X 10^6/dish and allowed to adhere overnight. Cells were subsequently transfected with 10 μg pcDNA3-hGR and 3 μg pTAT-GRE-E1b-LUC for 24 h. Cells were re-seeded at 2.5 X 10^4 cells/well in 96-well plates and allowed to adhere for 24 h. Ligands were prepared by serial dilutions in 100% ethanol from a 10 mM ligand stock. Cells were stimulated in triplicate wells with ligands at the indicated final concentrations (10 pM - 10 μM) in SF-DMEM for 24 h, then washed and harvested in Reporter Lysis Buffer. (a) Luciferase activity and total protein were measured, with luciferase activity normalized to average protein concentration in mg/mL. For the dose response curve, data were analysed with the maximal response (10 μM Dex) set to 100%. A non-linear regression model was carried out by plotting log agonist versus response with the Hill slope set to 1. (b) Cells seeded and transfected in parallel were harvested in SDS sample buffer and lysates were analyzed for GR levels by western blotting using GAPDH as a loading control. A representative western blot is shown. (c) Efficacy and potency for each ligand were calculated using GraphPad Prism 5. The results are pooled from 3 (R5020) or 4 (Dex, MPA, P_4) independent experiments where each point was in triplicate.

With Dex as the reference full GR agonist, dose response curves were plotted in order to determine the relative efficacy (maximal response) and potency (effective concentration required for 50% of maximal response) of each ligand (Fig. 5.6.2c). MPA reached a similar maximal response to Dex, however the efficacies for the highest concentration (10 μM) of P_4 and R5020 were only 28% and 4%, respectively, relative to Dex (Fig. 5.6.2a, c). The order of ligands in terms of potency was Dex > MPA > P_4 (Fig. 5.6.2c). These results suggest that the concentration of R5020 used in the gene expression studies (100 nM) is unlikely to activate the GR (Fig. 4.3.2.1-4.3.2.2). However the nuclear translocation of GR in response to R5020 warrants further investigation.

In order to obtain greater confidence in the co-localization results obtained with confocal microscopy, and to further indicate the close physical proximity of the GR and PR, nuclear co-localization was further investigated using super-resolution structured illumination microscopy (SR-SIM). This technique improves resolution by about 2-fold. Slides of COS1 cells used for the confocal microscopy were re-analyzed using SR-SIM (Fig. 5.6.3) and nuclear co-localization of GR and PR was quantified (Table 5.6.2). Channel alignment with commercial fluorescent beads was set up prior to imaging to calibrate the microscope.

In addition to the Manders co-localization coefficients, M1 and M2, and the Manders derivative overlap coefficient, O, the Pearson's correlation coefficient (P) was calculated using SR-SIM. P represents the correlation between the channels' distributions of intensity and is considered the simplest co-localization coefficient. Co-localization is indicated by a P value of 0.5 to 1, with no co-localization indicated by a value of -1 to 0.5.

Consistent with the confocal microscopy data, representative SR-SIM images indicate that GR and PR co-localized in the nucleus in response to Dex, R5020 and MPA, while GR was mostly cytoplasmic and PR appeared to be entirely nuclear for the vehicle control (Fig. 5.6.3).

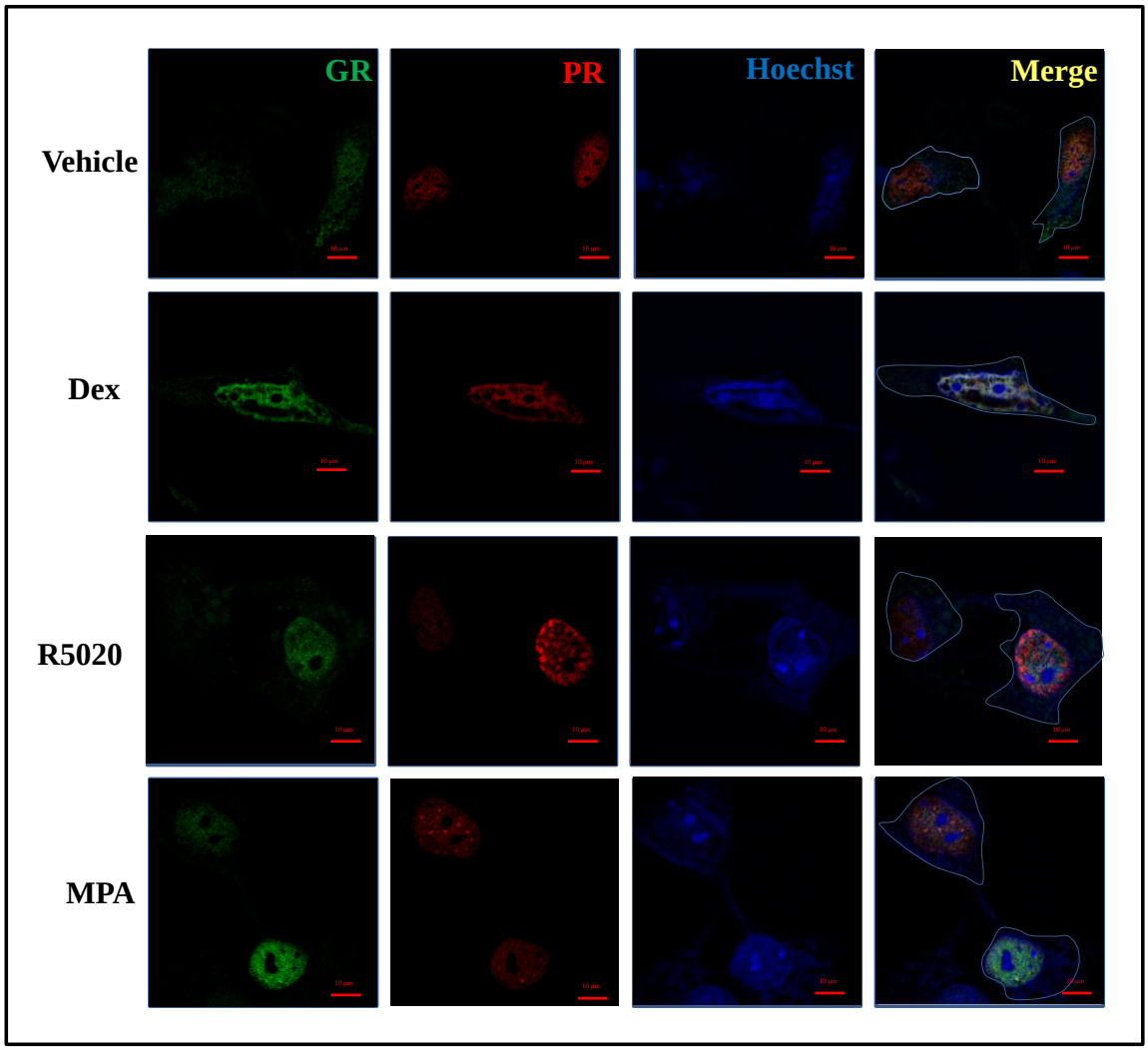


Figure 5.6.3. GR and PR co-localize in the nucleus in response to Dex, R5020 and MPA, unlike the vehicle control, in COS1 cells over-expressing both GR and PR. COS1 cells were prepared on glass slides as for Fig. 5.4 and visualized using super-resolution SIM. Images were captured and pre-processed and co-localization was quantified on two z-stacks per image (Table 5.6.2). Images shown are representative of 3 independent experiments, with 3-4 fields of view captured per slide and 1-3 cells per field of view. The scale bar (red) indicates 10 μm.

Co-localization was implied when both Manders coefficients (M1 and M2) were between 0.5 and 1. Based on this criteria, no co-localization between GR (M1: 0.437) and PR (M2: 0.792) was indicated in the absence of ligands (Table 5.6.2). Following ligand stimulation, however, the Manders coefficients indicated GR-PR co-localization in response to Dex (M1: 0.708, M2: 0.831), R5020 (M1: 0.629, M2; 0.836) and MPA (M1: 0.696, M2: 0.831, Table 5.6.2). However only M1 coefficients for Dex and MPA were statistically significant (Table 5.6.2). Interestingly, the Manders co-localization coefficients obtained here (M1, M2 and O) were lower than those determined by confocal microscopy (Table 5.6.1), suggesting that the extent to which GR and PR co-localize may be over-estimated by confocal microscopy.

The finding that both the Manders overlap coefficient (O) and the Pearson's correlation coefficient (P) were less than 0.5 indicated that no co-localization between GR and PR was observed in the absence of ligands (Table 5.6.2). In contrast, in the presence of ligands, O and P indicated significant GR-PR nuclear co-localization occurs in response to Dex, R5020 and MPA (Table 5.6.2).

Taken together, these co-localization parameters are consistent with a ligand-dependent nuclear co-localization between GR and PR, which suggests that GR and PR are likely to be within close physical proximity of each other in the nucleus of COS1 cells stimulated with Dex, R5020 or MPA.

Table 5.6.2. Quantification of co-localization of GR and PR in COS1 cells in response to Dex, R5020 and MPA using super-resolution microscopy.

	GR co-localized with PR (M1)		**PR co-localized with GR (M2)**		**Overlap coefficient (O)**		**Pearson's coefficient (P)**	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Vehicle (n=8)	0.437	0.087	0.792	0.0297	0.375	0.049	0.322	0.050
Dex (n=8)	0.708*	0.069	0.831	0.049	0.748***	0.072	0.734****	0.077
R5020 (n=8)	0.629	0.049	0.836	0.049	0.619*	0.054	0.604**	0.057
MPA (n=7)	0.696*	0.077	0.831	0.047	0.681**	0.041	0.667**	0.043

Co-localization coefficients were calculated using JACoP in ImageJ. The data are from 3 independent experiments, where 2-4 images were captured for each condition and a single z-stack was quantified per image. The n value indicates the number of GR/PR-expressing cells evaluated. Statistical comparisons were carried out using a parametric one-way ANOVA with Dunnett's post-test. Stars next to the mean value indicate significance compared to the vehicle control, with ****, ***, ** and * indicating $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

Upon closer examination of the enlarged images, there appeared to be regions within the nucleus that expressed only GR (green), only PR (red) or both together (yellow), and that these regions were expressed adjacent to each other throughout the nucleus (Fig. 5.6.4). The yellow regions were more evident with the ligand stimulations compared to the vehicle control (Fig. 5.6.4) but varied in frequency depending on the position of the z-stack (not shown). Furthermore there were pockets of the nucleus where only blue stain was observed, which most likely indicates regions of DNA inaccessible to transcription factors (Fig. 5.6.4).

These results suggest that although there are regions in the nucleus that contain only the GR or only the PR, there are indeed areas throughout the nucleus where GR and PR do co-localize in the presence of ligands. Taken together, the results in this chapter support the previous findings that PR-B modulates the activity of the GR, and that this potentially occurs through GR-PR association and co-localization in some regions of the nucleus, in a ligand-dependent manner.

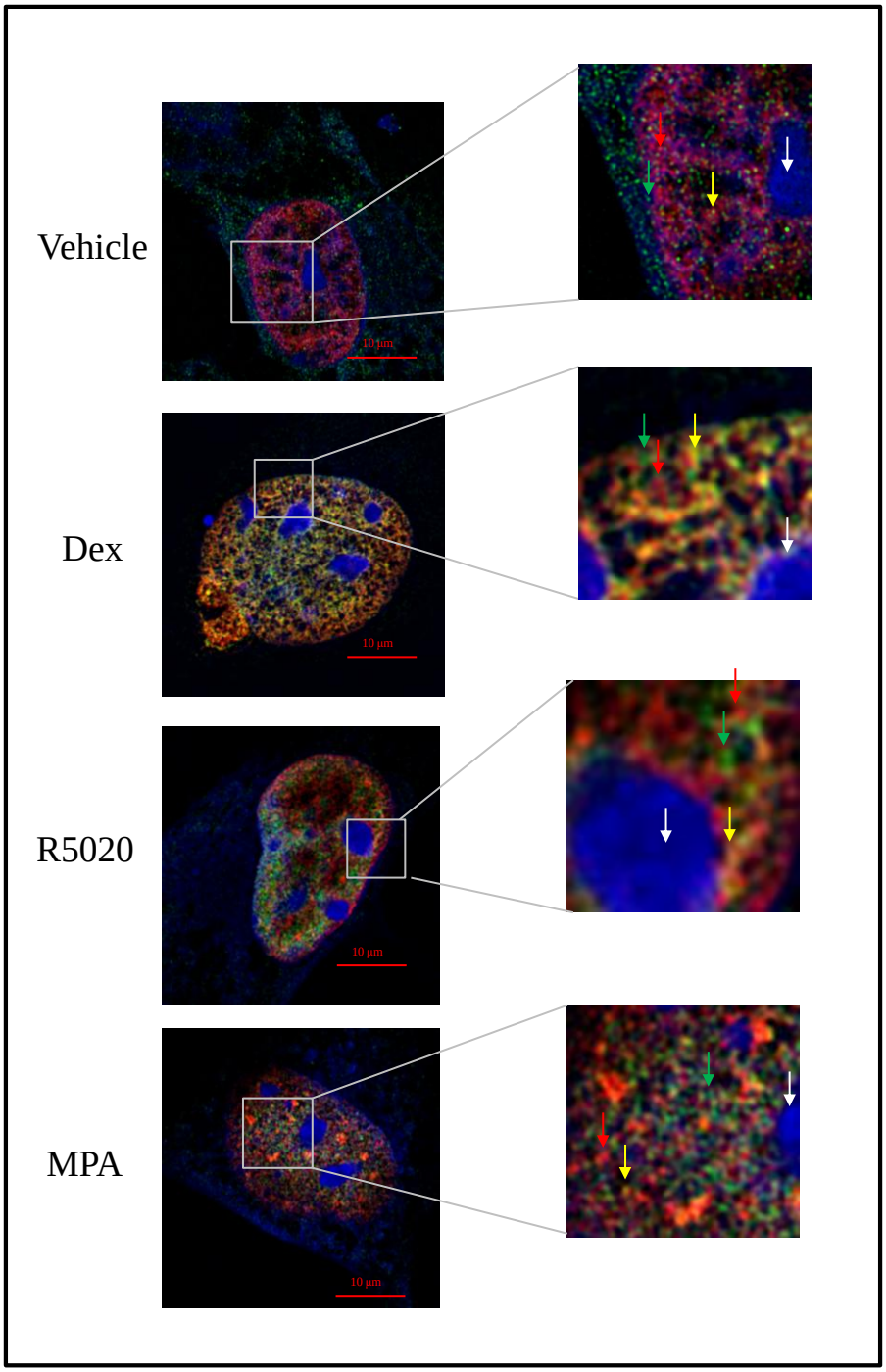


Figure 5.6.4. Super-resolution microscopy indicates GR- and PR-specific regions, as well as co-localized regions, within the nucleus of COS1 cells over-expressing GR and PR. COS1 cells were prepared on glass slides as for Fig. 5.4.1 and visualized using SR-SIM. Representative image of a COS1 cell (left) with enhanced zoom (right). Arrows indicate a representative GR-specific region (green), PR-specific region (red), co-localized region (yellow) and condensed DNA (white). The scale bar (red) indicates 10 µm.

Chapter 6

Discussion

Many women of reproductive age in Sub-Saharan Africa who are at the highest risk for HIV-1 acquisition also use the progestin-only injectable contraceptive DMPA (Butler et al. 2013). It is therefore crucial to understand whether, and how, MPA increases HIV-1 infection in HIV-1 target cells, in order to enable women at high HIV-1 risk to make informed contraceptive choices.

The lower FRT is the primary site for heterosexual HIV-1 transmission and provides a protective physical and immunological barrier to infection. The FRT has its own immune system compared to the circulatory blood system (Givan et al. 1997, Haynes and Shattock 2008, Wira and Fahey 2008, Jespers et al. 2010, Hafner et al. 2013, Nguyen et al. 2014, Wira et al. 2015). The immune cells predominantly targeted for HIV-1 infection are CD4+ T cells (Pope and Haase 2003), which represent a portion of the immune cells within both the circulating peripheral blood and the cervix (Prakash et al. 2001, Nguyen et al. 2014, Trifonova et al. 2014, Lee et al. 2015).

The present study used both primary cells and a cell line (PBMCs and TZM-bls) as models to investigate HIV-1 infection and mechanisms thereof between the P_4-high luteal phase of the menstrual cycle, the P_4-low follicular phase and contraception with the injectable MPA. Since MPA has been shown to activate the GR and the PR, the present study also expanded on the role of the GR and changing GR/PR levels on HIV-1 infection and regulation of genes relevant to HIV-1 infection. To this end, this study used HeLa and End1/E6E7 cells to investigate the effects of changing GR/PR levels on immunomodulatory gene expression, and COS1, U2OS and MCF-7 cells to explore the possible modulation of GR activity by the PR through protein association and nuclear co-localization.

6.1 MPA and luteal phase hormones both increase HIV-1 infection, but via potentially different mechanisms

Data from the present study show that while both MPA and luteal phase E_2/P_4 increase HIV-1 infection in PBMCs, MPA but not luteal phase E_2/P_4 changes the frequency of immune cell populations and expression of other markers relevant to HIV-1 infection. This suggests that MPA-induced HIV-1 infection may occur through different mechanisms to luteal phase E_2/P_4, unlike follicular phase E_2/P_4 which does not significantly alter HIV-1 infection in these models.

6.1.1 MPA and luteal phase E_2/P_4 increase HIV-1 infection

The present study showed that stimulation with near-peak concentrations of the contraceptive progestin MPA (100 nM) increased HIV-1 infection in both PBMCs and TZM-bl cervical cells. Specifically, MPA significantly increased HIV-1 infection in PBMCs after both 2 and 7 days' stimulation (Fig. 3.2.1, Fig 3.4.1.1a) and in TZM-bls after 2 days' stimulation (Fig. 4.2.1.1a).

The observation that MPA enhanced HIV-1 infection is consistent with other *in vitro* studies. For example, exogenous stimulation with MPA increased HIV-1 infection in activated PBMCs at 1 μM (Huijbregts et al. 2013) or 100 nM (Ray 2015), and non-activated PBMCs and ectocervical explants at 10 nM (Ray et al. 2017). Another study showed that 1-12.5 nM MPA increased single-cycle replication of HIV-1 pseudovirus (Sampah et al. 2015). One study showed that exogenous MPA did not increase productive HIV-1 replication, but did increase the uptake of HIV-1 in primary genital epithelial cells (GECs) (Ferreira et al. 2015). The same study showed that growth media supernatants from MPA-treated GECs increased HIV-1 replication in indicator TZM-bl cells (Ferreira et al. 2015). Similarly, supernatants from MPA-treated Vk2/E6E7 vaginal cells increased HIV-1 replication in indicator U1 cells (Irvin and Herold 2015).

Furthermore, this study is in line with non-human primate models showing that MPA increases SIV/SHIV in rhesus and pigtail macaques (Trunova et al. 2006, Smith et al. 2015, Butler et al. 2016). These molecular data are also in agreement with the latest clinical studies showing increased HIV-1 acquisition in predominantly DMPA users (Byrne et al. 2016, Tasker et al. 2017) as well as the most recent epidemiological observational meta-analyses showing a 40% increased HIV-1 acquisition risk in DMPA users compared to women using no hormone (Morrison et al. 2015, Ralph et al. 2015, Polis et al. 2016).

The observation that MPA increases infection in animal, clinical and both local (FRT) and systemic (PBMCs) models *in vitro*/*ex vivo* strongly implies that MPA can increase susceptibility to infection in multiple settings, independent of differences in species, cell populations, immune activation status, hormonal microenvironment and donor. This research is thus highly relevant to women in high-risk areas such as Kwa-Zulu Natal in South Africa, where DMPA is the most commonly used contraceptive.

A major concern with cell culture studies is whether the concentrations of MPA used truly represents physiological doses in DMPA users *in vivo*. Comparing the published serum MPA levels after the first 1-3 weeks post-administration showed there is a wide inter-individual and inter-study range of MPA serum levels (Fig. 1.4.1.1). The average detected peak MPA serum levels are about 5-40 nM and the

maximum levels reported reach up to 65-100 nM (Kirton and Cornette 1974, Koetsawang 1977, Hapgood et al. 2018). After about 1 month, MPA serum levels plateau at around 2.6 nM for 3 months (Mishell 1996). Therefore the MPA concentration used in the present study (100 nM) is still within the physiological range and represents the upper range of peak serum DMPA-IM levels detected shortly after injection. It should also be noted that MPA concentrations in different tissues are unknown and may be different to serum levels.

Additional evidence in support of MPA increasing HIV-1 infection at physiologically relevant doses comes from *in vitro* dose response experiments from the present author's laboratory. In these studies, MPA as low as 50 nM in PBMCs and 10 nM in ecto- and endocervical explants still significantly increases HIV-1 replication [(Ray et al. 2017) and unpublished]. These findings are consistent with a report showing that low concentrations of MPA (1-12.5 nM) increased HIV-1 pseudovirus infection in non-activated PBMCs (Sampah et al. 2015). Another study indicated that MPA increased HIV-1 replication in activated PBMCs at higher (1 μM) but not lower (10 nM) concentrations (Huijbregts et al. 2013). The discrepancy between the latter studies is likely to be influenced by the activation status of the PBMCs. Activated cells are already primed to produce T cell responses and are more susceptible to infection. The study by Sampah et al (2015) found that prior activation of PBMCs minimized the effect of MPA on HIV-1 infection. Additional differences between these studies, such as time of MPA incubation (18 versus 24 h), donor-specific effects, and relative immune cell populations between donors may explain the differences in HIV-1 replication.

While no studies have yet investigated HIV-1 acquisition risk in DMPA-SC users (Polis et al. 2017, Hapgood et al. 2018), the peak serum concentrations are in the range of 1.6-4.4 nM (Jain et al. 2004, Toh et al. 2004). This appears to be lower than peak DMPA-IM levels, but to date no studies have directly compared maximal peak levels for DMPA-IM and DMPA-SC in the same study. Thus it is difficult to predict whether or not DMPA-SC use would change biological responses relevant to HIV-1 susceptibility (Polis et al. 2017).

Collectively, the evidence from this study and others showing that MPA increases HIV-1 infection in PBMCs at both the middle and upper ranges of physiological MPA serum levels suggests that DMPA users are at risk of increased HIV-1 acquisition shortly after DMPA administration.

The present study also showed that hormones mimicking the luteal phase of the menstrual cycle, unlike the follicular phase hormones, enhanced HIV-1 infection in both PBMCs and TZM-bl cervical cells. Specifically, luteal phase E_2/P_4 significantly increased HIV-1 infection in PBMCs after 7 days' stimulation (Fig. 3.2.1, Fig. 3.4.1.1), while a non-significant increase was shown in TZM-bls after 2

days' stimulation (Fig. 4.2.1.1a). In PBMCs, the extent of HIV-1 infection with luteal phase E_2/P_4 was 1.4-5.4-fold lower than that with MPA (Fig. 3.2.1, Fig. 3.4.1.1).

Higher HIV-1 infection with luteal phase hormones in PBMCs in this study is in agreement with non-human primate models showing increased SIV/SHIV infection during the luteal phase in naturally cycling macaques (Sodora et al. 1998, Vishwanathan et al. 2011, Kersh et al. 2014, McNicholl et al. 2014, Radzio et al. 2014) or macaques treated with P_4 that reached serum levels similar to the luteal phase (Marx et al. 1996). This finding is also consistent with a report using cervical explants *ex vivo* from naturally cycling women in the luteal phase (Saba et al. 2013). There are not many studies using exogenous combinations of E_2/P_4 to assess HIV-1 infection *in vitro*. One study in activated PBMCs showed that exogenous stimulation with follicular phase hormones (100 pM E_2/1 nM P_4) increased HIV-1 infection in PBMCs, while luteal phase hormones (1 nM E_2/100 nM P_4) decreased infection (Asin et al. 2008). However their result could reflect donor-specific differences since only one donor was used, as well as differences in incubation period (4 days prior to infection), concentrations of E_2/P_4 used and activation state (Asin et al. 2008). Another study indicated that a combination of 20 nM E_2/500 pM P_4 had no effect on HIV-1 p24 or RNA levels in a T cell line, CEMss (Ragupathy et al. 2016). However, since different experimental protocols were used for these studies, the effect of exogenous E_2/P_4 on HIV-1 infection may critically depend on the donor/s used, frequency of immune cell types, differential activation states, order of hormone and virus incubation, type of virus used (IMC or pseudovirus, R5- or X4-tropic), duration of hormone stimulation and infection, and concentrations of E_2/P_4 used.

Follicular phase E_2/P_4 (400 pM E_2/1 nM P_4) did not significantly increase HIV-1 infection in PBMCs (Fig. 3.2.1, Fig. 3.4.1.1), in contrast to luteal phase E_2/P_4. This is in agreement with non-human primate studies showing that SIV/SHIV infection was higher during the luteal phase than the follicular phase (Marx et al. 1996, Sodora et al. 1998, Vishwanathan et al. 2011, Kersh et al. 2014). Limited information is available on exogenous follicular phase hormones and HIV-1 infection *in vitro*. One study showed follicular phase hormones (100 pM E_2/1 nM P_4) increased HIV-1 replication in activated PBMCs (Asin et al. 2008). However several experimental differences exist between their study and the present one, including but not limited to, different E_2 concentrations used, different hormone and virus incubation times and prior activation of the PBMCs in their study.

The concentrations of E_2/P_4 used in the present study to represent menstrual cycle phases are comparable to physiological E_2/P_4 levels in naturally cycling women. This study used 400 pM E_2 plus 10 nM P_4 to mimic the luteal phase of the menstrual cycle, but other studies have used different concentrations of E_2/P_4 to represent the luteal phase *in vitro* in other model systems. For example, 1 nM E_2/100 nM P_4 in PBMCs (Asin et al. 2008), 10 nM E_2/1 μM P_4 in primary endometrial stromal

cells (Ancelin et al. 2002), 1 nM E_2/100 nM P_4 in activated PBMCs (Huijbregts et al. 2013), 10 nM E_2/100 nM P_4 in immortalized endometrial stromal cells (Schutte and Taylor 2012), and 10 nM E_2/100 nM ORG2058, as a more stable progestogen than P_4, in primary endometrial stromal cells (Salamonsen et al. 1997). While the concentrations of luteal phase E_2/P_4 used in this study are lower than those used in the above studies, especially that of P_4, the concentrations nevertheless still fall within the physiological range of reference values in another report (Stricker et al. 2006), where P_4 levels range between 0.95-71.87 nM and the range for E_2 is 51.75-1163.02 pM across the luteal phase of the menstrual cycle (see section 1.3.1). Nevertheless it is possible that higher P_4 concentrations such as 100 nM may have shown more pronounced or different effects.

The effects of P_4 alone (that is, without E_2) on HIV-1 infection have been reported in some studies. For example, 500 pM P_4 increased HIV-1 infection as measured by p24 levels in both a T cell line and PBMCs, and reduced antiviral efficacy in the T cell line (Ragupathy et al. 2016). In another study, 10 nM P_4 did not increase X4 or R5-tropic HIV-1 replication in activated PBMCs, whereas 1 μM P_4 increased replication of an X4- but not R5-tropic virus (Huijbregts et al. 2013). Previous work in the present author's laboratory using available T cell lines, including Jurkat cells, showed that these cell lines appeared to be non-physiological due to the absence of active GR (data not shown).

While high P_4 has been linked to increased HIV-1 susceptibility, there is also some evidence to suggest exogenous E_2 is protective against HIV-1 infection. For example, 10 nM E_2 significantly decreased HIV-1 p24 levels in CD4+ T cells and macrophages (Rodriguez-Garcia et al. 2013a), while in other studies, topical E_2 protected against SIV in animal models (Smith et al. 2000b, Smith et al. 2004). In the present study, the combined stimulation of E_2 and P_4 makes it impossible to distinguish potential protective effects conferred by E_2. The concentration of E_2 used (400 pM) may also be too low to counteract the effects of P_4, but was used in order to more accurately represent the two main menstrual cycle phases. However, since the E_2 concentration is the same for both luteal and follicular phase in this study, the effects observed on HIV-1 infection and expression of immune markers are likely due to P_4.

In the present study, TZM-bl cells were used as a model of infectable cervical cells, since they express high levels of CD4 and CCR5 (Platt et al. 1998). Infection in TZM-bl cells measures the steps up to transcription of the LTR, including fusion, entry, reverse transcription, nuclear translocation and LTR transcription. Therefore any changes in HIV-1 infection in response to MPA in these cells suggest that the mechanism is not via downstream events in the HIV life cycle. TZM-bl cells thus provide a useful opportunity to gain insight into biological mechanisms of infection involving any of the abovementioned steps. Importantly, TZM-bl cells do not undergo activation like T cells and therefore lack the receptors and T cell-specific signalling pathways that occur in PBMCs. Therefore this model

provides additional insight into biological mechanisms that are independent of activation and T cell specific functions. However, the evidence from this study showing similar effects (that is, increased CCR5 levels) for both PBMCs and TZM-bl cells gives additional insight into biological mechanisms that are likely applicable to target cells expressing CD4 and CCR5. These target cells include many cell types in the FRT such as CD4+ T cells, macrophages and DCs.

Large variability in HIV-1 infection was observed between biological repeat experiments in both PBMCs and TZM-bls, although the inter-donor variability was greater for PBMCs. This variability in PBMC infection was typically observed in all PBMC assays in the present author's laboratory and is consistent with the literature (Sampah et al. 2015). Therefore PBMCs from each donor were pre-screened for infectability prior to infection assays (see section 2.8.2). However the large variability observed in TZM-bl cells was somewhat surprising, as a cell line is expected to be relatively homogeneous. The source of variation between experiments was not from the virus used, since these assays used aliquots from the same virus stock, nor was it from changes in cell viability in the presence of virus, since MTT assay results showed no major changes (Appendix B. Table B1.1). Therefore the cause for the differences in infection is most likely the biological variation in viral infection between independent biological assays for TZM-bls as well as PBMCs, in which inter-donor variability between responses is an additional source of variability.

Collectively the results from this study show, despite large variations in HIV-1 infection between PBMC donors, that exogenous stimulation with luteal phase hormones and MPA, but not follicular phase hormones, at levels within the physiological range, increases HIV-1 infection in PBMCs and/or TZM-bl cells. These findings may have major significance for susceptibility to HIV-1 infection in both naturally cycling women during the luteal phase and DMPA users during the first few weeks shortly after injection. These findings also suggest that the effects observed in clinical studies are likely due to direct effects on HIV-1 target cells expressing CD4 and co-receptors and offer opportunities to further investigate the mechanisms behind infection.

6.1.2 MPA- induced HIV-1 infection most likely occurs through increased frequency of CD4+ T cells, increased CD4/CD8 ratios and increased CCR5 and CD69 on CD4+ T cells, while luteal phase E_2/P_4 increases HIV-1 infection via a different, unidentified mechanism

Multiple plausible biological mechanisms have been suggested to explain how the injectable contraceptive MPA can increase HIV-1 infection in the FRT and in peripheral blood (see section 1.4.3). Important mechanisms most likely relevant to the present study are changes in adaptive

immunity (frequency of key immune cell types and their activation status) and increased levels of the CCR5 co-receptor, which would facilitate R5-tropic viral entry.

The present study showed that in PBMCs, MPA but not luteal phase E_2/P_4 significantly increased the frequency of CD4+ T cells (Fig. 3.3.1.1a), the primary target cells for HIV-1 infection, and furthermore increased the density of the activation marker CD69 on CD4+ T cells (Fig. 3.3.1.2b). Increased T cell activation correlates with increased T cell infection reported in some studies (Stevenson et al. 1990, Haase 2005, Meditz et al. 2011). The activation status of immune cells has been shown to render them more susceptible to HIV-1 infection (Greenhead et al. 2000, McKinnon et al. 2011, McKinnon and Kaul 2012). Together with higher levels of the CD4 receptor on the surface of host target cells, increased levels of the co-receptor CCR5 could facilitate HIV-1 entry into target cells. The present study showed that MPA but not luteal phase hormones increased the density of CCR5 on target CD4+ and CD3+ T cells (Fig. 3.3.2.2), without changing the frequency of CCR5-expressing cells (Fig. 3.3.2.1a). These results agree with some studies in PBMCs from DMPA users. A recent report showed a trend for increased activation and CCR5 density in CD4+ T cells from women at 1 or 3 months after DMPA injection, compared to before injection (Tasker et al. 2017). In the same study, PBMC infection increased from month 1 to month 3 in DMPA users (Tasker et al. 2017). Another study observed increased CCR5 expression in CD4+ and CD8+ T cells, without a concomitant increase in frequency in CCR5-expressing CD4+ or CD8+ T cells in DMPA users compared to women not using hormonal contraception (Sciaranghella et al. 2015). A third study showed higher CCR5 expression and frequency of CCR5-expressing T cells in the cervix but not matched PBMCs of injectable contraceptive (predominantly DMPA) users compared to women not using long-term contraceptives (Byrne et al. 2016). In this study, injectable contraceptive users were at a higher risk of HIV-1 acquisition compared to naturally cycling women (Byrne et al. 2016). In contrast, another study did not observe a difference in cervical CD4+CCR5+ T cell proportion in DMPA users compared to women not using hormonal contraception (Smith-McCune et al. 2017). In an *ex vivo* study, 1 μM but not 100 nM MPA prevented CXCR4 and CCR5 down-regulation in T cells from activated PBMCs (Huijbregts et al. 2013), which is not inconsistent with MPA increasing CCR5 density in T cells from the present study.

The finding that MPA but not luteal phase E_2/P_4 significantly increased CD4 and CCR5 mRNA levels in cervical TZM-bl cells (Fig. 3.3.2.3) further supports the PBMC results. Multiple studies with MPA in different FRT compartments are broadly consistent with the increased activation of CD4+ T cells in PBMCs and higher CCR5 levels in PBMCs and TZM-bls from this study. For example, a recent report in injectable contraceptive (predominantly DMPA) users showed higher frequency of activated cervical CD4+CCR5+ T cells compared to women not using a long-term contraceptive (Byrne et al. 2016), while another report in DMPA users showed higher frequency of activated endometrial T cells,

without an increase in CCR5 density on CD4+ T cells (Smith-McCune et al. 2017). The latter study also showed increased expression of the activation marker HLA-DR in endometrial CD4+ and CD8+ T cells (Smith-McCune et al. 2017). Higher numbers of cells expressing CD3, CD8, CCR5 and the activation marker HLA-DR were observed in vaginal biopsies from DMPA users compared to women in the follicular phase (Chandra et al. 2013). In line with these studies, a recent study has shown that HIV-1 preferentially targets cervical CD4+ T cells that also express increased CCR5, CD69 and mucosal integrins (Joag et al. 2016). In addition, DMPA-treated rhesus macaques exhibited a higher frequency of CCR5+CD4+ cells in vaginal tissue than E_2-treated macaques, although this effect was not statistically significant (Goode et al. 2014). In contrast to the above reports, one study showed decreased frequency of vaginal CD3+ and CCR5+CD3+ T cells in DMPA users (Mitchell et al. 2014), while another reported no change in vaginal CD4+ or CD8+ T cells in DMPA users (Michel et al. 2015). A report in primary DCs *ex vivo* showed that MPA at different doses did not change expression of the activation marker HLA-DR (Quispe Calla et al. 2015). Furthermore, a study using non-activated PBMCs stimulated *ex vivo* with exogenous MPA (1-12.5 nM) did not observe an effect on the frequency of CD4+ T cells expressing CCR5, CXCR4 or the activation markers CD25, CD69, HLA-DR and CD38 (Sampah et al. 2015).

Collectively these results suggest that increased CD4+ T cell activation and CCR5 levels are sometimes but not always detectable in peripheral blood and the FRT. The detectable effects of MPA may therefore depend on multiple variables, including but not limited to time of sampling post-injection, populations of other immune cells and their activation status, levels of other endogenous hormones *in vivo*, concentration of MPA used and duration of stimulation *in vitro*. This may in part translate to variable responses *in vivo* due to inter-individual differences, for example in immune activation status and serum levels, which is a possible reason for varying results over 40 years in HIV-1 acquisition studies.

Importantly, the present study showed that MPA but not luteal phase hormones simultaneously decreased the frequency of CD8+ T cells while increasing the frequency of CD4+ T cells (Fig. 3.3.1.1). Therefore the ratio of CD4/CD8 was significantly increased by 1.5-fold in both MPA-treated total CD3+ lymphocytes (Fig. 3.3.1.1c) and in cells expressing the activation marker CD69 (Fig. 3.3.1.1d). A higher CD4/CD8 ratio is consistent with other studies in PBMCs where CD4 cells outnumber CD8 cells (Byrne et al. 2016, Iyer et al. 2017). In addition, higher CD4/CD8 ratios have been observed in PBMCs of DMPA users (Sridama et al. 1992) and postmenopausal women using NET for HRT (Dogan et al. 2005) but not women using COCs (Prakash et al. 2002). Taken together with the increase in CD69 density on activated CD4+ T cells, these results suggest that MPA enhances HIV-1 infection in target cells by increasing the frequency of infectable cells (CD4+), while concomitantly decreasing the frequency of cytotoxic cells capable of combating infection (CD8+).

Decreased frequency of CD8+ T cells in response to MPA is consistent with a study showing decreased CD8+ T cell effector function (production of IFNγ and TNF) in murine trigeminal ganglia stimulated with 100 pM MPA *ex vivo* or from mice treated with MPA-containing pellets (Cherpes et al. 2008a).

The present study showed that MPA but not menstrual cycle hormones significantly decreased the frequency of activated CD69+CD14+ monocytes (Fig. 3.3.1.1b), although CD69 density (Fig. 3.3.1.2b), CCR5 density (Fig. 3.3.2.2) and frequency of CCR5-expressing cells (Fig. 3.3.2.1a) were not changed. Monocytes play an important role in activating CD4+ T cells by producing soluble mediators. In line with this, a study in non-activated PBMCs showed that HIV-1 replication in CD3+ cells and whole PBMCs was lower when CD14+ monocytes were depleted (Sampah et al. 2015). The results from this study could therefore suggest decreased monocyte function in response to MPA, but further investigation is needed.

Furthermore MPA has been shown to repress mRNA expression of the ligand for CCR5, RANTES, in PBMCs, Ect1/E6E7 and End1/E6E7 cells (Africander et al. 2011a, Govender et al. 2014, Hapgood et al. 2014b), which is in agreement with the literature that RANTES decreases HIV-1 infection through competition for CCR5 (Cocchi et al. 1995, Alfano and Poli 2005).

Several clinical, *ex vivo* and animal studies suggest that high P_4 levels, like those observed during the luteal phase, are associated with increased susceptibility to infection and/or increased CCR5 levels in the FRT (see section 1.3.2). This would suggest that there are common overlapping potential mechanisms for enhanced HIV-1 infection between DMPA usage and the luteal phase. However, the results from this study in PBMCs indicate that luteal phase hormones, unlike MPA, do not increase the frequency of CD3+, CD4+, CD8+ T cells or CD14+ monocytes (Fig. 3.3.1.1a), or frequency of activated CD69-expressing cells (Fig. 3.3.1.1b), or expression of CD69 (Fig. 3.3.1.2b), or frequency of CCR5-expressing cells (Fig. 3.3.2.1), or expression of CCR5 (Fig. 3.3.2.2, Fig. 3.3.2.3a). Limited studies are available comparing menstrual cycle phases to the injectable contraceptive MPA in the same study. One of these is a recent study in a cohort of South African women showing that the frequency of cervical CCR5+CD4+ T cells was about 4 times higher in injectable contraceptive (predominantly DMPA) users than naturally cycling women in the luteal phase (Byrne et al. 2016). Another study showed that IgG and IgA levels in CVL samples were increased in DMPA users but decreased in naturally cycling women post-ovulation (luteal phase) (Francis et al. 2016). However it is difficult to compare across *in vitro* and clinical studies due to methodological and biological differences. These results are likely to be influenced by time of sampling, immune cell frequencies, concentrations of endogenous hormones and/or MPA, how the menstrual cycle phases were classified, duration of contraceptive use and/or other variables. Taken together, the results from this study and

limited clinical studies support the conclusion that MPA and luteal phase E_2/P_4 increase HIV-1 infection via potentially different mechanisms.

The pro-inflammatory cytokine IL-6 was used in this study as a marker for inflammation. While a link for inflammation and enhanced HIV-1 susceptibility has been established, whether DMPA use is associated with increased or decreased inflammation has yielded conflicting results. Some clinical studies have reported an increase (Deese et al. 2015, Francis et al. 2016), decrease (Walong et al. 2016) or no change (Morrison et al. 2014, Ngcapu et al. 2015) in IL-6 protein levels from CVLs in DMPA users. In the present study, MPA but not luteal phase E_2/P_4 repressed IL-6 mRNA expression in both PBMCs and TZM-bl cells (Fig. 3.3.3.1). This finding is consistent with the immunosuppressive function of MPA on IL-6 mRNA or protein shown in PBMCs (Huijbregts et al. 2013, Hapgood et al. 2014b, Huijbregts et al. 2014, Michel et al. 2015), CD14+ monocytes (Hapgood et al. 2014b), primary endocervical fluid (Smith-McCune et al. 2017), primary ectocervical explants (Ray 2015), immortalized endocervical cells (Govender et al. 2014), and mouse fibroblast cells (Koubovec et al. 2004). Furthermore, previous research from the present author's laboratory has shown that 100 nM MPA gives different effects on select genes involved in immune function compared to 100 nM or 1 μM P_4 (Koubovec et al. 2004, Govender et al. 2014, Hapgood et al. 2014b). In addition, some of this work also shows that the repression of IL-6 and other pro-inflammatory genes by MPA requires the GR in immortalized endocervical cells and PBMCs (Govender et al. 2014, Hapgood et al. 2014b). In general, P_4 inhibits inflammatory responses and promotes an anti-inflammatory state *in vitro* (Hall and Klein 2017). Limited research on exogenous P_4 include one study showing that 10 nM or 1 μM P_4 did not change IL-6 protein levels in activated PBMCs (Huijbregts et al. 2013), in agreement with the present study. Other studies have shown that 1 μM P_4 represses LPS-induced IL-6 expression in primary uterine fibroblasts (Fukuyama et al. 2012, Kim et al. 2012), but increases TNFα-induced IL-6 mRNA expression in Vk1/E6E7 and Ect1/E6E7 cells *in vitro* (Africander et al. 2011a). The differences between studies are likely to reflect multiple differences in experimental design, such as cell type, activating compound and concentration of hormone used.

The present study showing enhanced HIV-1 infection in PBMCs in response to MPA may have significance for disease progression to AIDS in DMPA users. However, the available albeit limited studies from large populations suggest MPA and other hormonal contraceptives have no effects on disease progression (Heffron et al. 2013, Phillips et al. 2013, Phillips et al. 2016). This conclusion does not exclude the possibility that the effects of MPA may be relevant for some individual women and may depend on time post-injection, number of years using MPA and/or individual MPA pharmacokinetics.

Taken together, the present data suggests that MPA but not luteal phase E_2/P_4 increase HIV-1 infection in target cells through different potential mechanisms, with MPA increasing activated CD4+ T cells and CCR5 expression, while luteal phase hormones increase HIV-1 infection via an unidentified mechanism. This study appears to be the first to directly compare a combination of E_2/P_4 representing menstrual cycle phase hormones and MPA in terms of HIV-1 infection in PBMCs and TZM-bls and molecular mechanisms of infection. Collectively the results from PBMCs and TZM-bl cells provide insight into biological mechanisms by which MPA can increase HIV-1 infection in any target cells which express CD4 and the CCR5 co-receptor. The increase in CCR5 levels in both of these cell models suggests that there is a common mechanism that is likely to be clinically relevant to CCR5-expressing cells in both the peripheral blood and the FRT.

6.1.3 MPA- but not luteal phase E_2/P_4-induced HIV-1 infection is mediated by the GR

The present study showed that HIV-1 infection induced by MPA, but not luteal phase E_2/P_4, is mediated by the GR in PBMCs (Fig. 3.4.1.1b) and TZM-bl cells (Fig. 4.2.1.1a), using the GR/PR antagonist, RU486 and GR siRNA, respectively.

The levels of endogenous steroid hormone receptors were measured in representative female PBMC donors in this study. Since GR but not PR was detected (Fig. 3.4.2.1, Appendix A, Fig. A1.1), the effect of MPA on HIV-1 infection is most likely via the endogenous GR. The lack of detectable PR in PBMC samples is consistent with some (Neifeld et al. 1977, Kontula et al. 1983, Tabibzadeh and Satyaswaroop 1989, Mansour et al. 1994, Schust et al. 1996, D'Amore et al. 2013) but not other (Szekeres-Bartho et al. 1989, Asin et al. 2008, Cabrera-Munoz et al. 2012, Tan et al. 2015) reports. These differences could be due to multiple factors, such as donor gender, methods of detection, choice of antibody, menstrual cycle status (if female donors) and/or other variables. Although studies in MCF-7 breast cancer cells showed that stimulation with 1 nM E_2 up-regulated PR mRNA levels (Cho et al. 1994), attempts by the present author to up-regulate expression of the endogenous PR by prolonged incubation with low levels of E_2 (400 pM for up to 14 days) were not successful (data not shown). The presence of over-expressed PR-B in TZM-bl cells did not have a significant effect on MPA-induced HIV-1 infection (Fig. 4.2.1.1a). This supports the idea that MPA-induced HIV-1 infection in TZM-bl cells, like in PBMCs, is mediated by the GR. While no significant increase in HIV-1 infection was observed with luteal phase E_2/P_4 in TZM-bl cells, this was also not altered upon PR-B over-expression (Fig. 4.2.1.1a), further suggesting that PR-B is not involved in luteal phase hormone-associated HIV-1 infection.

Possible roles of SRs other than GR and PR in MPA-induced effects were considered. The representative female PBMC donors expressed ERα mRNA but not protein (Appendix A, Fig. A1.1). Other studies have shown ERα mRNA or protein expression in PBMCs (Cohen et al. 1983, Phiel et al. 2005, Asin et al. 2008, Pierdominici et al. 2010, D'Amore et al. 2013). Thus the effects of exogenous E_2 upon stimulation with luteal or follicular phase hormones are likely to be mediated by endogenous ER in PBMCs. However, MPA does not bind to the ER (Africander et al. 2011b, Stanczyk et al. 2013), and therefore the actions of MPA are most likely to not be mediated by the ER in PBMCs. While MR mRNA was detected in both PBMCs (Appendix A, Fig. A1.1) and TZM-bl cells (Appendix A, Fig. A2.1), MR protein levels were not detectable due to the non-specific nature of the available anti-MR antibody. The equilibrium dissociation constant (Ki, an indication of the ligand concentration at which 50% of receptors are occupied) for P_4 binding to the MR is 1.7 nM and P_4 has potent anti-mineralocorticoid activity (Wambach and Higgins 1978, Fagart et al. 1998, Africander et al. 2011b, Africander et al. 2013, Stanczyk et al. 2013). MPA binds MR with lower affinity than P_4 and a *Ki* of 197 nM (Africander et al. 2013). It is therefore possible that if MR protein was expressed, P_4 but not MPA would be expected to antagonize the MR at the concentrations used in this study. While neither AR mRNA nor protein was detected in representative PBMCs (Appendix A, Fig. A1.1), TZM-bl cells express both GR and AR protein (Appendix A, Fig. A2.1). Given that the Ki values for P_4 and MPA binding to the AR are 36.6 nM and 19.4 nM, respectively (Africander et al. 2014), some binding to the AR would be expected to occur using the concentrations of P_4 and MPA in TZM-bl cells in this study. However, since no AR was detected in PBMCs, the effects of MPA on HIV-1 infection observed in PBMCs are likely to be mediated by the GR only.

In the present study, co-stimulation with the GR/PR antagonist RU486 in PBMCs did not change luteal phase E_2/P_4-induced HIV-1 infection in PBMCs (Fig. 3.4.1.1b), and decreasing GR levels using siRNA did not change luteal phase E_2/P_4-induced HIV-1 infection in TZM-bl cells (Fig. 4.2.1.1). This suggests that the increase in HIV-1 infection in PBMCs with luteal phase hormones is not mediated by the GR. In line with this observation is binding affinity research showing that the Ki for P_4 binding to the GR (215 nM) is much higher than the Ki for MPA (10. 8 nM) (Koubovec et al. 2005), suggesting that the concentrations of P_4 used in the present study to mimic the luteal phase (10 nM) or the follicular phase (1 nM) are most likely too low to activate a significant percentage of the GR, unlike MPA. Further supporting this conclusion is the dose response assay from the present study, where P_4 activated GR-mediated reporter gene activity in COS1 cells over-expressing GR only at 1-10 μM (Fig. 5.6.2a). P_4 binding to the GR may therefore only be relevant at higher P_4 concentrations (Jones et al. 2008, Guo et al. 2012, Lei et al. 2012, Engler et al. 2017), such as pregnancy where P_4 serum levels reach 400-636 nM (Kuhl 1990, Sheffield et al. 2009) and P_4 is immunosuppressive in order to promote foetal tolerance (Piccinni et al. 1995, Chien et al. 2009, Tan et al. 2015).

Following the results showing that MPA-induced HIV-1 infection in PBMCs is mediated by the GR, it was of interest to measure GR protein levels in these cells. The present study showed that GR protein levels, in matched samples from the HIV-1 infection assay, appeared to be lower in MPA- but not luteal phase E_2/P_4-stimulated PBMCs compared to the vehicle control (Fig. 3.4.2.1a). While GR protein levels appeared to be decreased after 2, 7 and 12 days' exposure to MPA, the levels were only significantly lower after 7 days (Fig. 3.4.2.1b). This observation suggests that MPA requires the active GR for increased HIV-1 infection, but may also increase ligand-dependent GR turnover. This agrees with other work from the present author's laboratory showing MPA decreased GR protein levels in a time-dependent manner in PBMCs (Ray 2015), End1/E6E7 cells (Govender et al. 2014) and COS1 cells (Avenant et al. 2010b). Decreased GR protein is consistent with the glucocorticoid-like properties of MPA and the established observation that glucocorticoids like Dex induce GR turnover (Dong et al. 1988, Hoeck et al. 1989, Webster et al. 1997, Wallace and Cidlowski 2001, Deroo et al. 2002, Avenant et al. 2010a, Avenant et al. 2010b).

Taken together, these data support a role for GR, but not other steroid receptors, in mediating HIV-1 infection in response to MPA but not luteal phase E_2/P_4 at the concentrations used in this study.

6.2 HIV-1 infection in the absence of exogenous MPA or E_2/P_4 increases with low GR/high PR and may involve the regulation of soluble factors

The present study showed that in the absence of exogenous MPA or E_2/P_4, HIV-1 infection significantly increased when GR levels were decreased by siRNA in TZM-bl cells (Fig. 4.2.2.1a). Consistent with this, HIV-1 infection in PBMCs significantly increased upon stimulation with the GR/PR antagonist RU486 in the vehicle control condition (Fig. 3.4.1.1a). GR protein levels were not changed by PR-B over-expression (Fig. 4.2.2.1b, c). These results suggest that HIV-1 infection in the absence of exogenous MPA or E_2/P_4 is sensitive to changes in GR levels, with unliganded GR protecting against infection. However, due to the fluctuating levels of multiple endogenous hormones, there is unlikely to be a state of no hormones *in vivo*, at least for E_2/P_4. This suggests that GR and PR are unlikely to be truly unliganded. Nevertheless these data provide some insight into molecular mechanisms of cross talk between the GR and PR.

A potential mechanism for increased HIV-1 infection when GR levels were decreased in the absence of exogenous MPA or E_2/P_4 could be via a preferential increase in CD4 expression, since CD4 mRNA levels were significantly increased when GR levels were decreased, unlike CCR5 or CXCR4 (Fig. 4.3.1.1) mRNA levels which were unchanged. However the direct effects of GR on CD4 levels remain to be determined.

This potential mechanism of higher CD4 levels could also be applicable in the presence of MPA in TZM-bl cells, since CD4 mRNA levels were increased (Fig. 3.3.2.3b). In PBMCs, however, while MPA increased the frequency of CD4+ T cells (Fig. 3.3.1.1a), the densities of CD3, CD4 and CD8 on the surface of lymphocytes were significantly decreased (Fig. 3.3.1.2a). Usually the CD4 receptor is down-regulated during HIV-1 infection and cell differentiation (Paillard et al. 1990, Butera et al. 1991, Pimentel-Muinos et al. 1992, Geleziunas et al. 1994, Levesque et al. 2004). The results from the present study could indicate that despite the decrease in CD4 density, the increase in the number of CD4+ T cells in combination with the simultaneous increase in CCR5 levels on CD4+ T cells (Fig. 3.3.2.2), is sufficient to promote infection in the presence of MPA. Again, however, a direct effect for MPA on CD4 was not established in this study.

Together these data suggest that there may be different effects on HIV-1 infection and expression of markers relevant to infection depending on different levels of GR, and whether the GR is unliganded or bound to MPA. There is evidence from the literature to suggest a role for the GR and GR levels in HIV-1 pathogenesis, although this research has mostly focussed on the liganded GR, usually bound to Dex. Clinically, AIDS patients have been shown to develop GC hypersensitivity and reduced immunity, possibly through GC-induced repression of cytokines (Mirani et al. 2002, Kino et al. 2003, Hapgood and Tomasicchio 2010). In addition, skin samples from AIDS-associated Kaposi's sarcoma (AIDS-KS) patients have higher GR protein levels than healthy control donors (Guo et al. 1996). Using shRNA targeting the GR in the THP1 monocyte cell line, it was shown that ligand-activated GR was required for proviral integration (Wiegers et al. 2008). Thus changes in GR levels are likely to have physiological implications in HIV-infected individuals, as well as people exposed to high amounts of the endogenous GC cortisol during periods of chronic stress.

In addition, the present study suggests that HIV-1 infection in the absence of exogenous MPA or E_2/P_4 is sensitive to changes in PR levels. In TZM-bl cells, HIV-1 infection was significantly increased with both high GR/high PR and low GR/high PR (Fig. 4.2.2.1a). This increase in HIV-1 infection with high levels of unliganded PR-B occurs through an unknown mechanism, since there was no change in CD4, CCR5 or CXCR4 mRNA levels with over-expressed PR-B in TZM-bl cells (Fig. 4.3.1.1). There appears to be no evidence in the literature directly implicating a role for unliganded PR-B in HIV-1 infection. The effect of unliganded PR may be relevant to HIV-1 infection in the FRT, where PR protein is expressed in cervical stromal fibroblast cells and sometimes in the epithelium (Ayehunie et al. 2015). The PR detected in pre- and postmenopausal ecto- and endocervical explants in this study could therefore represent PR expression in stromal but not epithelial cells (Appendix B, Fig. B2.1, B2.2). Interestingly, a recent study showed that co-culture of endometrial stromal fibroblasts with PBMCs enhanced HIV-1 infection of CD4+ T cells, in the

absence of exogenous E_2/P_4 mimicking the luteal phase (Neidleman et al. 2017). This suggests that adjacent cells expressing PR can mediate HIV-1 infection of other cells.

An interesting observation from the present study was that, in the absence of exogenous MPA or E_2/P_4, HIV-1 infection in TZM-bl cells could be changed when co-cultured with supernatants from End1/E6E7 or HeLa cells in which GR/PR levels were altered from high GR/no PR to low GR/high PR (Fig. 4.4.1). This indirect effect is consistent with the result in TZM-bl cells in which GR/PR levels were directly changed (Fig. 4.2.2.1a). Supernatants from End1/E6E7 cells with low GR/no PR also increased HIV-1 infection in TZM-bl cells (Fig. 4.4.1a). These results suggest a role for secreted soluble mediators in HIV-1 infection in the absence of exogenous MPA or E_2/P_4 in TZM-bl cells. These soluble mediators could be the result of paracrine signalling from adjacent cells or autocrine signalling from the TZM-bl cells themselves. Whether or not the soluble factors are regulated directly or indirectly by changing GR/PR remains unanswered.

The present study aimed to investigate the role of the pro-inflammatory cytokine IL-6 as a soluble factor that could be mediating increased HIV-1 infection in the absence of exogenous MPA or E_2/P_4 in TZM-bl cells. IL-6 was an attractive candidate for two reasons. Firstly, some studies have shown a potential association between increased IL-6 levels and HIV-1 infection (see section 1.6). Secondly, IL-6 expression is regulated by the transcription factor NFκB, whose subunits have been shown to bind to and are inhibited by both GR and PR (see section 1.6). This puts forward IL-6 as a potential link between the secretion of soluble mediators, HIV-1 infection and changing GR/PR levels

The present study showed that basal IL-6 mRNA levels were significantly increased with low GR/high PR in both End1/E6E7 cells and HeLa cells (Fig. 4.3.3.1). IL-6 protein levels in HeLa cells followed the same pattern; with higher IL-6 protein levels of about 400 pg/ml with low GR/high PR compared to about 100 pg/ml with high GR/no PR (Fig. 4.4.2a). These levels are much lower than the 20 ng/ml IL-6 secreted from endocervical cells in one study (Fahey et al. 2005). Together these results suggest that IL-6 mRNA and/or protein levels are sensitive to changes in GR/PR levels, where GR favours an anti-inflammatory response and PR-B promotes a pro-inflammatory response.

The attempt to link GR/PR levels, increased IL-6 levels and HIV-1 infection in TZM-bl cells disappointingly did not show increased HIV-1 infection with increasing concentrations (0-200 pg/ml) of exogenous recombinant IL-6 (Fig. 4.4.3). This experiment did not have a positive control, which would in retrospect have been useful for the interpretation of the result. However, obtaining a positive control for the efficacy of recombinant IL-6 is not straight-forward and would have required finding a cell line system responsive to IL-6, that expresses an IL-6 receptor and generates a quantifiable IL-6 response. Such a system was not available at the time but might be worth considering in future work.

Nevertheless, there are some *in vitro* studies suggesting that exogenous IL-6 increases HIV-1 infection in different models. For example, stimulation with exogenous IL-6 (20 ng/ml) together with IFNγ increased susceptibility of MDMs to X4-tropic infection (Zaitseva et al. 2000). Another report showed that IL-6 (100 units/ml) increased HIV-1 replication in MDMs (Poli et al. 1990). However another study observed that recombinant IL-6 (40-1000 pg/ml) dose-dependently decreased HIV-BaL reverse transcriptase activity in macrophages (Rogez-Kreuz et al. 2005). Together these studies imply that the mechanism for IL-6 increasing HIV-1 infection is not clear. The differences between these studies and the present study could be attributed to multiple variables, including but not limited to different cell type, different concentrations of exogenous IL-6, duration of incubation, degradation of IL-6, and order of stimulation and infection. There appears to be no evidence in the literature on exogenous IL-6 and increased infection in T cells. However, the results from the present study do not exclude the possibility that, in line with the studies above, the increase in IL-6 mRNA/protein could be a potential mechanism for the observed increase in HIV-1 infection in TZM-bl cells, with low GR/high PR.

Taken together, the results from this study in the absence of exogenous MPA or E_2/P_4 support a role for changes in relative GR/PR levels, from high GR/no PR to low GR and/or high PR-B levels, in increasing HIV-1 infection in TZM-bl cells, and that this occurs through mediating changes in unidentified secreted factors.

6.3 GR-mediated gene regulation in response to MPA is altered in the presence of PR-B

GILZ and IL-6 were used in this study as model genes for GR-mediated transactivation and transrepression, respectively (see section 1.6). Consistent with results from the present study (summarized in Table 6.3), robust GR-mediated transcriptional responses to GILZ and IL-6 in response to Dex and/or MPA have previously been reported in other cell types (Ronacher et al. 2009, Verhoog 2010, Africander et al. 2011a, Verhoog et al. 2011, Govender 2014, Govender et al. 2014, Hapgood et al. 2014b, Ray 2015).

The cell type- and gene-specific differences between End1/E6E7 and HeLa cells may be due to variations in GR knockdown efficiency, with End1/E6E7 cells showing a 50% decreased in GR levels (Fig. 4.3.2.1c, d) and HeLa cells only having 25% decrease in GR levels (Fig. 4.3.2.2c, d). The GR levels in HeLa cells after GR knockdown may therefore still be sufficient to induce GILZ mRNA in response to MPA, while IL-6 repression still occurs in a GR-dependent manner possibly because IL-6 transrepression is more sensitive to GR levels.

The data from the present study suggest that the presence of PR-B changes the GR-mediated responses to MPA. This effect was observed for the endogenous gene IL-6 in both End1/E6E7 and HeLa cells, whereas no effect was observed for GILZ or in response to Dex (Fig. 4.3.2.1-3, Table 6.3). Thus the effect of PR-B on GR-mediated transcriptional responses appears to be gene- and ligand-specific.

It could be hypothesized that with high GR/high PR, MPA molecules are divided between GR and PR-B such that the GR is not sufficiently activated and does not repress IL-6 expression. Then, with low GR/high PR in End1/E6E7 cells, MPA binds to the more available PR-B and the lower numbers of GR-MPA cannot repress IL-6. In contrast in HeLa cells which have a slightly higher GR:PR ratio even in the low GR/high PR condition, the levels of GR are sufficiently high enough to be activated by MPA to repress IL-6. Together this suggests that MPA regulation of IL-6 is anti-inflammatory via the GR and either anti-inflammatory or not changed (i.e. no longer anti-inflammatory) via the PR, depending on the cell type and relative GR levels. These findings could have important implications for the inflammatory environment in the FRT of DMPA users, where the relative levels of GR/PR change in response to the menstrual cycle and in different FRT compartments (see section 1.5.4).

Table 6.3. Summary of Dex- and MPA-induced effects on GILZ and IL-6 transcriptional responses in End1/E6E7 and HeLa cells when the relative levels of GR/PR are altered.

			High GR No PR	**Low GR No PR**	**High GR High PR**	**Low GR High PR**
			GR GR GR GR	GR GR	GR GR PR PR GR GR	GR GR PR PR
Ligand	**Gene**	**Cell line**	**NSC**	**siGR**	**NSC**	**siGR**
			-PR-B		**+PR-B**	
Dex	GILZ	End1/E6E7	++++	++	++++	++
		HeLa	++++	++	++++	++
	IL-6	End1/E6E7	++++	+	++++	++
		HeLa	++++	+	++++	+
MPA	GILZ	End1/E6E7	++	-	++	-
		HeLa	++	++	+++	++
	IL-6	End1/E6E7	++	-	-	-
		HeLa	+	-	-	+

Number of + signs indicates the degree of induction for GILZ and repression for IL-6, - indicates no change relative to the vehicle control. Results are summarized from Figures 4.3.2.1, 4.3.2.2 and 4.3.2.3.

GR/PR protein levels relative to GAPDH were determined by western blotting in End1/E6E7, HeLa and TZM-bl cells. In all three cell lines, PR-B over-expression in the low GR condition was slightly but not significantly lower than in the non-silencing control (Fig. 4.2.1.1b, d, Fig. 4.2.2.1b, d, Fig. 4.3.2.1c, e, Fig. 4.3.2.2c, e). This might suggest that the siRNA oligonucleotides targeting the GR can also target the PR for knockdown. Therefore the results with PR-B over-expression should be interpreted with caution. Additionally, both GR and PR undergo ligand-dependent turnover (Dong et al. 1988, Hoeck et al. 1989, Webster et al. 1997, Lange et al. 2000) and therefore the relative levels of GR and PR would also be expected to be lower in response to their ligands, adding further complexity to the interpretation of these results. Furthermore, differences in PR-B over-expression between the cell lines are likely due to variations in transfection efficiency. This was also observed between experiments in the same cell line (Fig. 4.2.1.1b, d, Fig. 4.2.2.1b, d). However, transfection efficiency using a β-galactosidase expression vector was not used since previous work suggests its expression is regulated by steroid hormones in the cell line models used in the present author's laboratory.

This study also suggests that liganded PR-B can decrease liganded GR transcriptional activity on a reporter gene (Fig. 5.2.1a, Fig. 5.2.2a). In addition, unliganded PR-A repressed GR-mediated activity in response to Dex as well as PR-B-mediated activity in response to R5020 (Fig. 5.2.1a, Fig. 5.2.2a, Appendix B, Fig. B5), which is in line with previous studies (Vegeto et al. 1993, Pieber et al. 2001, Maruo et al. 2010). However, when both PR-A and PR-B were over-expressed, Dex induction of TAT-GRE-LUC was similar to that in the absence of either PR isoform (Fig. 5.2.1a, Fig. 5.2.2a). This could mean that when PR-A and PR-B are co-expressed in the presence of GR, PR-A preferentially represses PR-B-mediated activity such that neither PR-A nor PR-B can inhibit GR-mediated activity and therefore a higher Dex induction is observed. These results therefore suggest that the relative ratio of PR-A/PR-B could influence GR-mediated gene regulation, which may change the pro- or anti-inflammatory response. In line with this, a study showed that liganded PR-A increases pro-inflammatory responses while PR-B promotes anti-inflammatory responses on some cytokine/chemokine genes (IL-1β, IL-8) in immortalized myometrial cells expressing inducible PR isoforms (Tan et al. 2012).

Interestingly, while MPA induced TAT-GRE-LUC 48-fold in the presence of GR, this induction was further increased to 110-fold in the presence of GR and PR-B (Fig. 5.2.1a, Fig. 5.2.2a). This suggests that both GR and PR-B mediate up-regulation of TAT-GRE-LUC at the same time in response to MPA. Following the hypothesis from the earlier data on endogenous IL-6 that PR-B modulates GR-mediated activity in response to MPA, it is possible that MPA-bound PR-B inhibits the activity of MPA-bound GR. However this effect cannot be distinguished since MPA-bound PR-B also mediates

induction of TAT-GRE-LUC. Alternatively, perhaps only the unliganded PR is able to modulate GR activity, in the presence of Dex which does not bind PR.

PR-specific gene regulation was not fully explored in this study, for a number of reasons. Firstly, the study aimed to investigate expression of endogenous genes, which are likely to be more physiologically relevant than promoters such as MMTV which contain 4 GRE/PREs. Another criterion for candidate genes in this study was their relevance to immune function. It was therefore challenging to find a PR-regulated immune function gene that is not already regulated by the GR. It is likely that other transactivation and/or transrepression immune function genes are regulated by the PR only; however a thorough search for such genes was not conducted. For the purposes of this study it was decided that robust GR-mediated responses on GILZ and IL-6 would be sufficient for the current investigation into immune function in the FRT.

Secondly, results from this study suggest that over-expressed PR-B was not functional on endogenous genes, based on the observation that over-expressed PR-B regulated progestin induction of the reporter gene TAT-GRE-LUC but not the GR/PR-regulated endogenous gene MKP1 in both End1/E6E7 and HeLa cells (Appendix B, Fig. B4.3). This observation is consistent with a previous report that newly synthesized PR, unlike constitutively expressed PR, cannot activate the MMTV promoter in NIH 3T3-derived cells (Smith et al. 1993, Smith et al. 2000a). The same authors showed that deficient PR activity on an endogenous MMTV promoter was due to the inability of the PR to initiate the chromatin remodelling required for gene activation (Smith et al. 1997). In retrospect, a more suitable cell line model would have been the derivative HeLa cells stably over-expressing each of the PR isoforms (Richer et al. 1998). There are additional reasons why PR-B would be functional on a reporter gene but not an endogenous gene. For example, the high copy number of over-expressed promoter-reporter expression vectors, and the absence of adjacent condensed regions of chromatin could enable greater access for PR-B to the DNA and therefore could explain high PR-B activity on the TAT-GRE-LUC reporter gene but not the endogenous gene.

Thirdly, while characterizing the function of endogenous GR and PR in MCF-7 and T47D cell lines, it was found that PR was functional on the TAT-GRE-LUC reporter gene (Appendix B, Fig. B6.2a, c), but GR was only weakly functional on endogenous transactivation models (Appendix B, Fig. B6.2b, d, Fig. B6.3a, c) and not functional on endogenous transrepression models (Appendix B, Fig. B6.3b, d). It is tempting to speculate that the high levels of PR in T47D cells could be inhibiting endogenous GR activity. One study found that the absolute levels of GR protein were about 60-fold lower than PR in T47D cells (Horwitz et al. 1978), which could suggest preferential use of the more-available PR than GR, at least for ligands that can bind both receptors. Consistent with the present study, other studies have also reported a non-functional GR in T47D cells (Cato et al. 1986, Cato et al. 1988,

Chalepakis et al. 1988, Wu and Pfahl 1988, Nordeen et al. 1989). A T47D derivative cell line stably expressing comparable amounts of GR to those of PR, T47D-A(1-2), has been described (Nordeen et al. 1989). This cell line would also perhaps have been a better choice for investigating GR/PR reciprocal modulation on endogenous genes. However, attempts to transiently over-express increasing amounts of GR expression vector in T47D cells in this study were unsuccessful (Appendix B, Fig. B6.4). Interestingly, in T47D-A(1-2) cells the stably integrated MMTV promoter was found to be more inducible by GC than progestin (Archer et al. 1994a, Archer et al. 1994b), suggesting that when GR/PR levels are equivalent, a GR-mediated response is favoured. In line with this, a recent study in T47D-A(1-2) cells showed that GR knockdown restored PR-mediated gene regulation in response to R5020, suggesting that GR can modulate PR activity in this model (Ogara et al. 2015). It is tempting to speculate that stably expressed GR inhibits PR activity in these cells.

While the results of the present study suggest that PR-B can modulate the activity of liganded GR on some genes in some cells, other studies suggest that the presence of GR can inhibit PR activity. Collectively these observations suggest that GR and PR can reciprocally modulate each other's activity. These findings suggest that the relative levels of GR/PR may play an important role in determining the inflammatory and immune responses in the FRT of DMPA users.

6.4 Unliganded GR-mediated gene regulation is altered in the presence of unliganded PR-B

This study also provides some evidence that changing the relative levels of GR/PR alters the basal transcription of some but not other immunomodulatory genes in the absence of MPA (Fig. 4.3.3.1-2). These findings are summarized in Table 6.4. These results suggest that in the absence of MPA, changing GR/PR levels from high GR/no PR to low GR/high PR modulates the basal expression of some immunomodulatory genes, in a gene- and cell-specific manner.

While the genes investigated in this study (Table 6.4) are known to be regulated by the GR, their regulation by the PR is not well established. The exception is IL-8, for which ligand-mediated repression via both GR and PR has been demonstrated. IL-8 repression by GR and PR occurs through different mechanisms, where PR inhibits the recruitment of Pol II to the transcription initiation site and GR bound to SRC-2 inhibits the transcription elongation factor b complex (Rogatsky et al. 2002, Kobayashi et al. 2010). Interestingly, one study in immortalized myometrial cells with inducible PR isoforms showed that in the absence of ligands, PR-B-dominant cells expressed higher levels of IL-1β and IL-8 than the PR-A-dominant cells (Tan et al. 2012). Together the data on IL-6, IL-8, RANTES and GILZ in the present study could suggest that the unliganded GR generally favours an anti-

inflammatory state while the unliganded PR-B generally favours a pro-inflammatory state in cervical cells.

It is difficult to say whether or not unliganded GR modulates the activity of unliganded PR-B based on the results from this study. In HeLa cells over-expressing PR-B, basal IL-6 mRNA levels increased when GR levels were decreased (Fig. 4.3.3.1d, Table 6.3). In addition, in End1/E6E7 cells over-expressing PR-B, basal GILZ mRNA levels increased when GR levels were decreased (Fig. 4.3.3.1a, Table 6.3). It could be that PR-mediated regulation of these genes can only occur with low GR levels, but the effects appear to be gene- and cell type-specific. Further investigation is therefore needed, using genes shown to be directly regulated by the PR.

Table 6.4. Summary of basal transcriptional responses in End1/E6E7 and HeLa cells when the relative levels of GR/PR are altered

			High GR No PR	**Low GR No PR**	**High GR High PR**	**Low GR High PR**
			GR GR GR GR	GR GR	GR GR PR PR GR GR	GR GR PR PR
Ligand	**Gene**	**Cell line**	**NSC**	**siGR**	**NSC**	**siGR**
			-PR-B		**+PR-B**	
None (basal)	GILZ	End1/E6E7	+++	++	+	+
		HeLa	-	-	-	+
	IL-6	End1/E6E7	-	+	-	+
		HeLa	-	+	+++	++++
	IL-8	HeLa	-	-	-	+
	RANTES	HeLa	-	-	-	+
	IL-10	HeLa	-	+ (?)	+ (?)	-
	IL12-p35	HeLa	-	-	-	-
	IL12-p40	HeLa	-	+ (?)	-	+ (?)

Number of + signs indicates degree of induction for GILZ and IL-10, and repression for IL-6, IL-8, IL-12 and RANTES. - indicates no change relative to NSC-PR-B. (?) indicates a non-statistically significant effect. Results are summarized from Figures 4.3.3.1 and 4.3.3.2.

It is possible that the effects on immunomodulatory gene expression in this study from high GR/no PR to low GR/high PR are solely due to changes in GR levels (Gougat et al. 2002). Higher levels of unliganded GR can dimerize and regulate expression of GILZ in COS1 cells over-expressing GR (Robertson et al. 2013b). In addition, a portion of unliganded GR is localized to the nucleus and can activate transcription in the absence of hormone ligands, and in the presence of other signalling factors such as TNFα (Hapgood et al. 2016).

Alternatively, it could be that unliganded GR and unliganded PR-B modulate each other's activity through various mechanisms including changing chromatin remodelling in key regulatory regions. There are many studies using liganded receptors that support this hypothesis. For example, progestin-bound PR can activate transiently expressed but not stably integrated MMTV that has acquired an ordered chromatin formation, unlike glucocorticoid-bound GR (Archer et al. 1994a, Archer et al. 1994b, Smith et al. 1997). Another study showed that RU486-bound PR inhibited GR activation of the MMTV promoter by preventing GR's ability to promote assembly of a transcription initiation complex (Fryer et al. 1998). In line with this, the unliganded PR in breast cancer cells was shown to silence basal expression of target genes by recruiting a multi-protein repressor complex that was displaced upon hormone binding (Vicent et al. 2013). Additionally, R5020-activated PR has been shown to inhibit glucocorticoid-induced activation of β-casein by physically binding to the promoter and blocking GR binding (Buser et al. 2011). However, the chromatin remodelling capabilities, interacting partners and context-dependence of GR and PR are more complex than indicated here and have been reviewed by others (Kagoshima et al. 2001, Trotter and Archer 2007, Grontved and Hager 2012, King et al. 2012, Burd and Archer 2013, Ceballos-Chavez et al. 2015). Taken together, PR-B could repress GR activity by modifying the chromatin architecture and/or physically preventing GR binding to promoters.

The differential effects observed with changing GR/PR levels could also be a result of competition for available co-factors (co-activators, co-repressors and co-modulators). Hundred of co-regulators interact with the GR (Weikum et al. 2017b). Levels of exogenously expressed co-repressors NCoR and SMRT differentially affected the potency of agonists bound to GR versus PR (Song et al. 2001). Furthermore, PR and GR both interact with steroid receptor coactivator (SRC)-1 and SRC-2, but have different preferences, that is, PR preferentially recruits SRC-1, while GR prefers SRC-2 (Li et al. 2003). Co-activator expression and function also vary in a cell- and tissue-specific manner (Molenda et al. 2003, Lonard et al. 2007, York and O'Malley 2010, Stashi et al. 2014) and could therefore influence the ligand-independent transcriptional responses to changing GR/PR.

Taken together, over-expressed PR-B appears to modulate the GR-mediated regulation of some key immunomodulatory genes, but not others, in the absence of MPA, which may involve a number of mechanisms not further explored in this thesis. This provides useful insight into reciprocal modulation of GR- and PR-mediated transcriptional activity. These results could have implications for the baseline inflammatory and immune responses in naturally cycling women not using contraceptives, where relative levels of GR/PR change in the FRT across the menstrual cycle.

6.5 GR and PR may associate within the same protein complexes within the nucleus

Following the data above suggesting that GR and PR may reciprocally modulate each other's activity in regulation of some immunomodulatory genes, it was hypothesized that the mechanism of reciprocal modulation could be through physical association of the receptors. Using co-IP to infer an interaction (which could be either direct or indirect), PR appeared to be pulled down with anti-GR antibody, in both COS1 cells over-expressing GR/PR-B and MCF-7 cells expressing endogenous GR/PR (Fig. 5.3.1-2). There appeared to be greater PR pulled down in the presence of MPA in COS1 cells in some but not all experimental repeats (Fig. 5.3.1), suggesting that the GR-PR interaction may be ligand-dependent. However this was not clear in MCF-7 cells. Further investigation is therefore required to determine the ligand-dependency of this association.

Consistent with this, another study using reciprocal co-IP assays showed that PR-A interacts with GR in mouse lung cells, although they did not investigate whether GR interacts with PR-B (Shao et al. 2006). Furthermore a recent study in T47D cells showed that fluorescent tagged mCherryGR and eGFP-PR formed a complex in the presence of R5020 or Dex but not in the absence of ligand (Ogara et al. 2015). The same study showed using ChIP-seq in T47D-A(1-2) cells that 74% of Dex-bound GR mapped to the same binding regions as R5020-bound PR, and conversely that 48% of PR-bound regions were also bound by GR (Ogara et al. 2015). This supports the hypothesis that GR and PR can bind to the same target regulatory DNA regions as a possible heterodimer.

Following the indication of a potential association, the cellular localization of over-expressed GR and PR in COS1 cells was investigated using immunofluorescence with confocal microscopy (Fig. 5.5). GR and PR were shown to be mostly co-expressed within the same cells (Fig. 5.4). In addition, quantification of nuclear co-localization showed that GR and PR co-localized (that is, spatially co-occurred) in the nucleus in response to the ligands Dex, R5020 and MPA but not the vehicle control (Fig 5.6.1, Table 5.6.1). This finding was further supported using SR-SIM, which has about a 2-fold higher resolution than confocal microscopy (Fig. 5.6.3, Table 5.6.2). Further inspection of SR-SIM images indicated that ligand-dependent GR-PR co-localization occurred within some regions of the nucleus, but that there were regions expressing GR-only or PR-only (Fig. 5.6.4).

These results are consistent with other work showing that PR is mostly localized to the nucleus (Perrot-Applanat et al. 1985, Renoir et al. 1990, Guiochon-Mantel et al. 1991) and that GR translocates to the nucleus in a ligand-dependent manner (Picard and Yamamoto 1987, Vicent et al. 2002). It has also been shown that GR nuclear translocation can be increased by increasing GR levels (Robertson et al. 2013a). A previous report using immunohistochemistry showed that PR-A and GR

co-localize in the nucleus in mouse lung alveolar but not bronchial epithelium, although this is likely influenced by the low levels of endogenous corticosterone (Shao et al. 2006).

The nuclear translocation of GR in response to R5020 has to the present author's knowledge not been previously shown. R5020 is generally used in relative binding affinity studies as the reference ligand with 100% binding to the PR. There are limited reports suggesting that R5020 can bind to the GR with low affinity (Nordeen et al. 1981, Stromstedt et al. 1990), so this finding is perhaps unsurprising. The dose response assay in this study indicated that R5020 at 100 nM did not activate GR-mediated TAT-GRE-LUC reporter gene activity (Fig. 5.6.2). This suggests that while R5020 could induce GR nuclear translocation, R5020-bound GR may not be transcriptionally active. These results support the idea that R5020 is a poor PR-specific agonist, and future studies should perhaps focus on a PR agonist with no GR cross-reactivity, such as NET (Ronacher et al. 2009).

In light of the work showing that PR can physically block GR binding to a target promoter (Buser et al. 2011), there is likely a dynamic and temporal interplay where the GR and PR move on and off the same target promoters. This leads to interesting unanswered questions such as whether GR and PR bind to the same sites like promoters and/or regulatory regions of immunomodulatory genes, in a ligand-dependent or –independent manner. However, as mentioned above, this may depend on chromatin context, co-factor expression, promoter-specific effects and/or other factors.

Together the results from the co-IP assays suggest that GR and PR associate in a ligand-independent manner, while the immunofluorescence results suggest that GR and PR co-localize in a ligand-dependent manner. It is possible that a GR-PR association in the same complex in the nucleus modulates the ligand-dependent effects of different GR/PR ligands, while other non-genomic mechanisms may operate for ligand-independent effects. These non-genomic mechanisms could include modulation of chromatin remodelling and activation of signalling cascades. Non-genomic signalling pathways have been described for both the GR and PR (Ehring et al. 1998, Edwards et al. 2002, Leonhardt et al. 2003, Stellato 2004, Vallejo et al. 2005, Chien et al. 2006, Lowenberg et al. 2008, Gellersen et al. 2009, Ordonez-Moran and Munoz 2009). It is also possible that the GR-PR association in the absence of MPA observed by co-IP occurs with a very small percentage of GR/PR such that these effects are not observed with immunofluorescence. This effect would especially not be observed if this association occurs in the cytoplasm, since SR molecules are more spread out than in the nucleus. Taken together, the results from this study support the hypothesis that GR and PR associate with each other. This closer physical proximity may be a mechanism by which GR and PR reciprocally modulate each other's activities. This may be relevant in the FRT where GR/PR ligands induce the nuclear co-localization and association of GR and PR, in addition to possible non-genomic

effects in the cytoplasm, which may have important consequences on GR- and PR-mediated immunomodulatory gene regulation and/or HIV-1 infection.

Chapter 7

Conclusions and Future Perspectives

7.1 Conclusions

The results in PBMCs and TZM-bl cells support the first central hypothesis in this study, which was that MPA, at a concentration representing the upper range of serum levels in DMPA users, and E_2/P_4, mimicking the luteal phase of the menstrual cycle, increase HIV-1 infection, and that this may occur through potentially different biological mechanisms. These mechanisms of enhanced infection in PBMCs with MPA, unlike luteal phase E_2/P_4, are most likely through an increased CD4/CD8 ratio, increased expression of CCR5 co-receptor on the surface of CD4+ T cells, and increased expression of the activation marker CD69 on CD4+ T cells. The results in cervical TZM-bl cells showing that MPA increases HIV-1 infection and mRNA levels of both the CD4 receptor and the CCR5 co-receptor strongly support the PBMC results. The results in both PBMCs and TZM-bl cells also provide evidence of a role for the GR and GR levels in modulating HIV-1 infection in the presence of MPA. The results also support other studies suggesting that DMPA users and women in the luteal phase of the menstrual cycle are at higher risk of HIV-1 acquisition than women in the follicular phase.

While the TZM-bl cervical cell model does not fully represent the FRT, it provides a useful opportunity to study HIV-1 infection, particularly viral entry, and provides insight into biological mechanisms relevant to HIV-1 target cells. While there are known differences in immune cells (such as frequency of T cells) in the FRT compared to PBMCs, the results from the two models used in this study combined (PBMCs and TZM-bls) suggest that these mechanisms are likely relevant to any HIV-1 target cells in the body. These HIV-1 target cells include many cell types in the FRT that express CD4 and the CCR5 co-receptor, such as CD4+ T cells and macrophages. This thesis therefore provides important evidence for the proof of concept for biological mechanisms of infection. Currently the biomarkers for infectability have not been defined (Hapgood et al. 2018). However the results from this study suggest that CCR5 levels may be a useful biomarker. Furthermore the finding that luteal phase hormones increase HIV-1 infection via a different mechanism to MPA suggests that the effects are not due to P_4 but rather to activation of the GR by MPA.

The present author is aware that there are limitations translating *ex vivo* research to *in vivo* effects. However this study aimed to find evidence of biological mechanisms for MPA and HIV-1 infection,

which cannot be done *in vivo*. Another limitation of the current study is that the cell models used were non-FRT target cells, so the immune system of the FRT was not fully represented. Furthermore the concentrations of exogenous hormones used *ex vivo* in this study were possibly too high for MPA and too low for P_4 representing the luteal phase. Nevertheless the molecular mechanisms and insights provided by these data are consistent with many clinical observations, and provide strong support for a role of increased CCR5 levels as a key biological mechanism by which MPA can increase HIV-1 infection in any HIV-1 target cells that express CD4 and CCR5, in both peripheral blood and the local FRT.

In line with the growing body of epidemiological and molecular evidence that MPA increases susceptibility to HIV-1 infection, the WHO has recently changed the medical eligibility criteria for MPA and other injectables from Category 1 (no restriction) to Category 2 (where the benefits of using this method "generally outweigh the theoretical or proven risks") (World Health Organization 2017). Identifying progestin contraceptives with no effects on HIV-1 infection, such as NET-EN or LNG, that may be more suitable alternatives to DMPA in high HIV-1 risk areas, is a subject of ongoing research (Hapgood 2013).

The results of this thesis also provide proof of concept in support of the second central hypothesis of this study, which was that changing the relative levels of GR/PR can influence HIV-1 infection and the regulation of immunomodulatory genes in the FRT. This is relevant to DMPA users *in vivo* since MPA activates both the GR and the PR. Together the results in cervical cell lines suggest that MPA acting via the GR increases HIV-1 infection and promotes immunosuppressive regulation of key GR-mediated immunomodulatory genes. Furthermore the presence of PR-B increases HIV-1 infection in the absence of MPA and modulates GR-mediated immunomodulatory gene regulation in both the absence and presence of MPA. However the effect of PR-B on immunomodulatory gene expression appeared to be gene-, ligand- and cell type-specific, which suggests that in these models the main contribution to increased HIV-1 infection is not via different expression of immunomodulatory genes. This appears to be consistent with the current clinical studies suggesting that overall MPA may have no effect on immunomodulatory gene expression. Nevertheless the *in vitro* evidence of immunosuppressive effects of MPA via the GR suggest that other progestin-based contraceptives that do not act via the GR, such as NET (Ronacher et al. 2009), administered as the injectable Nur-isterate, may be more suitable contraceptives to use in high HIV-1 risk areas (Hapgood 2013). The use of NET instead of MPA may thus be less likely to enhance sexual HIV-1 transmission in these high-risk areas.

The third central hypothesis of the current study, stating that GR and PR reciprocally modulate each other's activity through a possible interaction, was supported by the results suggesting that PR-B may modulate the activity of GR in some contexts and that there is a ligand-dependent association between

GR and PR in the nucleus. Although cell lines and not primary FRT cells were used, the results supporting the second and third hypotheses are likely to have important physiological relevance to changing relative GR/PR levels in FRT tissues *in vivo*. This may be particularly relevant for immune function and susceptibility to HIV-1 acquisition in the ectocervix, where both GR and PR are expressed. PR expression in the ectocervix has been shown to be higher during the luteal phase, which would result in conditions of either high GR/high PR or low GR/high PR *in vivo*. This would depend on GR levels which also fluctuate in response to circadian and chronic stress-induced variations in endogenous GCs like cortisol (Cohen et al. 2012, Patterson et al. 2013, Contoreggi 2015, Cain and Cidlowski 2017). However, the high inter-donor variability in GR and PR protein expression in cervical samples *ex vivo* suggests that the effects of changing relative GR/PR levels on HIV-1 infection and immunomodulatory gene regulation may be more pronounced in some women than others.

Based on the results from this study, it could be hypothesized that HIV-1 infection increases in the presence of MPA as a result of two concurrent biological mechanisms, which are represented schematically in Figure 7.1. The main mechanism is that MPA increases CCR5 levels on the surface of target cells and increases activation of CD4+ T cells while simultaneously increasing the ratio of CD4/CD8 T cell frequency (Fig. 7.1: 1). This effect is likely to be relevant to all HIV-1 target cells in the body, including the FRT. The second mechanism is that MPA activates the GR which regulates GR-dependent transactivation of GILZ and transrepression of IL-6 (Fig. 7.1: 2, 3). GR-mediated regulation of immunomodulatory genes creates a more anti-inflammatory, immunosuppressive state which could promote HIV-1 infection by suppressing the adaptive immune responses in the local FRT (Fig. 7.1: 2). The presence of PR-B, however, does not affect HIV-1 infection but modulates GR-mediated regulation of key immunomodulatory genes, such as increasing expression of IL-6, via a GR-PR association (Fig. 7.1: 4, 5). Therefore the presence of PR-B promotes a more pro-inflammatory state. However the balance of pro- or anti-inflammatory effects depends on the relative levels of GR/PR, which are likely to vary in the FRT from different women *in vivo*.

In conclusion, the results from this thesis provide insight into likely biological mechanisms for MPA-induced HIV-1 infection in target cells expressing CD4 and CCR5. In addition, the results from this study suggest that HIV-1 infection critically depends on GR but not PR-B levels, while regulation of immunomodulatory genes depends on changing GR/PR levels. Taken together, it is likely that DMPA use may increase HIV-1 infection in some but not other individuals, depending on multiple factors such as CCR5 levels on target cells, CD4+ T cell frequency, varying GR/PR levels, metabolism, serum concentrations, immune activation status and endogenous hormone levels. The complexity of these factors may be a molecular explanation for why so much variability has been observed in clinical studies of MPA and HIV-1 acquisition.

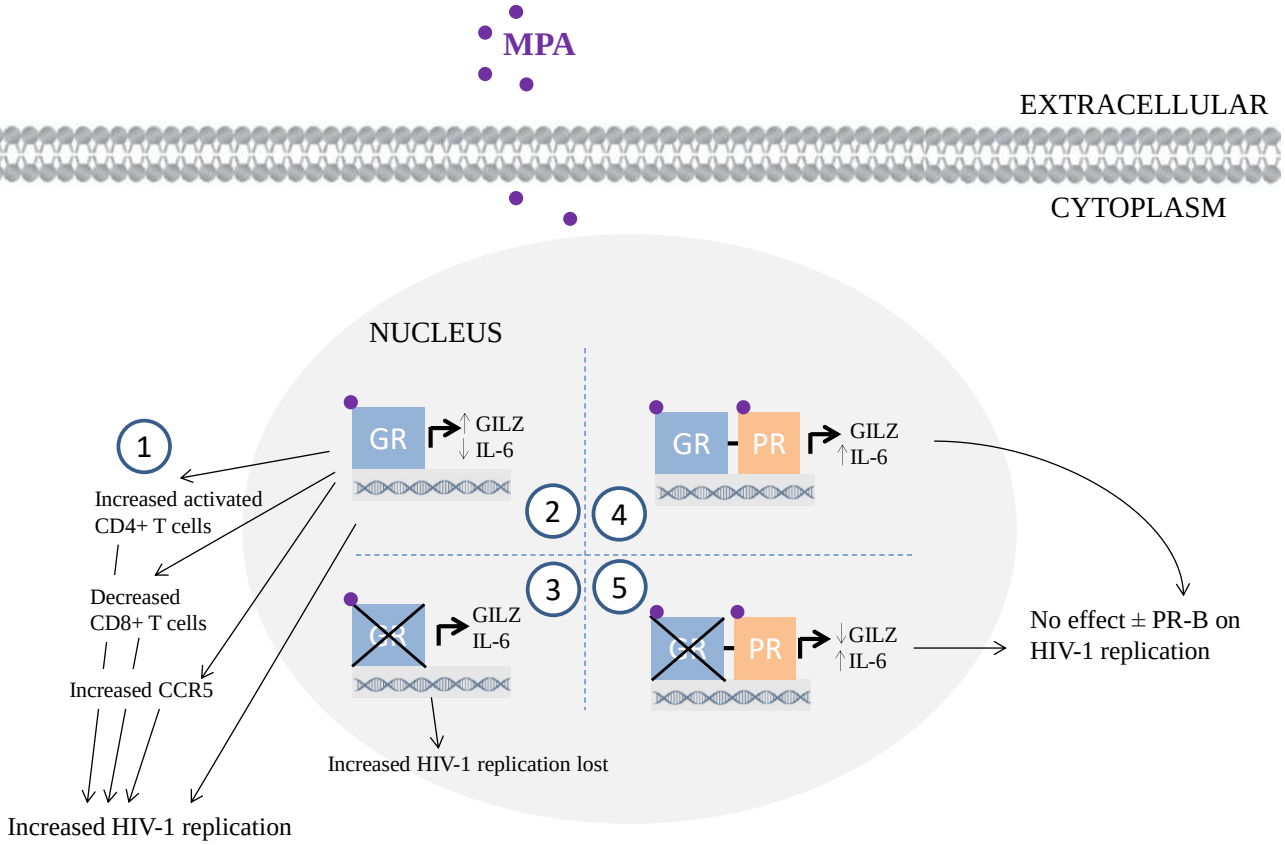


Figure 7.1. Model representing possible biological mechanisms by which MPA increases HIV-1 infection in target cells and the effects of changing GR/PR levels on HIV-1 infection and immunomodulatory gene regulation in the presence of MPA. MPA is lipophilic and diffuses across the cell membrane. (1) MPA increases CCR5 levels on the surface of target cells, increases activation of CD4+ T cells and increases the frequency of CD4+ T cells while simultaneously decreasing the frequency of CD8+ T cells. These biological mechanisms combined result in increased HIV-1 infection in target cells. (2) With high GR/no PR, MPA induces GR-mediated transactivation of GILZ and transrepression of IL-6. In addition, MPA increases HIV-1 infection, most likely via the biological mechanisms indicated in (1). (3) With low GR/no PR, the GR-mediated regulation of GILZ and IL-6 is lost. Furthermore HIV-1 infection with low GR/no PR is abolished compared to high GR/no PR, suggesting that high GR levels are required for MPA-induced HIV-1 infection. (4) With high GR/high PR and (5) low GR/high PR, MPA induces IL-6 mRNA expression, suggesting that PR modulates GR-mediated activity. This occurs via a ligand-dependent GR-PR association, indicated by the line joining GR and PR. The presence of PR-B does not change MPA-induced HIV-1 infection, suggesting that infection depends on GR but not PR levels. For simplification, GR and PR are represented as monomers that directly bind to target gene DNA and regulate transactivation of GILZ or transrepression of IL-6, IL-8 and RANTES. Figure layout adapted from (Hapgood et al. 2016).

7.2 Future perspectives

The results from this thesis led to several interesting unanswered questions that could be explored further. A few of these are discussed below.

Importantly, this study provides *in vitro* proof of concept of relevant biological mechanisms by which MPA can increase HIV-1 infection. Whether these biological mechanisms are clinically relevant *in vivo* remains to be determined. Therefore important future work could test whether some or more of these mechanisms are also observed using PBMC and FRT samples from women on MPA compared to women using no hormonal contraceptives and either in the luteal or the follicular phase of the menstrual cycle.

The PBMCs used in the present study were from female donors at unknown phases of the menstrual cycle. It remains unanswered whether or not the endogenous E_2/P_4 levels in the serum of these women, who may already be in either the luteal or follicular phase, could be masking the effects of exogenous E_2/P_4 in this study. Future work could therefore include quantifying endogenous E_2/P_4 serum levels, thereby categorizing the PBMC donors as in the luteal or follicular phase, prior to HIV-1 infection assays. This study also did not investigate whether more pronounced or different effects on HIV-1 infection and regulation of markers relevant to infection would be observed using exogenous P_4 at higher concentrations. Peak levels of P_4 in the luteal phase as high as 71-200 nM have been reported (Goebelsmann 1986, Vassiliadou et al. 1999, Stricker et al. 2006). Therefore another approach for *in vitro* PBMC experiments would be to use different combinations of E_2/P_4 with higher P_4 concentrations, in order to better mimic the early, mid- and late luteal and follicular phases. Co-stimulation with exogenous LH and FSH could also be used to mimic the natural cycle more closely.

It could be that different responses might be observed if E_2 was co-stimulated with MPA. Therefore, in order to more closely mimic the hormonal environment in the serum of DMPA users, future PBMC experiments could use exogenous stimulation of MPA together with E_2/P_4 at concentrations observed in DMPA users. Whether the effects on HIV-1 infection with MPA are also observed at lower physiological doses of exogenous MPA is a subject of ongoing study in the present author's laboratory. These experiments also use *ex vivo* cervical explant models to gain insight into mechanisms of HIV-1 infection in the FRT. Infection assays from the current study used an R5-tropic IMC where infection was indicated by LTR-driven expression of *Renilla* luciferase. However, since the findings from this study are in the context of the laboratory-adapted strain HIV-$_{1BaL\_Renilla}$, and since different strains of HIV-1 behave differently, some of this data may change if other strains were to be tested. Whether this infection model is physiologically relevant could be tested using clinical isolates of R5- and X4-tropic viruses. It remains unanswered whether the GR plays a direct role in the

regulation of CCR5 expression. This is also a subject of ongoing research in the present author's laboratory.

The question also remains whether GR/PR levels change in tissues from the FRT *in vivo*. Future work could therefore investigate GR/PR levels in FRT samples from women using MPA and women during different stages of the menstrual cycle. An approach for measuring relative GR/PR levels in primary FRT cells could be immunohistochemistry analysis of archived sections taken from these women.

Another unanswered question is whether endogenous or stably expressed PR could have different effects on immunomodulatory gene expression *in vitro* compared to transiently over-expressed PR, as used in this study. Further research could employ the use of other cell line models expressing both GR and PR, for example, the HeLa derivative stably expressing PR-B (Richer et al 1998, Mori et al 2009), the MDA-MB-231 breast cancer cell lines stably expressing each of the PR isoforms and the T47D(A1-2) breast cancer cell line which expresses high levels of endogenous PR and equivalent levels of stably expressed GR (Nordeen et al. 1989). In these cell line models, the relative levels of GR/PR could be altered using siRNA knockdown only, thereby eliminating the confounding effects of variable PR over-expression. In addition to these cell lines, another approach to investigate changing GR/PR levels in a primary cell model could be to use nucleofection to over-express PR in PBMCs.

It is unclear whether the GR and PR may be in close enough physical proximity to interact and form a heterodimer. The work of this study showing ligand-dependent GR/PR nuclear co-localization and protein association, and of Ogara et al (2015), enticingly suggest that GR and PR may interact in a ligand-dependent manner to form a heterodimer. This would be interesting to follow up using Forster Resonance Energy Transfer (FRET) in MPA-treated cells, in order to suggest close physical proximity. FRET and GR/PR co-localization in response to MPA could also be investigated in other cell line models expressing both GR and PR, such as the cell lines stably expressing PR derived from HeLa and MDA-MB-231 cells and the T47D(A1-2) breast cancer cell line.

One of the unanswered questions from the present study is whether there is a causative link between one or more of the immunomodulatory genes regulated by GR/PR and increased HIV-1 infection. It is tempting to speculate that IL-6, showing higher mRNA and/or protein levels with low GR/high PR, could increase HIV-1 infection in TZM-bl cells. However, this link was not established in this study. Future work could therefore investigate different combinations of soluble factors, such as IL-6 and other cytokines, on mediating HIV-1 infection indirectly in TZM-bl cells. However there are likely to be complex combinations of soluble factors involved, making it difficult to identify *in vitro*. Therefore another experiment for future consideration is the high-throughput screening for genes relevant to immune function that are differentially expressed with changing GR/PR levels, for example using

RNA-Seq. If a candidate(s) is identified and exogenous stimulation of its product can enhance HIV-1 infection *in vitro*, the causal link can be demonstrated if co-incubation with an antibody specific to this factor abolishes HIV-1 infection. A candidate gene for further investigation could be the chemokine IL-8. Previous work has shown that repression of NFκB-driven IL-8 expression is regulated by both GR and PR (Kunsch and Rosen 1993, Mukaida et al. 1994, Nissen and Yamamoto 2000, Horie et al. 2005, Tsaprouni et al. 2007). There is also some conflicting evidence linking IL-8 and HIV-1 infection (Denis and Ghadirian 1994, Meddows-Taylor et al. 1998, Tiemessen et al. 2000, Lane et al. 2001, Promadej-Lanier et al. 2010, Mamik and Ghorpade 2014), further suggesting that IL-8 could be a promising GR/PR-regulated gene involved in HIV-1 infection. Whether GR and PR occupy the same promoter(s), such as IL-8, at the same time, in a ligand-dependent manner, could be tested *in vitro* using chromatin immunoprecipitation assays. Regarding the choice of receptor-specific agonists, there are some hints from this thesis suggesting that R5020 may be a poor PR-specific agonist. Therefore future assays could instead use NET or other progestins which do not bind to the GR.

The mechanism by which unliganded PR-B increased HIV-1 infection in TZM-bl cells was not identified in this study. Interestingly, there is evidence that the HIV-1 long terminal repeat (LTR) promoter contains binding sites for the GR (Mitra et al. 1995, Hapgood and Tomasicchio 2010). Owing to their ability to recognise and bind to similar DNA target sequences, the PR may also bind to these sites in the LTR promoter. It would therefore be interesting to determine whether PR-B could also modulate HIV-1 infection via regulating LTR promoter activity. However there are conflicting reports in the literature (Hapgood and Tomasicchio 2010) showing that liganded GR can mediate either increased (Furth et al. 1990, Wiegers et al. 2008) or decreased (Laurence et al. 1989, Mitra et al. 1995, Kino et al. 2000, Hanley and Viglianti 2011) HIV-1 LTR transcription. Thus investigating a role for GR/PR and changing relative GR/PR levels on LTR-mediated transcription could be an avenue for further research.

Appendix A

Supporting data from other researchers in the Hapgood laboratory

Previous research from former PhD students in the Hapgood laboratory has established the basal steroid receptor mRNA and/or protein levels in the model cell systems used within this study.

A1. PBMCs:

PBMCs from two representative female donors express only detectable GR protein (Fig. A1.1b), but also express ERα, GR and MR mRNA (Fig. A1.1a). In this experiment, the same anti-PR antibody was used as for the present study, while MR was excluded due to the non-specific property of the available anti-MR antibody.

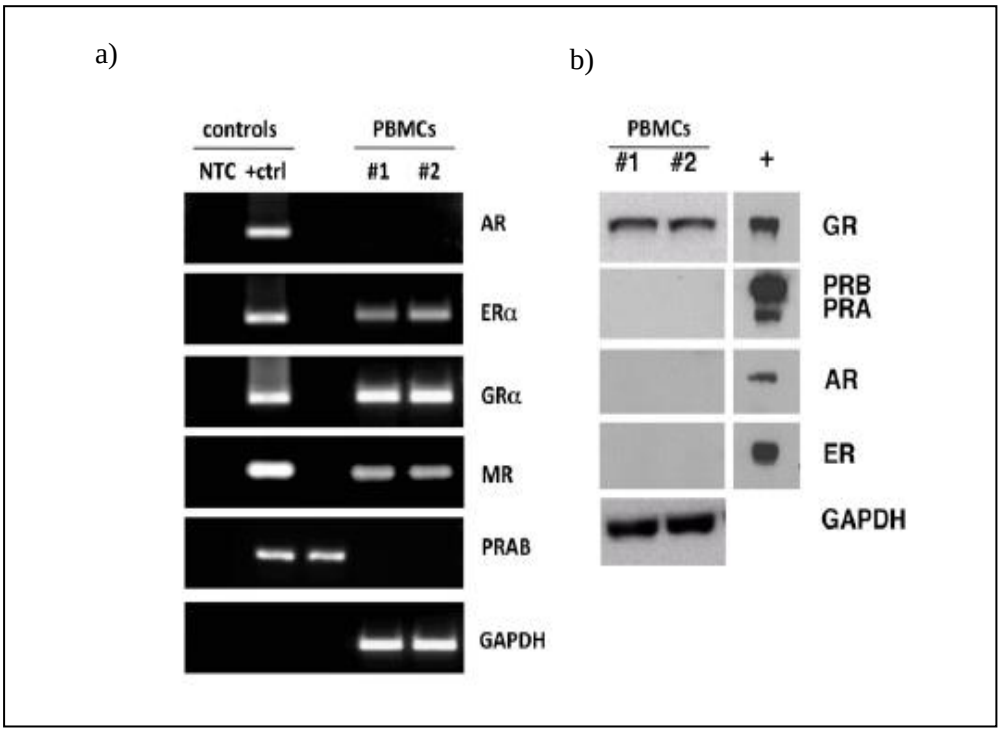


Figure A1.1. Basal steroid receptor mRNA (a) and protein (b) levels in two representative female PBMC donors (R. Ray, PhD thesis 2015, pg 74). (a) Steroid receptor mRNA levels were determined using conventional PCR with cDNA from two PBMC donors (#1 and #2) and receptor-specific primers (Tomasicchio et al. 2013), using GAPDH as a control for mRNA levels. Positive controls (+ctrl) for the steroid receptors were created by PCR amplification of the relevant plasmid DNA. PR (PRAB) has two positive controls, with PR-B loaded first, then PR-A. NTC denotes the no template control (negative control). PCR products were analyzed by gel electrophoresis. (b) Total protein isolated from two PBMC donors using TAPS

buffer was electrophoresed on an 8% SDS-PAGE gel followed by western blotting using antibodies specific to each steroid receptor. Positive controls (+) were created using COS1 cells, and were visualized using shorter exposure times on the same blots (Ray 2015). This figure also appears in (Tomasicchio et al. 2013).

A2. Cell Lines

HeLa cervical carcinoma cells express only detectable GR protein (Fig. A2.1b), but also express GR, AR and MR mRNA (Fig. A2.1a). The modified HeLa cell line, TZMBL, expresses GR and AR protein (Fig. A2.1b), and GR, AR and MR mRNA (Fig. A2.1a). The antibody against MR used here appears to be non-specific, but since MR mRNA is expressed, it may be possible that MR protein is expressed in HeLa and TZMBL cells at levels below the western blot detection limit.

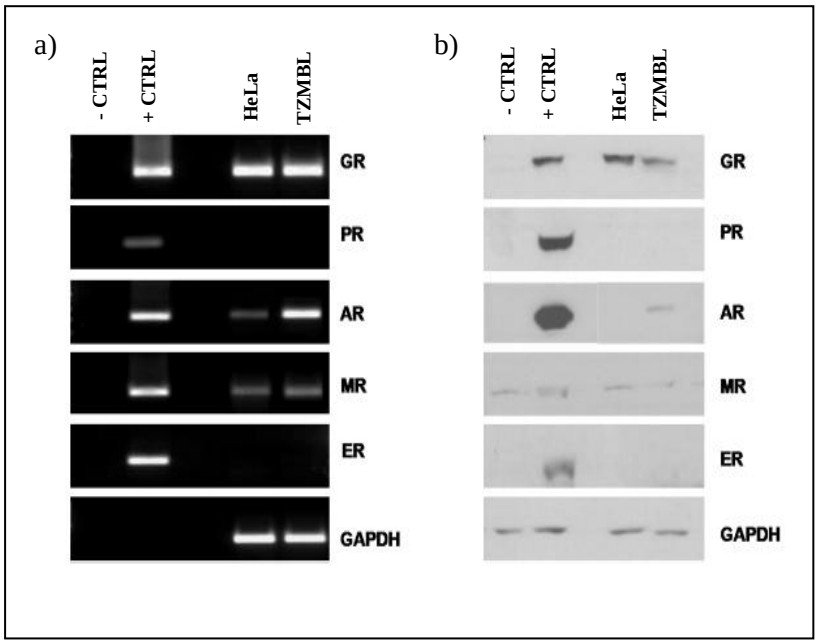


Figure A2.1. Basal steroid receptor mRNA (a) and protein (b) levels in HeLa and TZMBL cells (Y. Govender, PhD thesis 2014, pg 73). HeLa (1.5 X 10^5) and TZMBL (1 X 10^5) cells were seeded in 12 well plates and incubated overnight. Cells were washed with cold 1 x PBS and harvested with TRI Reagent for total RNA (a) or with SDS sample buffer for total protein (b). Steroid receptor gene expression (a) was measured using real-time qPCR with receptor-specific primers (Tomasicchio et al. 2013) and primers for the housekeeping gene GAPDH as a loading control. PCR products were confirmed by gel electrophoresis. Controls were created by PCR amplification of the relevant steroid receptor expression vector (pcDNA3-hGR, pMT-PR-B, pSV-hAR, pRS-hMR and pSG5-hER) plasmid DNA (+CTRL) or empty vector pcDNA3 plasmid DNA (-CTRL). Steroid receptor protein levels (b) were confirmed by SDS-PAGE with equal volumes of cell lysate and western blotting using specific antibodies, including anti-MR (H-300, sc-11412), anti-AR (441, sc-7305), anti-PR (C-20, sc-539) anti-ERα (MC-20, sc-542, all from Santa Cruz Biotechnology) and GAPDH as the loading control. Controls were created in COS1 cells by transient transfection for 24 h with empty vector pcDNA3 (-CTRL) or with steroid receptor expression vectors (+CTRL), followed by harvesting in SDS sample buffer (Govender 2014). This figure also appears in (Govender et al. 2014).

End1/E6E7 immortalized endocervical epithelial cells express GR, ERα and low levels of AR but not MR or PR-B protein (Fig. A2.2). The same non-specific antibody against MR was also used here. Both Fig. A2.1 and A2.2 used different anti-PR antibodies to what was used in the present study.

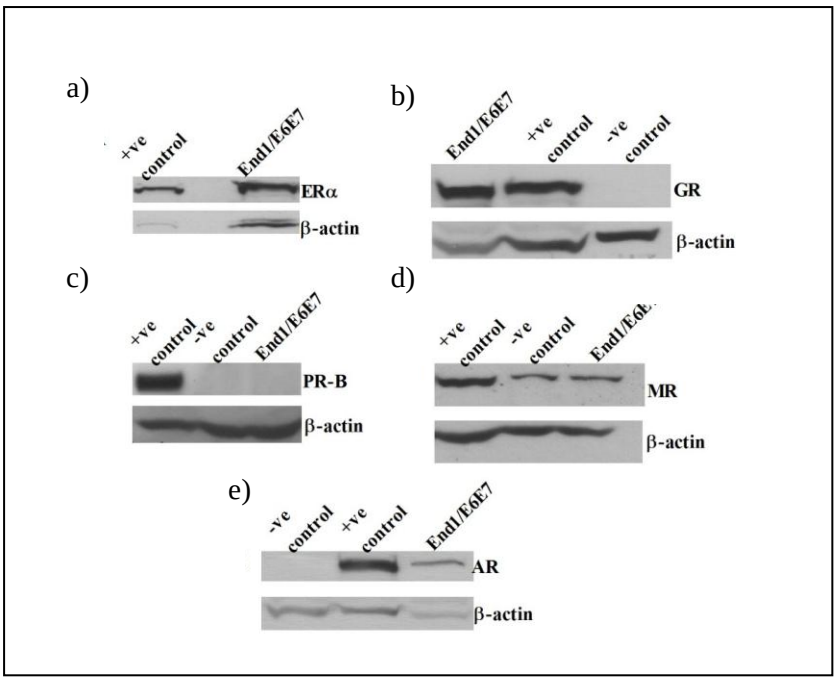


Figure A2.2. Basal steroid receptor protein levels in End1/E6E7 cells (N. Verhoog, PhD thesis 2010, pg 62). Equal volumes of whole cell lysates from End1/E6E7 cells, untransfected COS1 cells (-ve control) or COS1 cells transiently transfected with steroid receptor expression vectors (pSG5hPR-B, pCMV-HA-hGR, pSVhARo, and pRShMR) (+ve control) were analyzed by western blotting using antibodies against (a) ERα, (b) GR, (c) PR (B-30, sc-811, Santa Cruz Biotechnology), (d) MR and (e) AR, using β-actin (#4967, Cell Signaling, South Africa) as the loading control. Other antibodies used were the same as for Fig. A1b. For ERα (a), the positive control was an ER-expressing HeLa cell lysate (Verhoog 2010).

A3. Cervical explants

Ectocervical explants from normally cycling premenopausal women express detectable protein levels of GR, PR-A, PR-B, ERα and AR, in both the luteal (n=6) and follicular (n=6) phases of the menstrual cycle, with high inter-individual variability in protein levels (Fig. A3.1). In this experiment, the same anti-PR antibody was used as for the present study, while MR was excluded due to the non-specific property of the available anti-MR antibody. A slightly different method of protein isolation from cervical explant tissue (TissueRuptor probe homogenizer) was used compared to the present study (TissueLyser).

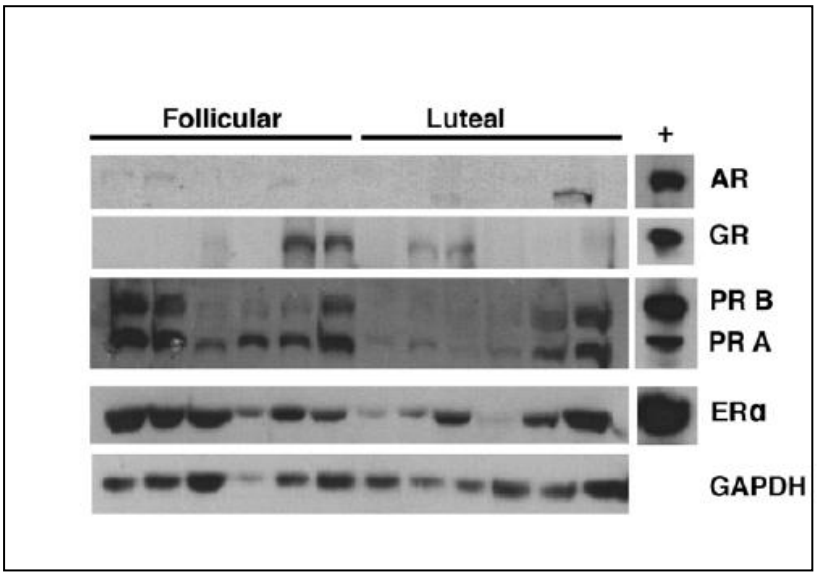


Figure A3.1. Basal steroid receptor protein levels in ectocervical explants from premenopausal women in the luteal or follicular phase of the menstrual cycle (R. Ray, PhD thesis 2015, pg 114). Cervical explants were homogenized in 800 µl Buffer RLT (Qiagen) with a probe homogenizer (TissueRuptor, Qiagen) and total protein was quantified using the Bradford assay. Western blots were carried out using 20 µg total protein loaded onto 8% SDS-PAGE gels and western blotting using antibodies specific to each steroid receptor. Positive controls (+) were created using COS1 cells, and were visualized using shorter exposure times on the same blots (Ray 2015).

Appendix B

Supporting data from the present author

Pilot assays for HIV-1 infection and the establishment of model cell line systems in which to study changing GR/PR, carried out by the present author, are presented as supplementary data in this appendix.

B1. Confirmation of HIV-1 infection in PBMCs

The ability of exogenous MPA or hormones mimicking the menstrual cycle phases to activate the HIV-1 LTR in the absence of HIV-$1_{BaL\_Renilla}$ (that is, the no-virus control) was measured in PBMCs. No significant differences were observed for any of the stimulation conditions (Fig. B1.1), suggesting that the exogenous hormones do not regulate the HIV-1 LTR. The presence of HIV-$1_{BaL\_Renilla}$ significantly increased the relative HIV-1 infection of the vehicle control, after both 2 and 7 days' stimulation with ligands (Fig. B1.1).

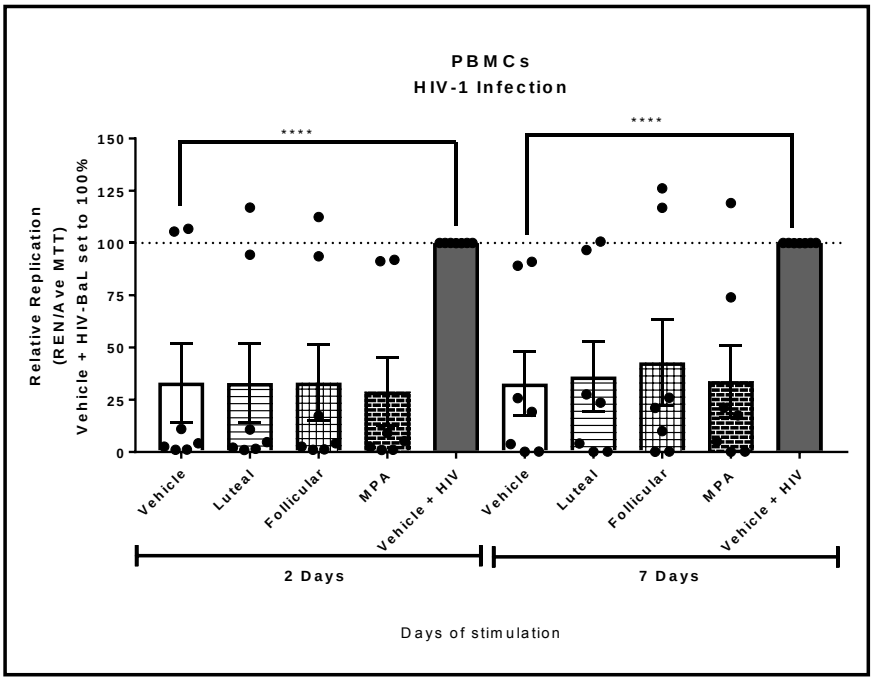


Figure B1.1 Stimulation with MPA or hormones mimicking the menstrual cycle phases does not change basal HIV-1 LTR activity. PBMCs were stimulated and infected as described in Fig. 3.2.1. The results show pooled data from at least 3 independent experiments, with 2-3 PBMC donors each, for a total of 7 independent donors. Relative infection was calculated as *Renilla* luciferase (RLU) divided by average absorbance at 595nm (MTT) for the quadruplicate wells. Infection was plotted relative to each day's vehicle control plus virus set to 100%. Statistical comparisons were carried out using a two-way ANOVA with Tukey's multiple comparisons post test. Significance is indicated by lines between samples with **** indicating $p<0.0001$.

For the same experiment, absolute HIV-1 infection was significantly higher in the vehicle control after 7 days, compared to the vehicle control plus virus after 2 days (Fig. B1.2), implying that the PBMCs were productively infected. These results are supplementary figures from the experiment reported in Fig. 3.2.1.

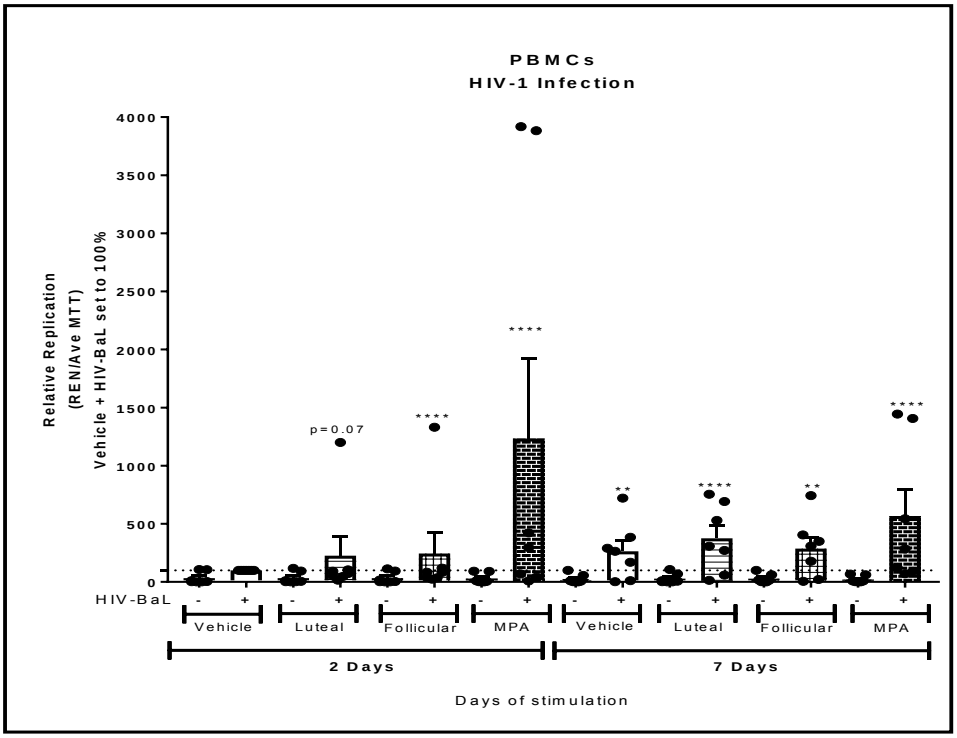


Figure B1.2. HIV-1 infection increases between 2 and 7 days of stimulation with exogenous hormones mimicking the menstrual cycle phases or contraception with MPA. PBMCs were stimulated and infected as described in Fig. 3.2.1. The results show pooled data from at least 3 independent experiments, with 2-3 PBMC donors each, for a total of 7 independent donors. Relative infection was calculated as *Renilla* luciferase (RLU) divided by average absorbance at 595nm (MTT) for the quadruplicate wells. Infection was plotted relative to each day's vehicle control plus virus set to 100%. Statistical comparisons were carried out using a two-way ANOVA with Dunnett's multiple comparisons post test. Stars above bars indicate significant differences compared to the Day 2 Vehicle control plus HIV-BaL, where **** indicates $p<0.0001$.

The effect of the GR antagonist RU486 on MPA-induced HIV-1 infection was measured in PBMCs (Fig. 3.4.1.1b). When the PBMC donors in which MPA increased HIV-1 infection relative to the vehicle control (that is, the MPA-responders) were plotted separately, the same decrease in MPA-induced HIV-1 infection in the presence of RU486 observed for all donors pooled together (Fig. 3.4.1.1b) was also shown. These results imply that the MPA-induced increase in HIV-1 infection is mediated by the GR.

In order to depict the biological variation in HIV-1 infection but not cell viability in response to HIV-1 infection, raw luciferase and MTT values are shown for two representative experiments for both PBMCs and TZM-bls (Table B1).

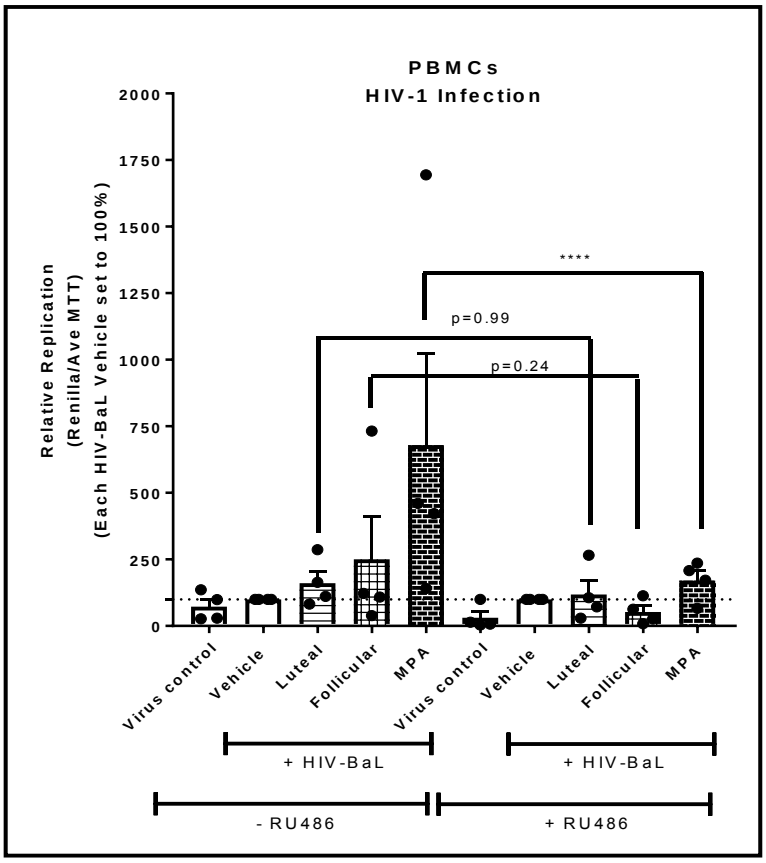


Figure B1.3. MPA-induced HIV-1 infection is mediated by the GR in MPA-responder PBMC donors. PBMCs were stimulated and infected as described in Fig. 3.4.1.1. The results show pooled data from 4 PBMC donors. Relative infection was calculated as *Renilla* luciferase (RLU) divided by average absorbance at 595nm (MTT) for the quadruplicate wells. Infection was plotted relative to both vehicle controls plus virus set to 100%. Statistical comparisons were carried out using a two-way ANOVA with Tukey's multiple comparisons post test. Significance is indicated by lines between samples with **** indicating $p<0.0001$.

Table B1. Variation in HIV-1 infection in PBMCs and TZM-bls arises from biological variability in luciferase and not cell viability between replicate wells.

			PBMCs							
Experiment	**Infection**	**Stimulation**	**Raw LUC (RLU)**				**Raw MTT (A595)**			
#2058 (Day 7)	**No-virus control**	Vehicle	60.0002	56.0002	76.0003	62.0002	0.098	0.106	0.099	0.101
		Luteal	60.0002	62.0002	64.0002	76.0003	0.073	0.1	0.078	0.088
		Follicular	74.0003	44.0001	58.0002	62.0002	0.084	0.09	0.077	0.093
		MPA	62.0002	66.0002	48.0001	70.0002	0.082	0.096	0.083	0.093
	HIV-BaL	Vehicle	282.004	208.002	366.007	136.001	0.073	0.068	0.074	0.088
		Luteal	238.003	1290.08	182.002	102.001	0.078	0.069	0.076	0.081
		Follicular	148.001	182.002	106.001	198.002	0.075	0.077	0.073	0.092
		MPA	968.046	984.048	1416.1	898.04	0.066	0.062	0.066	0.068
#9922 (Day 7)	**No-virus control**	Vehicle	90.0004	62.0002	66.0002	82.0003	0.071	0.09	0.074	0.086
		Luteal	66.0002	76.0003	54.0001	64.0002	0.056	0.07	0.062	0.077
		Follicular	70.0002	70.0002	80.0003	86.0004	0.055	0.067	0.065	0.07
		MPA	94.0004	90.0004	70.0002	68.0002	0.055	0.067	0.066	0.077
	HIV-BaL	Vehicle	82.0003	56.0002	66.0002	64.0002	0.054	0.07	0.061	0.075
		Luteal	2214.24	500.012	2532.31	1628.13	0.055	0.066	0.061	0.073
		Follicular	160.001	86.0004	172.001	132.001	0.054	0.063	0.06	0.059
		MPA	2232.24	2234.24	1600.13	1890.18	0.059	0.062	0.053	0.07
			TZM-bls							
Experiment	**Infection**	**Transfection**	**Raw LUC (RLU)**				**Raw MTT (A595)**			
#2	**No-virus control**	NSC-PR-B	28854.7	30140.4	31326	29787.4	0.532	0.558	0.694	0.694
		NSC+PR-B	29235.8	33011.3	32957.1	29693.1	0.459	0.469	0.531	0.595
		siGR-PR-B	20965.5	25756.5	24260.8	21238.1	0.226	0.239	0.308	0.283
		siGR+PR-B	23150.2	25622.1	20801.2	22107.9	0.365	0.271	0.336	0.293
	HIV-BaL	NSC-PR-B	586966	205754	329188	380737	0.493	0.669	0.602	0.43
		NSC+PR-B	733567	512358	515511	723883	0.531	0.466	0.587	0.542
		siGR-PR-B	354830	719678	483164	671821	0.324	0.322	0.327	0.351
		siGR+PR-B	498183	643268	568996	621761	0.354	0.194	0.3	0.226
#3	**No-virus control**	NSC-PR-B	42713.2	54350.4	52681.6	51455.4	0.29	0.396	0.372	0.235
		NSC+PR-B	33517	48806.4	55134.6	51294.6	0.308	0.328	0.264	0.367
		siGR-PR-B	22126	36854.4	41237.2	38821.7	0.372	0.248	0.316	0.353
		siGR+PR-B	18717.2	32925	33878.1	33930.3	0.218	0.309	0.372	0.344
	HIV-BaL	NSC-PR-B	711256	905192	933196	816823	0.319	0.49	0.42	0.479
		NSC+PR-B	761815	1085240	887348	664797	0.274	0.322	0.407	0.401
		siGR-PR-B	436981	535677	554797	623917	0.333	0.33	0.33	0.284
		siGR+PR-B	437437	554517	480423	747691	0.294	0.199	0.176	0.16

Raw values for Renilla luciferase (HIV-1 infection/LTR activation) and cell viability (MTT assay) are shown for two representative experiments each for PBMCs and TZM-bls. Each condition was measured in quadruplicate wells.

B2. GR and PR are expressed in pre- and post-menopausal cervical explant tissue

In order to replicate the pattern of PR-A, PR-B and GR protein expression between the luteal and follicular phase of the menstrual cycle shown previously (Appendix A, Fig. A3.1), total protein was extracted from additional ectocervical explants from normally cycling premenopausal donors and was probed for GR and PR protein levels by western blotting (Fig. B2.1). Donor information such as age and levels of endogenous reproductive hormones are shown in Appendix D. While no clear pattern between the luteal and follicular phase was observed for these samples due to the high variability in protein detection, both GR and PR were detectable in both phases of the menstrual cycle (Fig. B2.1).

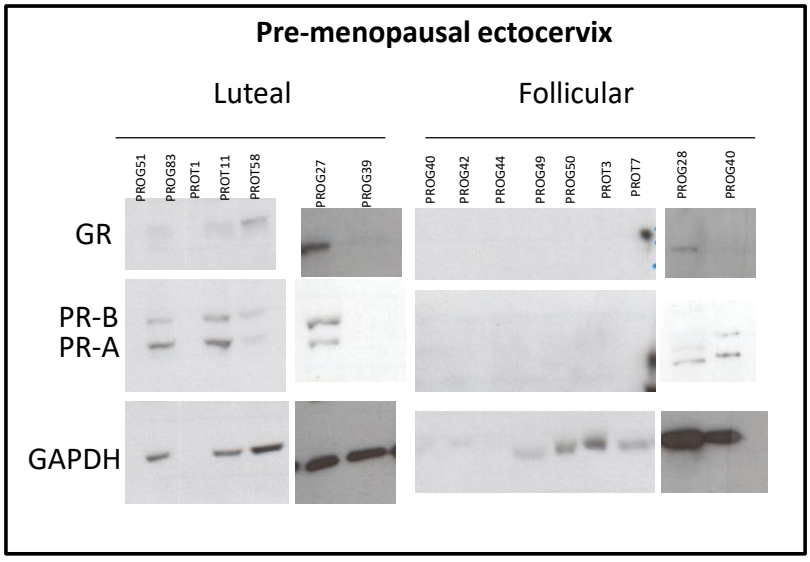


Figure B2.1. Both GR and PR are detectable in ectocervical explants from women in the luteal and follicular phase of the menstrual cycle. Cervical explants of about 3 x 3 mm were thawed from samples archived on the day of surgery. Explants were homogenized in RIPA Buffer using the TissueLyser. Total protein was quantified using the BCA assay. Western blots were carried out using antibodies specific to GR, PR or the loading control GAPDH. Luteal phase donors n=7; Follicular phase donors n=9.

In addition to premenopausal cervical explants, samples from postmenopausal donors were screened for endogenous GR/PR protein expression by western blotting in matched endocervical and ectocervical lysates from the same donors. Both GR and PR were detectable in cervical explants from postmenopausal donors, with GR levels appearing to be greater in the ectocervix while PR levels were greater in the endocervix (Fig. B2.2).

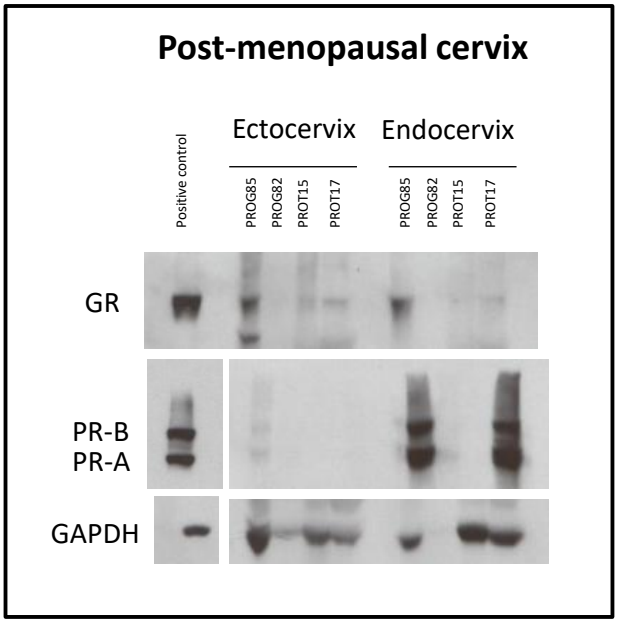


Figure B2.2. Both GR and PR are detectable in endo- and ectocervical explants from postmenopausal women (n=4). Cervical explants of about 3 x 3 mm were thawed from samples archived on the day of surgery. Explants were homogenized in RIPA Buffer using the TissueLyser. Total protein was quantified using the BCA assay. Western blots were carried out using antibodies specific to GR, PR or the loading control GAPDH. Positive controls were created using COS1 cells transiently transfected with 1 µg GR/1 X 10^5 cells and T47D breast cancer cells expressing endogenous PR-A and PR-B.

B3. Establishing GR knockdown and immune function gene regulation by the GR in End1/E6E7 and HeLa cells

An important aspect of investigating the effects of changing the relative ratios of GR/PR on transcriptional responses in cervical cells was to establish independent GR- and PR-mediated gene regulation. Known for their robust responses to GCs, the anti-inflammatory mediator GILZ and the pro-inflammatory cytokine IL-6 were chosen as a model gene for GR-mediated transactivation and transrepression, respectively.

In pilot experiments in End1/E6E7 cells, GILZ mRNA was induced 7.4-fold by Dex but not MPA, and this up-regulation was reduced to 2.7-fold when GR levels were decreased by siRNA targeting the human GR (Fig. B3.1a). IL-6 mRNA was repressed by Dex 5.3-fold and MPA 2.4-fold, and when GR levels were decreased, this repression was lifted back to basal levels (Fig. B3.1b). However these differences were not significant as only two independent repeats were analyzed. Decreased GR protein levels after 72 h post-transfection with siRNA were confirmed by western blotting (Fig. B3.1c).

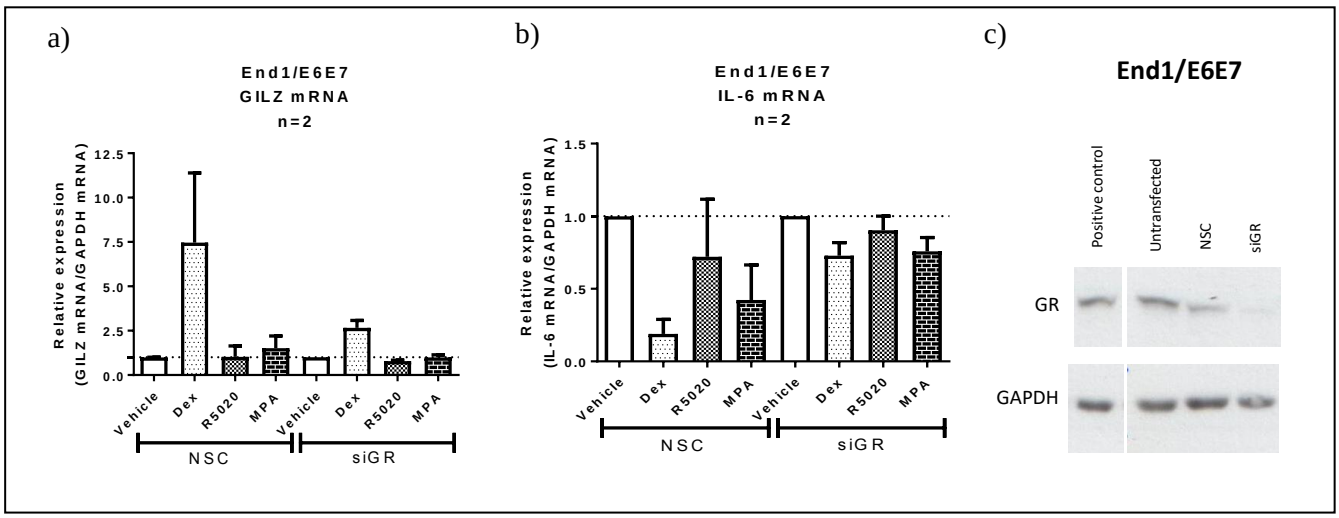


Figure B3.1. The effects of Dex and/or MPA on GILZ and IL-6 gene expression is mediated by the GR in End1/E6E7 cells. Cells were seeded at 1.5 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 48 h. Cells were stimulated with 100 nM final concentration of the indicated ligands in SF-KSFM for a further 24 h, then cells were washed and RNA was extracted. cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of GILZ (a) and IL-6 (b) were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to the vehicle control set to 1. The results are pooled from 2 independent experiments. (c) Cells seeded and transfected in parallel were harvested in SDS sample buffer and lysates were analyzed for GR levels by western blotting using GAPDH as a loading control. Lysates from COS1 cells over-expressing 1 μg pcDNA3-hGR were used as a positive control. A representative blot is shown.

In pilot experiments in HeLa cells, GILZ mRNA was induced by both Dex (4.6-fold) and MPA (3.9-fold); however when GR levels were decreased, GILZ was only reduced by 1.5/1.3-fold (Dex/MPA) (Fig. B3.2a), which was much less than the 2.8-fold difference observed in End1/E6E7 cells (Fig. B3a). IL-6 mRNA was repressed only by Dex (2.7-fold) but not MPA; this repression was lifted when GR levels were decreased (Fig. B3.2b). Decreased GR protein levels after 72 h post-transfection with siRNA were confirmed by western blotting (Fig. B3.2c).

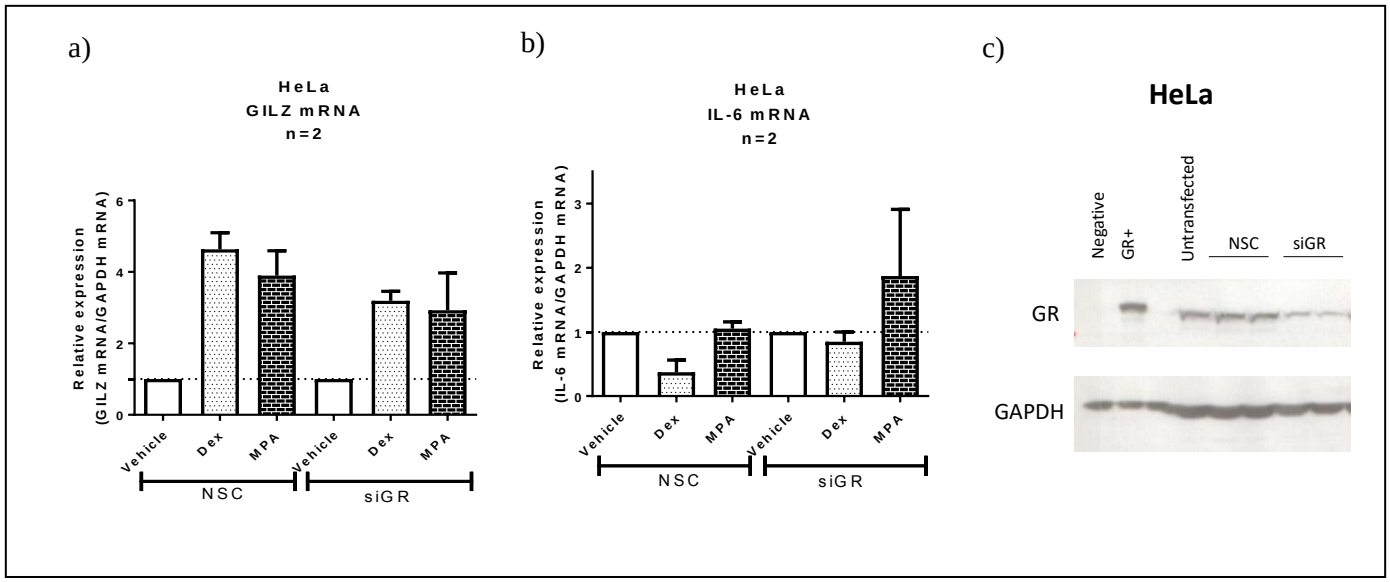


Figure B3.2. The effects of Dex and/or MPA on GILZ and IL-6 gene expression is mediated by the GR in HeLa cells. Cells were seeded at 1 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 48 h. Cells were stimulated with 100 nM final concentration of the indicated ligands in SF-DMEM for a further 24 h, then cells were washed and RNA was extracted. cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of GILZ (a) and IL-6 (b) were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to the vehicle control set to 1. The results are pooled from 2 independent experiments. (c) Cells seeded and transfected in parallel were harvested in SDS sample buffer and lysates were analyzed for GR levels by western blotting using GAPDH as a loading control. Lysates from COS1 cells over-expressing 1 µg pcDNA3-hGR were used as a positive control. A representative blot is shown.

These results suggest that Dex-induced GILZ and IL-6 responses are regulated via the GR in both End1/E6E7 and HeLa cells, and that MPA-induced GILZ expression in HeLa cells and IL-6 expression in End1/E6E7 cells are regulated via the GR. The discrepancy between End1/E6E7 and HeLa cells may in part be due to different endogenous GR levels (Fig. B6.1) or other cell- and/or promoter-specific differences.

B4. Establishing PR-B over-expression and TAT-GRE-LUC reporter gene regulation by the PR-B in End1/E6E7 and HeLa cells

Over-expression of PR-B was achieved in End1/E6E7 and HeLa cells by transient transfection of different amounts of the pMT-hPR-B expression vector.

End1/E6E7 cells appeared to be sensitive to transfection with PR-B, as observed by less cell growth or perhaps cell death seen under the microscope when amounts greater than 250 ng/1.5 X 10^5 cells were transfected (not shown). In a pilot assay, transfection of 250 ng PR-B/1.5 X 10^5 cells showed a 460-fold PR-B response on TAT-GRE-LUC (not shown). By looking at the total protein levels, an indication of cell number, only a small, acceptable reduction in cell number was observed (not shown). Therefore 250 ng PR-B was used in all subsequent experiments in End1/E6E7 cells.

PR-B was successfully over-expressed without changing endogenous GR levels in both End1/E6E7 (Fig. B4.1b) and HeLa (Fig. B4.2b) cells. A greater amount of pMT-hPR-B expression vector could be transfected into HeLa cells (500 ng/ 1 X 10^5 cells, Fig. B4.3b). In western blots, relative PR-B signal strength from over-expressed samples was not greater than those from samples expressing endogenous PR (data not shown), suggesting that over-expressed PR levels were within the physiological range. In End1/E6E7 cells, transactivation of the PR-responsive TAT-GRE-LUC reporter gene by the progestins R5020 (75-fold), LNG (149-fold) and MPA (61-fold) was observed only in the presence of over-expressed PR-B (Fig. B4.1a). While the fold increase only tended towards statistical significance for R5020, LNG- and MPA-induced fold increase was significant (Fig. B4.1a).

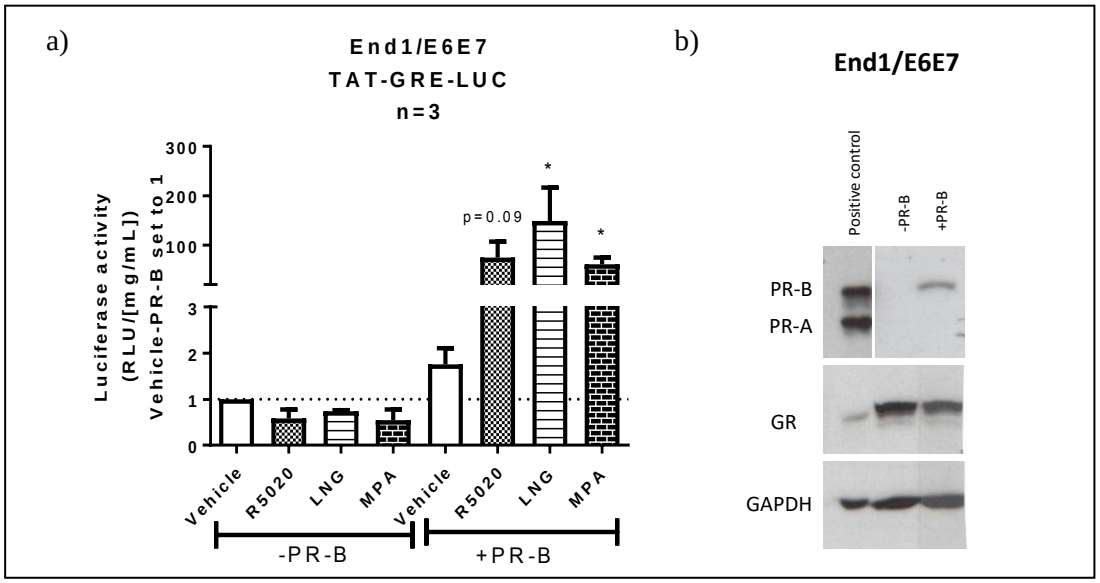


Figure B4.1. Over-expressed PR-B mediates progestin responses on the TAT-GRE-LUC reporter gene in End1/E6E7 cells. (a) End1/E6E7 cells were seeded at 8 X 10^4/well in 24-well plates and

allowed to adhere overnight. Cells were subsequently transfected with 125 ng pcDNA3 or pMT-hPR-B and 47 ng pTAT-GRE-E1b-LUC for 24 h. Cells were stimulated in triplicate wells with 100 nM final concentration of the indicated ligands in SF-KSFM for 24 h, then washed and harvested in Reporter Lysis Buffer. Lysates were analyzed for luciferase activity and total protein. Luciferase activity was normalized to average protein concentration in mg/mL. Fold change was determined relative to the –PR-B vehicle control. The results are pooled from 3 independent experiments where each point was in triplicate. Statistical comparisons were carried out using a two-way ANOVA with Tukey's multiple comparisons post-test (+PR-B Vehicle vs LNG), or an unpaired t-test (+PR-B Vehicle vs R5020/MPA). Stars above bars indicate significant differences compared to the +PR-B vehicle control, where * indicates $p<0.05$. (b) Cells seeded in 12-well plates and transfected in parallel were harvested in SDS sample buffer and lysates were analyzed for GR and PR-B levels by western blotting using GAPDH as a loading control. Blots were probed first for PR-B then stripped and re-probed for GR. Lysates from T47D cells expressing endogenous PR-A/PR-B or COS1 cells over-expressing 1 µg pcDNA3-hGR were used as positive controls. A representative blot is shown.

In HeLa cells, the GR/PR-agonist MPA activated TAT-GRE-LUC only 2.5-fold in the absence of PR-B, presumably via the endogenous GR, but a massive and significant 492-fold response was observed in the presence of PR-B (Fig. B4.2a). The progestin R5020 only significantly activated TAT-GRE-LUC in the presence of PR-B (553-fold, Fig. B4.2a).

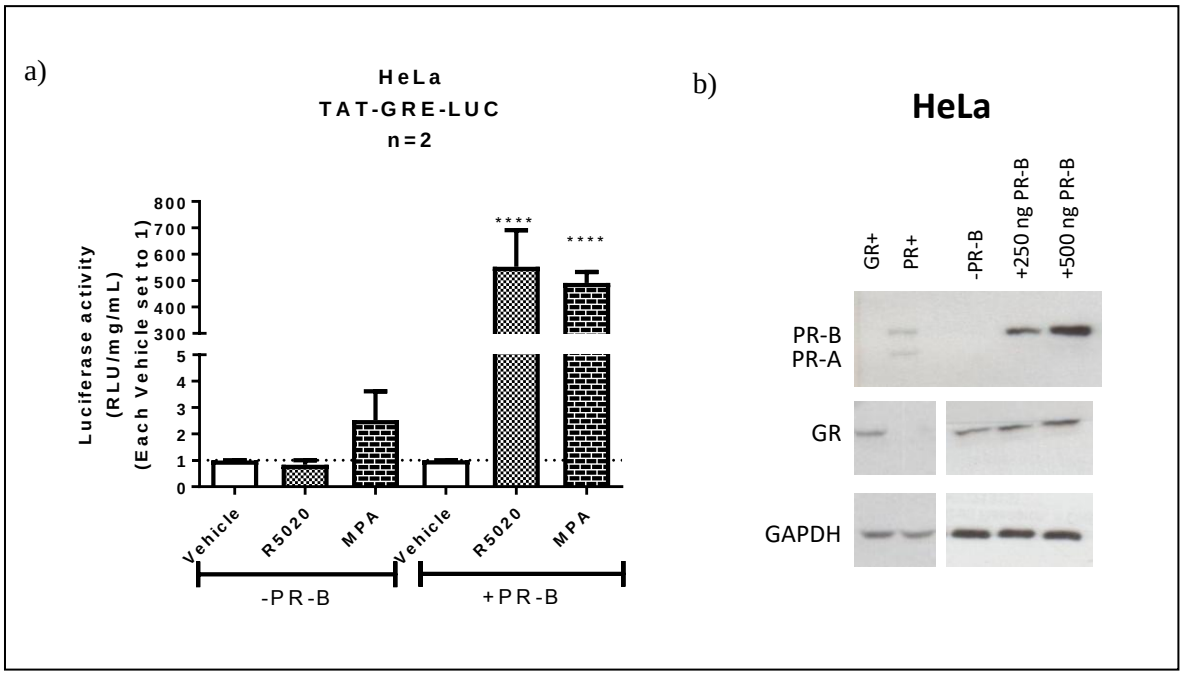


Figure B4.2. Over-expressed PR-B mediates progestin responses on the TAT-GRE-LUC reporter gene in HeLa cells. (a) HeLa cells were seeded at 5 X 10^4/well in 24-well plates and allowed to adhere overnight. Cells were subsequently transfected with 250 ng pcDNA3 or pMT-hPR-B and 47 ng pTAT-GRE-E1b-LUC for 24 h. Cells were stimulated in triplicate wells with 100 nM final concentration of the indicated ligands in SF-DMEM for 24 h, then washed and harvested in Reporter Lysis Buffer. Lysates were analyzed for luciferase activity and total protein. Luciferase activity was normalized to average protein concentration in mg/mL. Fold change was determined relative to the –PR-B vehicle control. The results are pooled from 2 independent experiments where each point was in triplicate. Statistical comparisons were carried

out using a two-way ANOVA with Tukey's multiple comparisons post-test. Stars above bars indicate significant differences compared to the +PR-B vehicle control, where **** indicates p<0.0001. (b) Cells seeded in 12-well plates and transfected with 250 or 500 ng pMT-hPR-B in parallel were harvested in SDS sample buffer and lysates were analyzed for GR and PR-B levels by western blotting using GAPDH as a loading control. Blots were probed first for PR-B then stripped and re-probed for GR. Lysates from T47D cells expressing endogenous PR-A/PR-B or COS1 cells over-expressing 1 μg pcDNA3-hGR or pMT-hPR-B were used as positive controls. A representative blot is shown.

Over-expressed PR-B was assessed for transcriptional activity on the endogenous transactivation gene, MKP1, reported to be regulated by the PR (Gizard et al. 2005, Chen et al. 2011). R5020 and/or LNG were used as PR-specific agonists in order to determine PR-specific effects, since the PR agonist MPA is also an agonist for the GR.

In End1/E6E7 cells, Dex up-regulated MKP1 5-fold, presumably via the endogenous GR (Fig. B4.3a), which is consistent with previous reports that glucocorticoids increase MKP1 expression (Kassel et al. 2001, Chen et al. 2002, Lasa et al. 2002, Clark 2003). This shows that endogenous GR is still active in the presence of over-expressed PR-B, which is also consistent with PR-B over-expression not changing GR protein levels (Fig. B4.1b). MKP1 expression did not increase in response to the progestins R5020 or LNG in the presence of over-expressed PR-B but increased 2-fold in response to MPA (Fig. 4.3a). The absence of progestin-induced up-regulation suggests that MPA-induced MKP1 up-regulation was most likely via the endogenous GR.

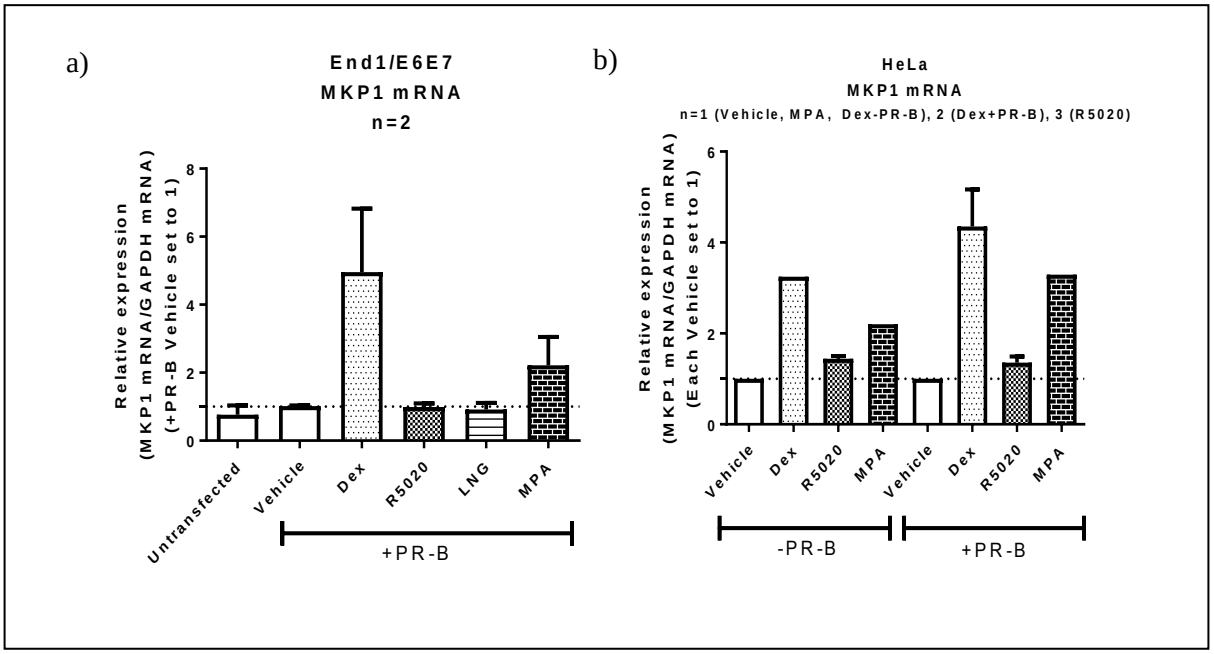


Figure B4.3. PR-B-mediated responses were not observed on endogenous MKP1 in End1/E6E7 and HeLa cells. Cells were seeded at 1.5 X 10^5 cells/well (a, End1/E6E7) or 1 X 10^5 cells/well (b, HeLa) in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 250 ng (End1/E6E7)

or 500 ng (HeLa) pMT-hPR-B or equivalent pcDNA3.1 for 24 h. Cells were stimulated with 100 nM final concentration of the indicated ligands in SF-KSFM (End1/E6E7) or SF-DMEM (HeLa) for a further 24 h, then cells were washed and RNA was extracted. cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels of MKP1 were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to the vehicle control set to 1. The results are pooled from 2 (End1/E6E7) or 1-3 (HeLa) independent experiments.

In HeLa cells, both Dex and MPA up-regulated MKP1 (Fig. B4.3b) in both the presence and absence of PR-B, with Dex-induced up-regulation greater than for MPA and the up-regulation appearing to be greater in the presence of PR-B. R5020 had no effect on MKP1 gene expression in either the absence or presence of PR-B. These results suggest that PR-mediated regulation of endogenous MKP1 does not occur via over-expressed PR-B in End1/E6E7 or HeLa cells. Since GR-mediated transcriptional responses on immunomodulatory genes were a primary focus of this project, further investigations into endogenous PR-mediated gene regulation were not continued. Subsequently, the effects of detectable levels of over-expressed PR-B protein on GR-mediated gene regulation were investigated.

Since the relative GR/PR levels change *in vivo* within different compartments of the FRT and in response to fluctuating menstrual cycle hormones, the molecular strategies of decreased GR levels and PR-B over-expression were combined in End1/E6E7, HeLa and TZM-bl cells order to represent changing GR/PR levels in an *in vitro* model of the FRT. Decreased GR protein levels using siRNA and transient transfection of PR-B resulted in four combinations: high GR/no PR, low GR/no PR, high GR/high PR and low GR/high PR. The consequences of changing relative GR/PR levels on HIV-1 infection (Fig. 4.2.1.1-4.2.2.1), HIV-1 CD4 receptor and CCR5/CXCR4 co-receptor expression (Fig. 4.3.1.1) and immunomodulatory gene regulation (Fig 4.3.2.1-4.3.3.2) were investigated in Chapter 4.

The gene expression results in Fig. 4.3.2.1 (End1/E6E7 cells) and Fig. 4.3.2.2 (HeLa cells) suggest that the presence of PR-B alters the GR-mediated transcriptional responses to MPA on GILZ and IL-6, when analysing the fold change relative to the vehicle control. Supplementary to these figures, these data were plotted relative to the NSC-PR-B (high GR/no PR) vehicle control in order to assess the effect of PR-B over-expression on GILZ and IL-6 gene regulation (Fig. B4.4-B4.5).

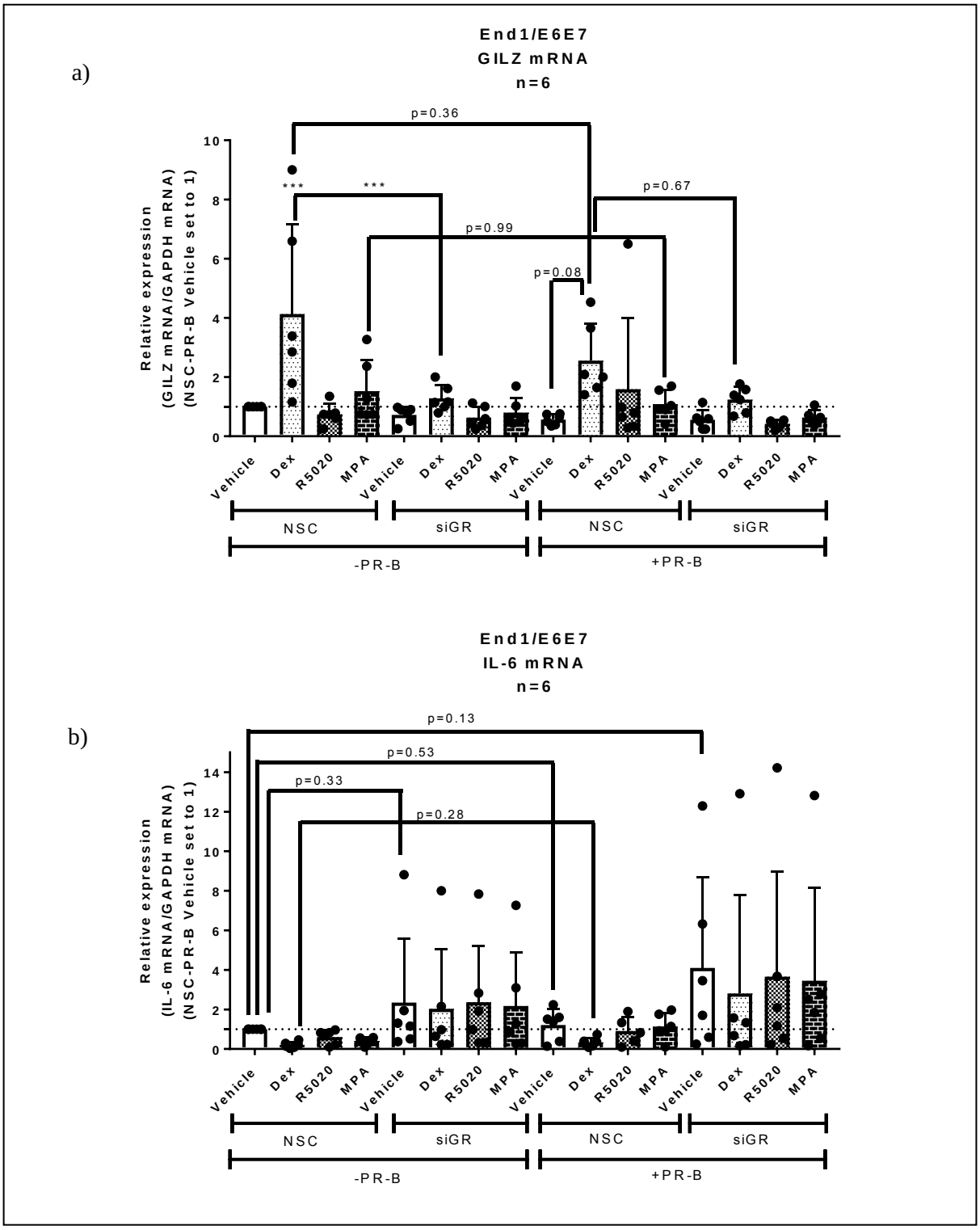


Figure B4.4. The presence of PR-B alters basal GILZ and IL-6 expression, and potentially dampens the GR-mediated transcriptional response to Dex on GILZ in End1/E6E7 cells. Cells were seeded at 1 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 24 h, then transiently transfected with 250 ng/well empty vector pcDNA3 or pMT-hPR-B for 24 h. Cells were stimulated with 100 nM final concentration of the indicated ligands in SF-KSFM for a further 24 h, then cells were washed. RNA was extracted and cDNA was synthesized from 500 ng RNA then used in real-time qPCR. Relative mRNA levels of GILZ (a) and IL-6 (b) were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to the NSC-PR-B vehicle control set to 1. The results are pooled from 6 independent experiments. Statistical comparisons were

carried out using a two-way ANOVA (a, b) with Tukey's multiple comparisons post test (a) or unpaired t tests (b). Stars above bars indicate significant differences compared to the NSC-PR-B vehicle control unless otherwise indicated by lines, where *** indicates $p<0.001$. Decreased GR expression and PR-B over-expression were confirmed previously by western blotting in parallel samples (Fig. 4.3.2.1c-e).

The results in End1/E6E7 cells show that Dex-induced up-regulation of GILZ mRNA was mediated by the GR, since the induction was lost with low GR/no PR (significantly) and low GR/high PR (near-significantly, Fig. B4.5a). Despite this, GILZ induction by Dex was not as elevated with high GR/high PR (2.6-fold) compared to high GR/no PR (4.1-fold, Fig. B4.4a). Although this was not significant, this may suggest a possible dampening of the Dex-induced, GR-mediated response by the presence of PR-B. However, this effect was not observed in HeLa cells (Fig. B4.5a), suggesting that the effect is cell type-specific. Furthermore no such observation was made for MPA on GILZ in either End1/E6E7 (Fig. B4.4a) or HeLa (Fig. B4.5a) cells, suggesting that the dampening effect is also ligand-specific.

These results also revealed that the basal levels (in the absence of ligands) of GILZ and/or IL-6 mRNA changed when the relative levels of GR/PR were altered. Although not significant, basal GILZ levels in End1/E6E7 cells decreased with high GR/high PR and low GR/high PR compared to high GR/no PR (Fig. B4.4a). Conversely, basal IL-6 levels appeared to increase with low GR/no PR and low GR/high PR compared to high GR/no PR in End1/E6E7 cells (Fig. B4.4b). No change was observed for basal GILZ levels in HeLa cells (Fig. B4.5a), but basal IL-6 levels significantly increased with high GR/high PR and low GR/high PR, compared to high GR/no PR (Fig. B4.5b). These initial experiments led to the further investigation of MPA-induced and basal responses to changing relative GR/PR levels in End1/E6E7 and HeLa cells.

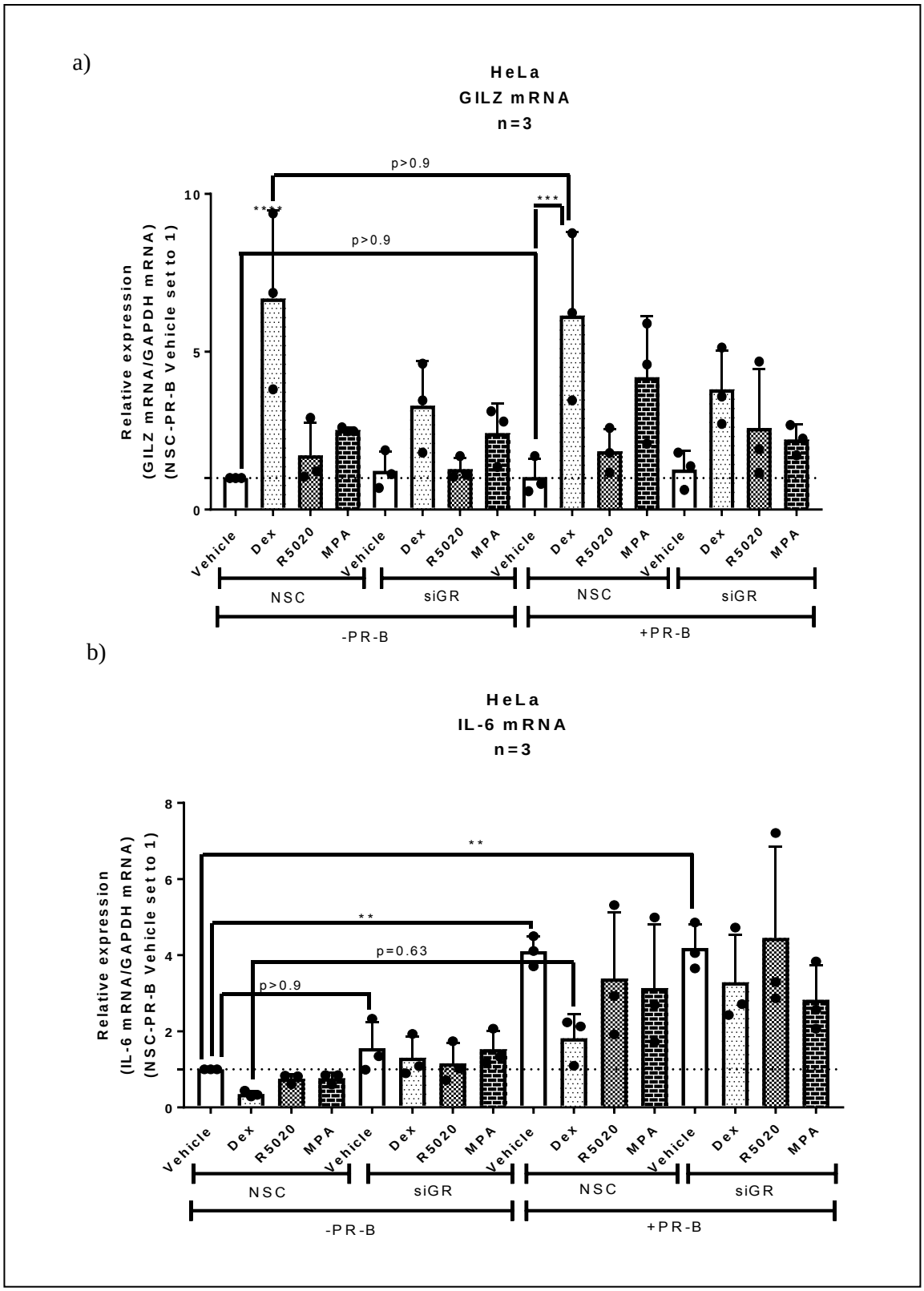


Figure B4.5. The presence of PR-B alters basal IL-6 but not GILZ expression in HeLa cells. Cells were seeded at 1 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with 10 nM NSC or siGR for 24 h, then transiently transfected with 500 ng/well empty vector pcDNA3 or pMT-hPR-B for 24 h. Cells were stimulated with 100 nM final concentration of the indicated ligands in SF-DMEM for a further 24 h, then cells were washed. RNA was extracted and cDNA was synthesized from 500 ng RNA then used in real-time qPCR. Relative mRNA levels of GILZ (a) and IL-6 (b)

were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to the NSC-PR-B vehicle control set to 1. The results are pooled from 6 independent experiments. Statistical comparisons were carried out using a two-way ANOVA with Tukey's multiple comparisons post test. Stars above bars indicate significant differences compared to the NSC-PR-B vehicle control unless otherwise indicated by lines, where *** and ** indicate $p<0.001$ and $p<0.01$ respectively. Decreased GR expression and PR-B over-expression were confirmed previously by western blotting in parallel samples (Fig. 4.3.2.2c-e).

B5. Over-expression strategies in U2OS cells do not generate good GR-mediated responses

In parallel to using cells expressing endogenous GR in which PR-B can be over-expressed (End1/E6E7, HeLa) as physiologically relevant models of the FRT, another strategy to investigate the transcriptional effects of changing the relative levels of GR/PR was the use of receptor-negative cell lines in which both GR and PR could be over-expressed. U2OS human osteosarcoma cells were chosen due to their low endogenous expression of GR. The initial approach was to investigate the effects of over-expressed GR- and PR-regulated immunomodulatory gene expression individually, then to combine over-expressed GR and PR-B in different ratios (GR>PR, GR=PR, GR<PR) in order to assess whether there was a preference for GR- or PR-mediated transcriptional responses when stimulated with the GR/PR ligand, MPA. However, this strategy did not reach that far.

U2OS cells could successfully over-express PR-A and PR-B (Fig. B5b). Using the PR agonist R5020 to activate PR-mediated regulation of the TAT-GRE-LUC reporter gene, it was confirmed in U2OS cells that PR-B is more transcriptionally active than PR-A since the response to R5020 was greater for cells over-expressing PR-B (79-fold) than PR-A (5.6-fold, Fig. B5a). Furthermore, it was established that R5020-induced activation of the TAT-GRE-LUC reporter gene by PR-B could be reduced by 1.5-fold (from 79-fold to 53-fold), although not significantly, by co-expression of PR-A (Fig. B5a). This observation is consistent with previous reports that PR-A acts as a transdominant repressor of PR-B (Vegeto et al. 1993).

U2OS cells could also successfully over-express GR (Fig. 5.2.2). However, while a GR-mediated response to Dex was observed for the transactivation model gene GILZ, from 1.5-fold in the absence to 4.8-fold in the presence of GR, the GR did not significantly repress the model transrepression gene IL-6 (only 1.4-fold) and did not significantly repress TNFα-regulated induction of IL-6 (not shown). Since the classical regulation of IL-6 by the GR was not shown in U2OS cells, it was decided that U2OS cells were not a suitable model for GR-mediated gene regulation.

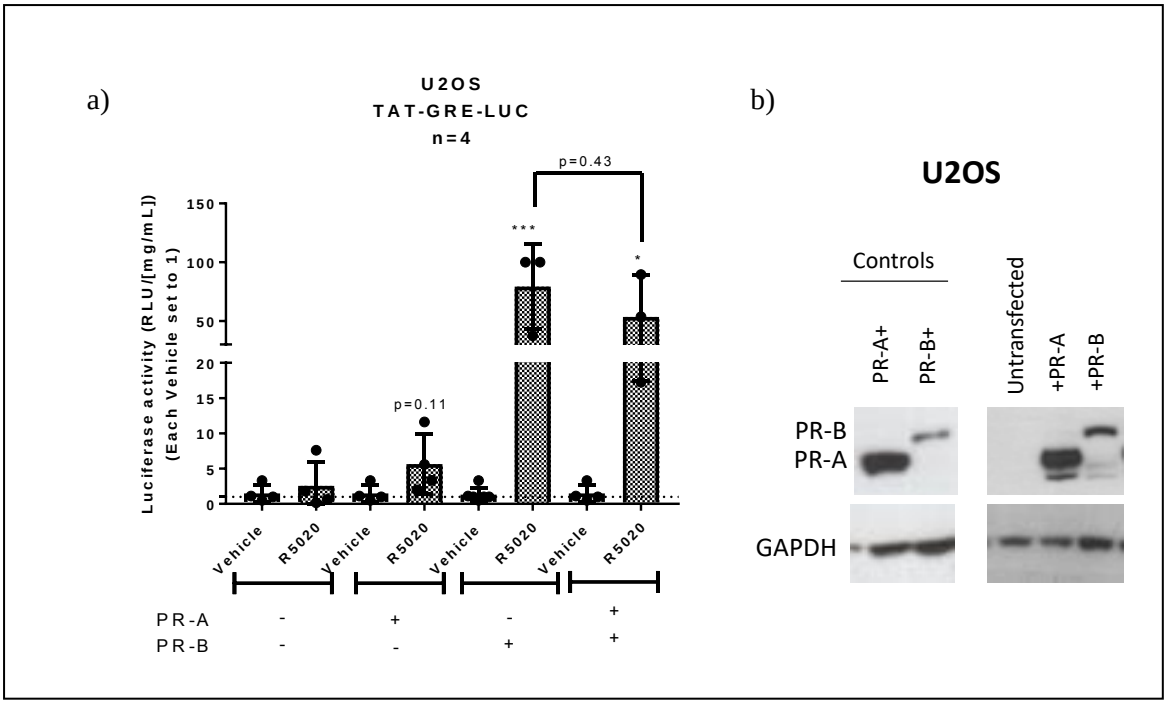


Figure B5. PR-A represses the R5020-induced activity of PR-B on the TAT-GRE-LUC reporter gene in U2OS cells. (a) U2OS cells were seeded at 7 X 10^4/well in 24-well plates and allowed to adhere overnight. Cells were subsequently transfected with 125 ng pcDNA3, pSG5-hPR-A and/or pMT-hPR-B and 47 ng pTAT-GRE-E1b-LUC for 24 h. Cells were stimulated in triplicate wells with 100 nM final concentration R5020 in SF-DMEM for 24 h, then washed and harvested in Reporter Lysis Buffer. Lysates were analyzed for luciferase activity and total protein. Luciferase activity was normalized to average protein concentration in mg/mL. Fold change was determined relative to each vehicle control. The results are pooled from 4 independent experiments where each point was in triplicate. Statistical comparisons were carried out using unpaired t-tests.Stars above bars indicate significant differences compared to each vehicle control unless otherwise indicated by lines, where *** and * indicate $p<0.001$ and $p<0.05$ respectively. (b) Cells seeded in 12-well plates and transfected in parallel with 125 ng pSG5-hPR-A or pMT-hPR-B for 48 h were harvested in SDS sample buffer and lysates were analyzed for PR levels by western blotting using GAPDH as a loading control. Lysates from COS1 cells over-expressing 1 µg pSG5-hPR-A or pMT-hPR-B were used as positive controls. A representative blot is shown.

B6. Endogenously GR- and PR-expressing cell lines, MCF-7 and T47D, do not generate good GR responses

In parallel to receptor-negative cell lines in which GR and PR could be over-expressed, a third strategy in which to study GR/PR levels was to investigate GR- and PR-mediated responses in endogenous GR- and PR-positive cell lines. Although not directly relevant to the site of HIV-1 transmission in the FRT, the breast cancer cell lines MCF-7 and T47D were promising candidates since both GR and PR were detectable by western blot (Fig. B6.1). This would eliminate any confounding effects arising from transient transfections of GR/PR expression vectors or siRNA. GR protein levels were highest in End1/E6E7 and HeLa cells, but were greater in MCF-7 and T47D cells

than in the "receptor-negative" COS1 and U2OS cells (Fig. B6.1a). While MCF-7 and T47D cells were the only cell lines to express endogenous PR (Fig. B6.1b), T47D cells expressed more endogenous PR than MCF-7 cells (Fig. B6.1b).

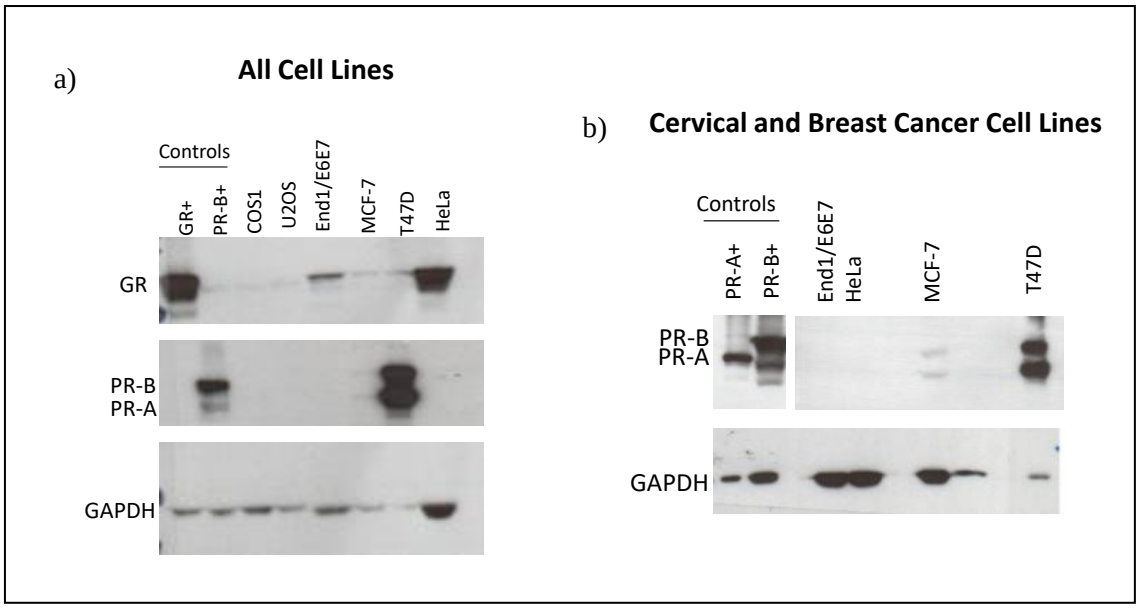


Figure B6.1. End1/E6E7, HeLa, MCF-7, T47D, COS1 and U2OS cells express detectable GR protein but only MCF-7 and T47D express endogenous PR. Cells from the indicated cell lines were seeded at varied densities in 12 well plates and allowed to grow until ~80% confluent. Cells were washed and total protein was harvested in SDS sample buffer and analyzed for GR and PR levels by western blotting using GAPDH as a loading control. Lysates from COS1 cells over-expressing 1 μg pcDNA3-hGR, pSG5-hPR-A or pMT-hPR-B were used as positive controls.

Functional activity of the endogenous GR and PR was assessed in MCF-7 and T47D cells by their ability to activate the TAT-GRE-LUC reporter gene, which is recognized and activated by both GR and PR.

Over-expression of PR-B activated the TAT-GRE-LUC reporter gene in response to the PR-specific agonist R5020 in both MCF-7 (significantly, 169-fold, Fig. B6.2a) and T47D (40-fold, Fig. B6.2c) cells, although in the absence of PR-B there was still an increase in R5020-induced TAT-GRE-LUC reporter gene activation in both MCF-7 (significantly, 20-fold, Fig. B6.2a) and T47D (64-fold, Fig. B6.2c) cells.

Over-expression of GR activated the TAT-GRE-LUC reporter gene in response to the GR-specific agonist Dex in both MCF-7 (significantly, 4989-fold, Fig. B6.2b) and T47D (575-fold, Fig. B6.2d) cells. In the absence of over-expressed GR there was a significant increase in Dex-induced TAT-

GRE-LUC reporter gene activation in only MCF-7 (3.2-fold, Fig. B6.2b) but not T47D (1.12-fold, Fig. B6.2d) cells. These results suggest that both endogenous GR and PR are functional on the TAT-GRE-LUC reporter gene in MCF-7 cells, but only endogenous PR is functional on the TAT-GRE-LUC reporter gene in T47D cells.

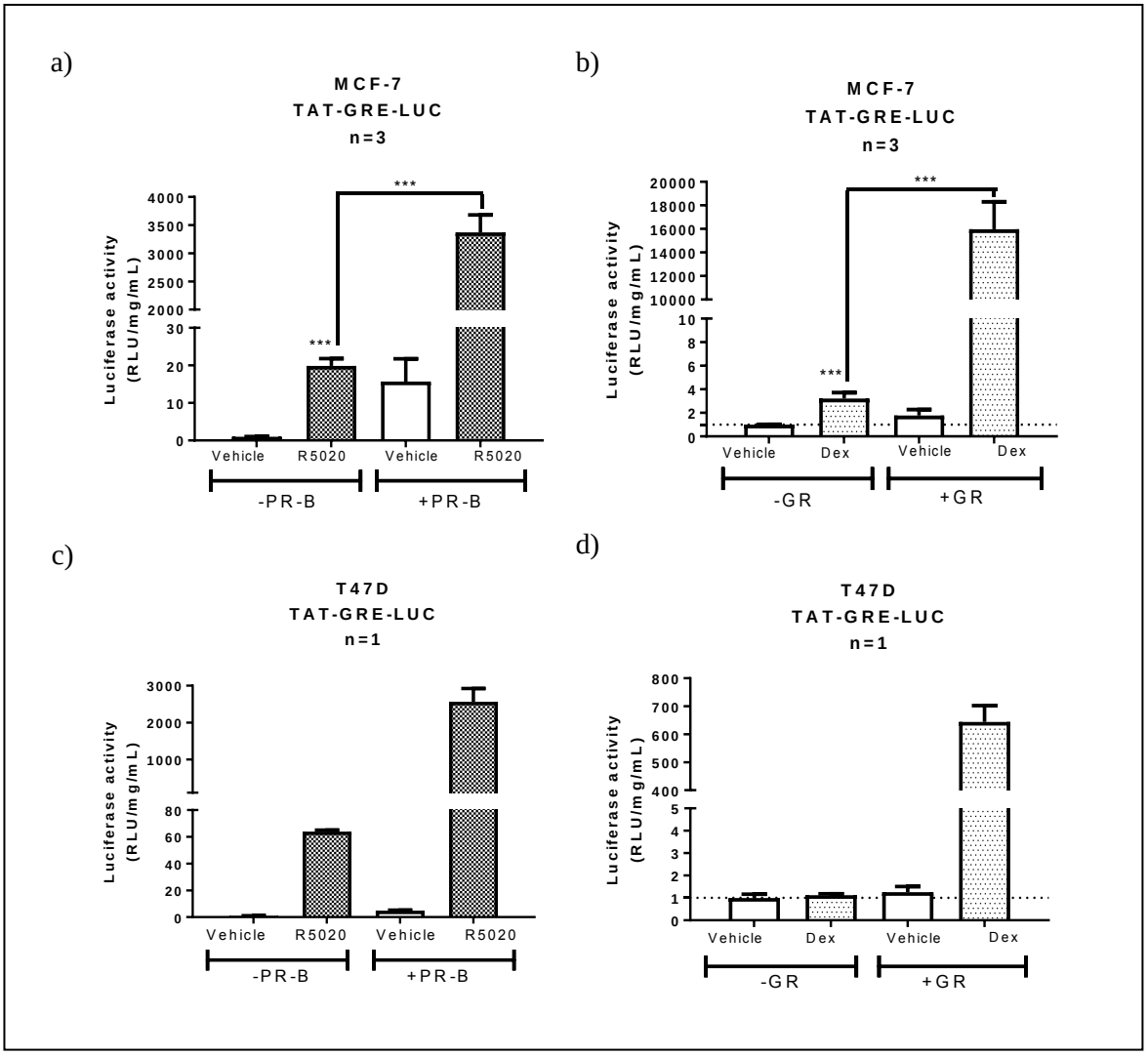


Figure B6.2. Endogenous GR and PR are functional on the TAT-GRE-LUC reporter gene in MCF-7 cells, while only endogenous PR is functional on the TAT-GRE-LUC reporter gene in T47D cells. MCF-7 (a, b) or T47D (c, d) cells were seeded at 7 X 10^4/well in 24-well plates and allowed to adhere overnight. Cells were subsequently transfected with 125 ng pcDNA3, pMT-hPR-B or pcDNA3-hGR and 47 ng pTAT-GRE-E1b-LUC for 24 h. Cells were stimulated in triplicate wells with 100 nM final concentration Dex or R5020 in SF-DMEM for 24 h, then washed and harvested in Reporter Lysis Buffer. Lysates were analyzed for luciferase activity and total protein. Luciferase activity was normalized to average protein concentration in mg/mL. Fold change was determined relative to each vehicle control in the absence of GR/PR-B. The results are pooled from 3 (MCF-7) or 1 (T47D) independent experiments where each point was in triplicate. Statistical comparisons were carried out using a one-way ANOVA with Tukey's multiple comparisons post test (a, b). Stars above bars indicate significant differences compared to the –PR-B vehicle control unless otherwise indicated by lines, where *** indicates $p<0.001$.

Further investigation into endogenous GR/PR activity was carried out by assessment of endogenous gene expression. The up-regulation of the model transactivation genes GILZ, MKP1, TAT and IκB was measured in response to Dex in MCF-7 cells. Surprisingly, only GILZ was up-regulated 1.5-fold in response to Dex, while MKP1 expression did not change (Fig. B6.3a). Selected model transrepression genes were assessed for their ability to be repressed by Dex in both the absence and presence of TNFα. However, Dex did not significantly repress IL-6, IL-8 or RANTES via the endogenous GR in MCF-7 cells, in either the absence or presence of TNFα (Fig. B6.3b).

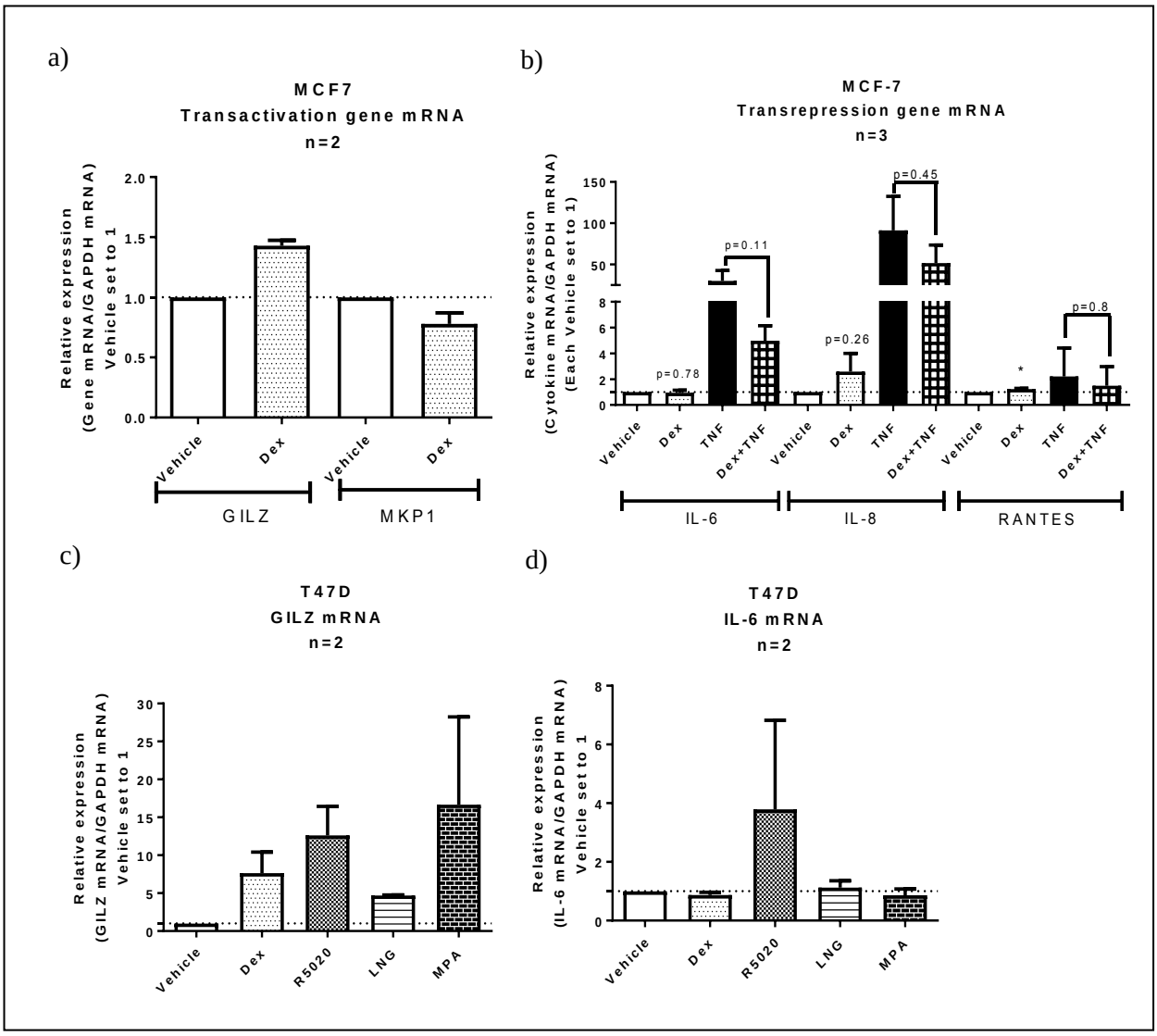


Figure B6.3. Endogenous GR does not regulate Dex-induced transrepression genes in MCF-7 or T47D cells. MCF-7 (a, b) or T47D (c, d) cells were seeded at 1 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were stimulated with 100 nM final concentration of the indicated ligands and/or 20 ng/mL TNFα (b) in SF-DMEM for a further 24 h, then cells were washed and RNA was extracted. cDNA was synthesized from 500 ng RNA then used in quantitative RT real-time qPCR. Relative mRNA levels

of GILZ (a, c), IL-6 (b, d), MKP1 (a), IL-8 (b) and RANTES (b) were normalized to GAPDH mRNA levels. Relative expression was determined by normalizing to the vehicle control set to 1. The results are pooled from 2 (a, c, d) or 3 (b) independent experiments. Statistical comparisons were carried out using an unpaired t-test (b). Stars above bars indicate significant differences compared to each gene's vehicle control (b) unless otherwise indicated by lines, where * indicates $p<0.05$.

In T47D cells, the expression of only one transactivation (GILZ) and transrepression (IL-6) gene was investigated. The endogenous GR appeared to up-regulate GILZ in response to Dex 7.6-fold, and surprisingly GILZ was up-regulated in response to the progestins R5020 (12.6-fold), LNG (4.7-fold) and MPA (16.7-fold, Fig. B6.3c). IL-6 mRNA was marginally repressed 1.15-fold in response to Dex, via the endogenous GR, and presumably via the endogenous PR, R5020 but not LNG or MPA up-regulated IL-6 by 3.8-fold (Fig. B6.3d).

These results suggest that both MCF-7 and T47D cells, although expressing detectable GR protein, do not exhibit robust GR-mediated responses that would be required to establish GR/PR-mediated effects on immunomodulatory genes.

This observation of a non-functional GR in T47D cells is consistent with other reports (Cato et al. 1986, Cato et al. 1988, Chalepakis et al. 1988, Wu and Pfahl 1988, Nordeen et al. 1989). Therefore attempts were made to over-express GR in T47D cells by transient transfection: this would be an attractive model in which to study GR/PR levels, given that T47D cells already express large amounts of endogenous PR which could be knocked down using siRNA (not shown). However, transient transfection of increasing amounts of GR expression vector did not translate into greater levels of GR protein (Fig. B6.4).

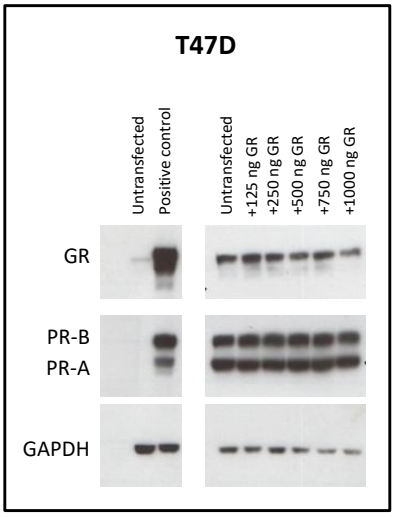
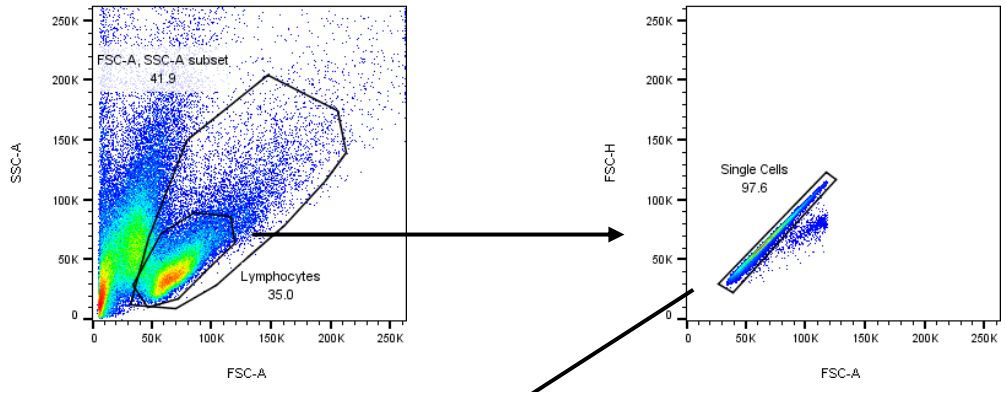


Figure B6.4. GR protein cannot be over-expressed in T47D cells. Cells were seeded at 1 X 10^5 cells/well in 12 well plates and allowed to adhere overnight. Cells were subsequently transfected with increasing amounts of pcDNA3-hGR for 48 h. Cells were washed and total protein was harvested in SDS sample buffer and analyzed for GR and PR levels by western blotting using GAPDH as a loading control. Western blots were scanned and quantified for relative GR (b) or PR-A/B (d) levels by calculating integrated density values.

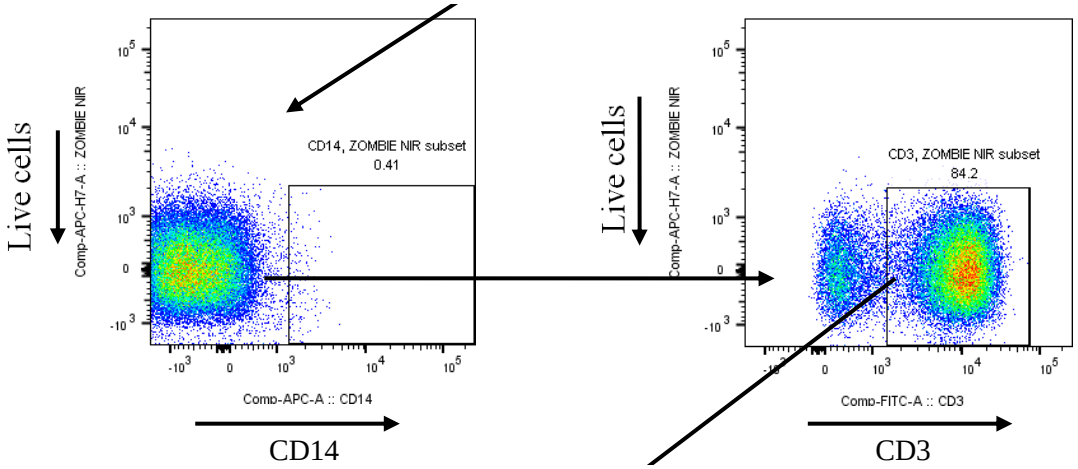
Since the hypotheses of this study required robust GR-mediated responses, especially via transrepression mechanisms on immune function genes, for the same reasons as U2OS cells, the endogenous GR/PR-expressing model cell strategy was left in favour of the GR-positive cervical End1/E6E7 and HeLa cell lines in which the relative levels of GR/PR could be altered by knockdown and over-expression approaches.

Appendix C
Gating strategy used for flow cytometry

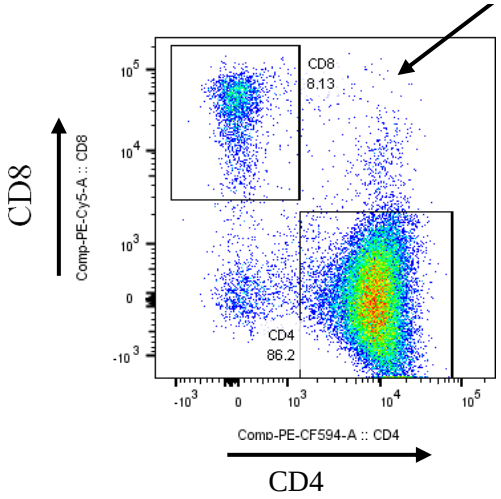
The selection of live, single cell populations and gating strategy using MFO controls for CD14, CCR5 and CD69 are indicated in the figures below. A representative PBMC donor (#2864) is shown.



Single cell populations were identified from the lymphocyte population, from which live (ZOMBIE-) cells were selected.

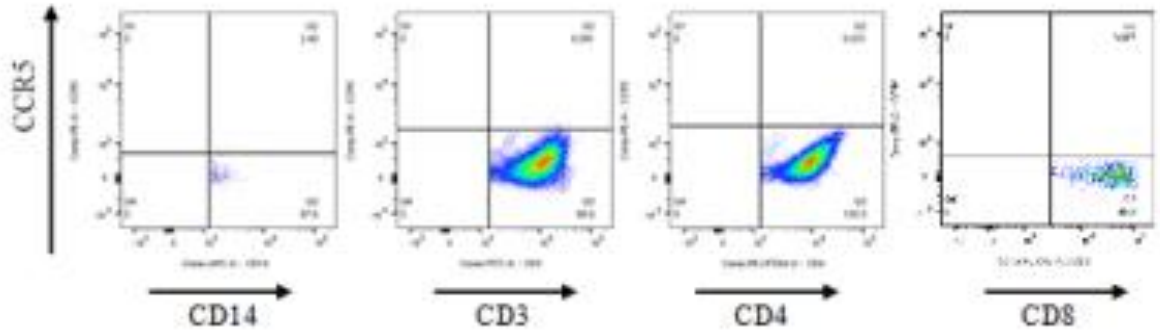


CD14+ monocytes were identified from the live single cells using the CD14 MFO control. CD3+ lymphocytes were selected from the CD14- cells.

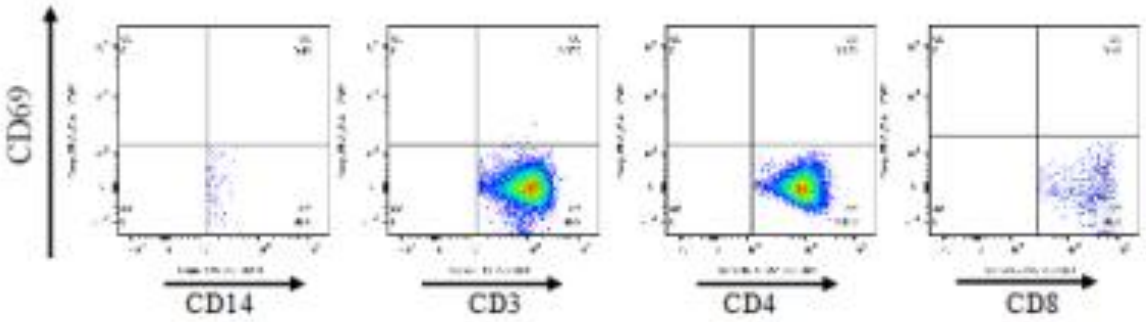


CD4+ and CD8+ lymphocytes were identified from the CD3+ lymphocyte population.

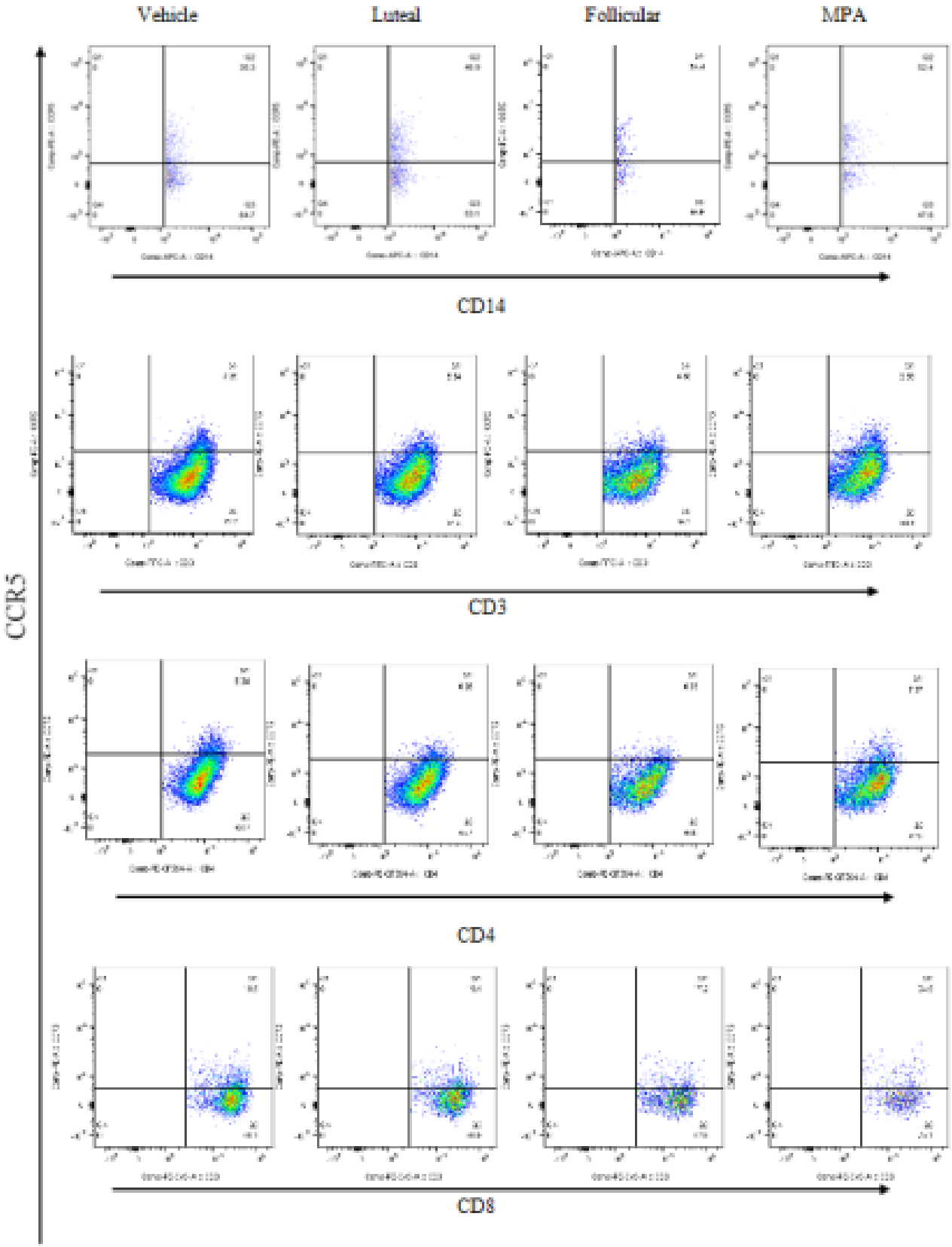
The CD14 MFO gating was applied to all samples. Next, the CCR5 MFO control was used to identify CCR5+ populations (CD14+CCR5+, CD3+CCR5+, CD4+CCR5+, CD8+CCR5+) and this gate was applied to all samples.

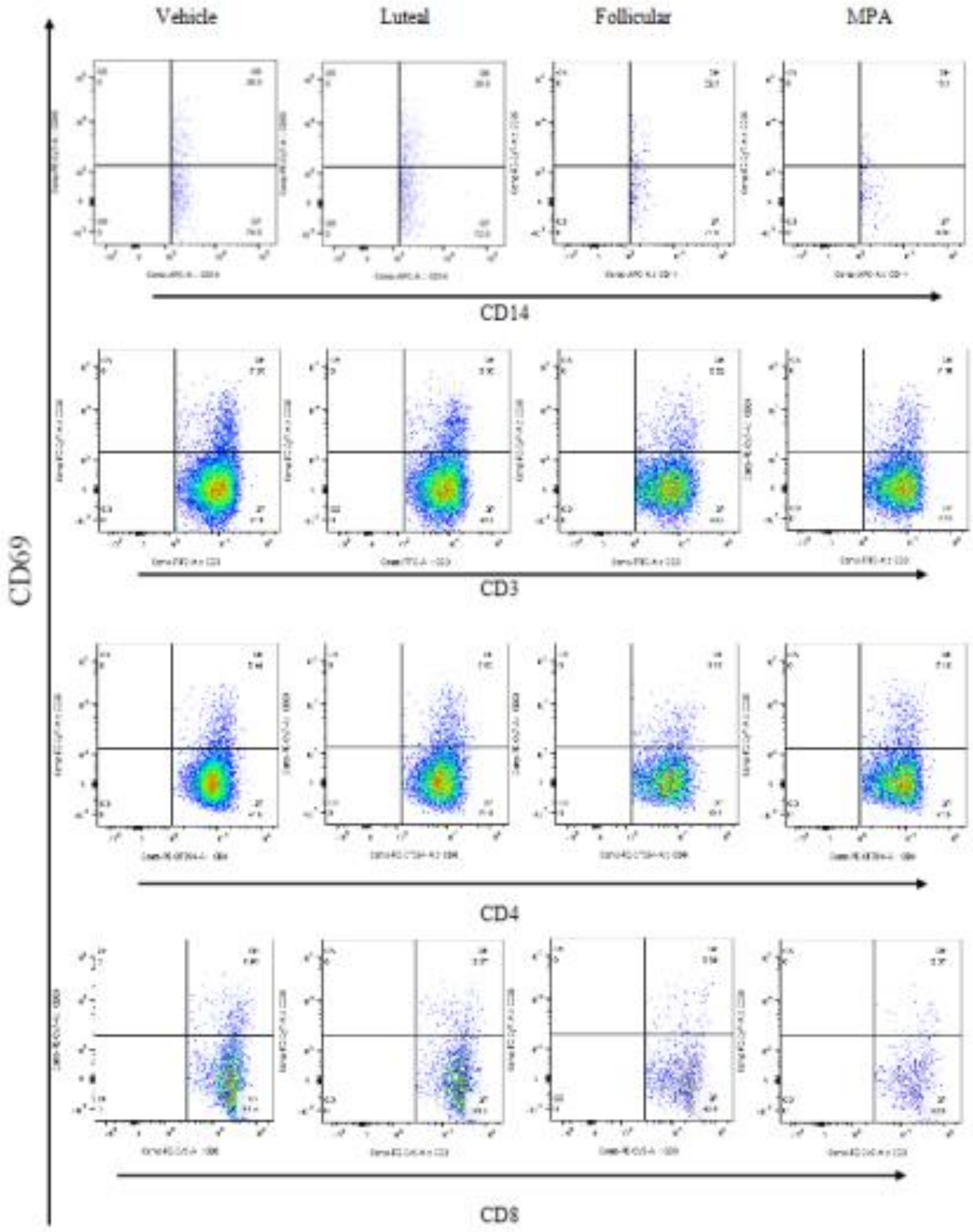


Finally, the CD69 MFO control was used to identify CD69 populations (CD14+CD69+, CD3+CD69+, CD4+CD69+, CD8+CD69+) and this gate was applied to all samples.



The gates for frequencies of double-positive cell populations (CD3/4/8/14+ with CD69+/CCR5+) for each stimulation (Vehicle, Luteal, Follicular, MPA) are shown for a representative PBMC donor (#2684) below.


Vehicle
Luteal
Follicular
MPA
CCR5
CD14
CD3
CD4
CD8


Vehicle
Luteal
Follicular
MPA
CD69
CD14
CD3
CD4
CD8

Appendix D

Cervical explant donor information

Table D1: Endogenous reproductive hormone levels, age and HSV status of the pre- and postmenopausal donors of cervical explant tissue

Patient no.	Date of collection	LH (IU/L)	FSH (IU/L)	E_2 (pM)	P_4 (nM)	Age	Menstrual cycle phase	HSV-1	HSV-2
LUTEAL PHASE									
PROG0027	10-Feb-14	2.4	2.8	341.7	7.3	36	Luteal	+	-
PROG0039	9-Jun-14	3.8	2.8	408.6	12	49	Luteal	+	-
PROG0051	15-Dec-14	0.6	3.3	212.4	0.5	41	Late luteal	+	+
PROG0083	6-Jan-16	5.1	2.8	390	7.2	45	Luteal	*	*
PROT001	30-Jul-13	0.6	1.9	370.5	32.1	43	Luteal – day 30	+	+
PROT0011	26-Jun-15	6.9	14.7	395.2	16.5	41	Early luteal		+
PROT0058	12-Jul-16	3.4	2.9	260	16	40	Luteal	+	+
FOLLICULAR PHASE									
PROG0028	10-Feb-14	5.3	4	1186	0.2	45	Follicular	+	-
PROG0040	10-Jul-14	3.1	7.2	125.9	0.3	43	Follicular	+	-
PROG0042	18-Sept-14	10.4	6.1	230.4	1.6	44	Follicular	+	+
PROG0044	9-Oct-14	9	6	2629	0.9	48	Follicular	+	-
PROG0049	5-Nov-14	3	5.9	84.2	0.5	44	Follicular	+	-
PROG0050	2-Dec-14	5.6	6.3	476.3	0.9	38	Follicular	-	-
PROT003	3-Dec-13	5.5	8.7	83.7	9.1	51	Follicular – day 7	+	+
PROT007	17-Apr-15	9.3	17.5	99.9	<0.7	46	Follicular – day 5	*	+
POSTMENOPAUSAL									
PROG0082	6-Jan-16	35.2	92.9	<19	1.4	69	Postmenopausal	+	-
PROG0085	14-Jan-16	37.2	77.5	43	0.6	51	Postmenopausal	+	+
PROT0015	25-Jan-16	*	*	*	*	58	Postmenopausal	*	+
PROT0017	28-Jan-16	*	*	*	*	72	Postmenopausal	+	+

PROG: Groote Schuur Hospital; PROT: Tygerberg Hospital. *: information unavailable. All laboratory tests were carried out at the NHLS. Menstrual cycle status was classified according to the NHLS guidelines (Table D2), by considering the levels of all hormones (LH, FSH, E_2, P_4).

Table D2: NHLS guideline reference ranges of endogenous sex hormone levels by menstrual cycle phase

	Reference ranges			
	LH (IU/l)	**FSH (IU/l)**	**E_2 (pM)**	**P_4 (nM)**
Follicular phase	2.4-12.6	3.5-12.5	46-608	0.6-4.7
Ovulatory phase	14.0-95.6	4.7-21.5	315-1828	2.4-9.4
Luteal phase	1.0-11.4	1.7-7.7	161-775	5.3-86.0
Postmenopausal	7.7-58.5	25.8-134.8	<18-201	0.3-2.5

Appendix E

Approval letters from the Human Research Ethics Committee

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room ES2-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682 • **Facsimile** [021] 406 6411
Email: posi.tsama@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

22 February 2017

HREC REF: 076/2017

Prof J Hapgood
Molecular and Cell Biology
Upper Campus

Dear Prof Hapgood

PROJECT TITLE: THE ROLE OF SYNTHETIC PROGESTINS IN EXPRESSION OF KEY HOST IMMUNOMODULATORY AND SIGNALLING GENES, HIV-1 REPLICATION AND TRANSMISSION, IN PRIMARY CERVICAL EPITHELIAL CELLS AND EXPLANTS AND PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs): INSIGHTS TOWARDS CHOICE OF CONTRACEPTIVE (PhD CANDIDATE: MS A BICK)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 28th February 2018.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student Ms A Bick will be involved in this study.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

pp T Burgess
PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.

HREC 076/2017

Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI),and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

HREC 076/2017

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS017: Annual Progress Report / Renewal

Record Reviews/Audits/Collection of Biological Specimens/Repositories/Databases/Registries

HREC office use only (FWA00001637; IRB00001938)			
This serves as notification of annual approval, including any documentation described below.			
☐ Approved	Annual progress report	Approved until/next renewal date	30/09/18
☐ Not approved	See attached comments		
Signature Chairperson of the HREC		Date Signed	21/9/17

Principal Investigator to complete the following:

HUMAN RESEARCH ETHICS COMMITTEE
20 SEP 2017
HEALTH SCIENCES FACULTY UNIVERSITY OF CAPE TOWN

1. Protocol information

Date (when submitting this form)	14 September 2017		
HREC REF Number	210/2011	Current Ethics Approval was granted until	2017
Protocol title	The role of synthetic progestins, in expression of key host immunomodulatory and signalling genes, HIV-1 replication and transmission in primary cervical and endometrial epithelial cells and tissue explants and peripheral blood mononuclear cells: insights towards choice of contraceptive.		
Principal Investigator	Prof Janet Patricia Hapgood		
Department / Office Internal Mail Address	Room 208, Dept. Molecular and Cell Biology (MCB), MCB Building, Upper Campus, University Ave., Science faculty.		
1.1 Does this protocol receive US Federal funding?		√ Yes	☐ No

2. Protocol status (tick √)

√	Research-related activities are ongoing
☐	Data collection is complete, data analysis only

Please indicate (in the block below) the titles and HREC reference numbers of any projects currently making use of the Database/registry/repository.

N/A

3. Protocol summary

Total number of records or specimens collected, reviewed or stored since the original approval		181
Total number of records or specimens collected, reviewed or stored since last progress report		49
Have any research-related outputs (e.g. publications, abstracts, conference presentations) resulted from this research? If yes, please list and attach with this report.	√ Yes	☐ No

4. Signature

Signature of PI	JHapgood	Date	20th September 2017

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