

**Role of the glucocorticoid receptor and HIV-1 Vpr in inflammatory gene
expression and HIV-1 LTR transcription in response to dexamethasone
and progestogens**

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously submitted any part of it at any university for a degree.

Signature.....

Date.....

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Role of the glucocorticoid receptor and HIV-1 Vpr in inflammatory gene expression and HIV-1 LTR transcription in response to dexamethasone and progestogens

Y. Govender (November 2014)

Abstract

The relationship between progestin-only injectable contraception and risk of HIV-1 acquisition is controversial. Most clinical data suggests that the injectable contraceptive medroxyprogesterone acetate (MPA), unlike norethisterone enanthate (NET-EN), increases susceptibility to infections such as HIV-1. The first part of this thesis investigated the differential effects, molecular mechanisms of action and steroid receptor involvement in gene expression by MPA as compared to NET and progesterone (P4) in the End1/E6E7 and HeLa cell line models for the endocervical epithelium, a key point of entry for pathogens in the lower female genital tract (FGT). Quantitative real-time PCR analysis showed that MPA, unlike NET-acetate (NET-A) and P4, increases mRNA expression of the anti-inflammatory GILZ and I κ B α genes. Similarly, MPA unlike NET-A, decreases mRNA expression of the pro-inflammatory IL-6, IL-8 and RANTES genes, and IL-6 and IL-8 protein levels. The predominant steroid receptor expressed in the cervical cell lines and primary endocervical epithelial cells is the glucocorticoid receptor (GR), and GR siRNA experiments show that the anti-inflammatory effects of MPA are mediated by the GR. Chromatin-immunoprecipitation results suggest that MPA, unlike NET-A and P4, represses pro-inflammatory cytokine gene expression in cervical epithelial cells via a mechanism involving recruitment of the GR to cytokine gene promoters, like the GR agonist dexamethasone (DEX). This is at least in part consistent with direct effects on transcription, without a requirement for new protein

synthesis. This is the first study to show direct proof for a GR-mediated mechanism of action in anti-inflammatory effects of MPA. Dose response analysis shows that MPA has a potency of ~24 nM for transactivation of the anti-inflammatory GILZ gene and ~4 – 20 nM for repression of the pro-inflammatory genes, suggesting that these effects are likely to be relevant at injectable contraceptive doses of MPA. These findings suggest that MPA effects on genital mucosal immune function and susceptibility to infections are likely to be very different to those of NET and P4, when mediated by the GR

The second part of this thesis investigated the effects of the virion associated HIV-1 protein, Vpr, on GR-regulated inflammatory genes in the presence of the ligands. Based on evidence in the literature it was hypothesized that Vpr may act as a co-activator of the GR in the regulation of inflammatory genes in the presence of DEX. Additionally, since the GR has been shown to be activated by MPA and P4, it was hypothesized that Vpr may also modulate progestogen-mediated inflammatory gene regulation. Promoter-reporter gene assays in HeLa cells suggested that Vpr acts as a co-activator of the GR in the presence of both DEX and MPA but not P4 or NET-A. However, Vpr did not enhance ligand-mediated transactivation of the endogenous GRE-containing genes, GILZ and $I\kappa B\alpha$. Furthermore, Vpr did not affect ligand-mediated transrepression of the endogenous NF- κ B/AP-1 containing genes, IL-6 and IL-8. Results suggest that the effect of Vpr on the GR in the presence of ligands may be promoter-specific, depend on presence of native chromatin and/or be sensitive to the relative concentrations of Vpr. Interestingly Vpr appeared to only regulate basal expression of the pro-inflammatory genes, suggesting an effect of Vpr on the unliganded GR. Taken together, results suggest that depending on the HIV-1 life cycle and Vpr concentration that Vpr may hijack host GR to promote either an anti-inflammatory or pro-inflammatory response.

In addition to its role in host inflammatory gene regulation, the GR has been reported to be involved in the regulation of HIV-1 LTR transcription in the presence of DEX. However, a direct role for the GR in this DEX-mediated modulation has not been previously shown. Literature reports suggest that Vpr plays an important role in HIV-1 LTR transcription. However, there are limited data on role of Vpr in the regulation of HIV-1 LTR transcription in the presence of DEX and no data for the synthetic progestins. The role of GR and Vpr in the regulation of HIV-1 LTR transcription in the presence of ligands was investigated. It was found that DEX and the progestogens all repressed Tat-activated LTR transcription in HeLa cells. Similar results were found with HIV-1 pseudovirus-activated LTR in TZM-bl cells. GR reduction by siRNA revealed that the LTR gene may be hypersensitive to GR and require small amounts of GR protein to mediate an effect. The rank-order of the effects of the ligands suggests that the GR is involved in the ligand-mediated repression of the LTR and Vpr plays no role in this regulation. These results suggest consequences for HIV-1 replication and disease progression. Depending on the stage of HIV-1 disease, the progestogens and DEX may either have anti-viral effects or possibly aid viral latency and thereby HIV-1 pathogenesis.

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List of Abbreviations

ADP	adenosine diphosphate
AIDS	acquired immunodeficiency syndrome
ALD	aldosterone
ANOVA	analysis of variance
AP-1	activator protein 1
AR	androgen receptor
ATCC	american type culture collection
bp	base pair
CCR5	chemokine receptor type 5
cDNA	complementary DNA
C/EBP	CCAAT enhancer binding protein
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CMV	cytomegalovirus
CVL	cervico-vaginal lavage
CXCR4	chemokine receptor 4

DBD	DNA-binding domain
DEPC	diethylpyrocarbonate
DEX	dexamethasone
DMEM	dulbecco's modified eagles medium
DNA	deoxyribonucleic acid
E2	estradiol
ECL	enhanced chemiluminescence
EC ₅₀	effective concentration required for 50% of maximal response
EDTA	ethylenediaminetetra-acetic acid
ER	estrogen receptor
ERE	estrogen response element
EtOH	ethanol
EtBr	ethidium bromide
ETG	etonogestrel
FCS	fetal calf serum
FGT	female genital tract
GAPDH	glyceraldehyde phosphate dehydrogenase
GC	glucocorticoid
GILZ	glucocorticoid induced leucine zipper

GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRIP-1	glucocorticoid receptor interacting protein type 1
HA	haemagglutinin
HIV-1	human immunodeficiency virus subtype 1
hrs	hours
HRT	hormone replacement therapy
HSV	herpes simplex virus
I κ B α	inhibitor of NF- κ B type α
IL	interleukin
IM	intra-muscularly
IFN	interferon
K _D	equilibrium dissociation constant
ker-sfm	keratinocyte serum-free medium
kDa	kilodalton
LBD	ligand-binding domain
LNG	levonorgestrel
LTR	long terminal repeat
Luc	luciferase

M	Molar
MIB	mibolerone
MIP	macrophage inflammatory protein
mins	minutes
MMTV	mouse mammalian tumour virus
MOPS	4-morpholine-propanesulfonic acid
MPA	medroxyprogesterone acetate
mRNA	messenger RNA
MR	mineralocorticoid receptor
NET	norethisterone
NET-A	norethisterone acetate
NET-EN	norethisterone enanthate
NFAT	nuclear factor of activated t-cell
NF- κ B	nuclear factor kappa B
NRE	negative regulatory element
NTD	N-terminal transactivation domain
OC	oral contraceptive
P4	progesterone
PARP-1	poly(ADP-ribose) polymerase- 1

PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PR	progesterone receptor
qRT-PCR	quantitative reverse transcription PCR
R5020	promegestone
RANKL	receptor of activated NF- κ B ligand
RANTES	regulated upon activation, normal T cell expressed and secreted
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
secs	seconds
SEM	standard error of the mean
SHIV	simian-human immunodeficiency virus
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
SQ	sub-cutaneously
SR	steroid receptor
SRE	steroid response element

Stat5	signal transducer and activator of transcription 5
STI	sexually transmitted infection
TAE	tris-acetate
TBS	tris-buffered saline
TBST	tris-buffered saline-tween
TAR	trans-acting responsive element
Tat	trans-activator of transcription
TF	transcription factor
TFRE	transcription factor response element
TNF- α	tumour necrosis factor- α
TAT	tyrosine aminotransferase
VMMC _s	vaginal mucosal mononuclear cells
Vpr	viral protein R
v/v	volume per unit volume
WHO	world health organisation
wt	wild type
w/v	weight per unit volume

Thesis Outline

Please note the following are publications arising from this thesis:

Govender Y, Avenant C, Verhoog N, Ray RM, Grantham NJ, Africander D and Hapgood JP. (2014) The injectable-only contraceptive medroxyprogesterone acetate, unlike norethisterone acetate and progesterone, suppresses inflammatory genes in endocervical cells via the glucocorticoid receptor. *PLoS ONE*. 9(5): e96497. doi:10.1371/journal.pone.0096497

Hapgood JP, Ray RM, **Govender Y**, Avenant C, Tomasicchio M. (2014) Differential glucocorticoid receptor-mediated effects on immunomodulatory gene expression by progestin contraceptives: implications for HIV-1 pathogenesis. *American Journal of Reproductive Immunology*. doi: 10.1111/aji.12214

Avenant C, Kotitschke A, Tomasicchio M, **Govender Y**, Kemp CD and Hapgood JP. HIV-1 Viral Protein R (VPR) highjack's the host Glucocorticoid receptor (GR) to modulate host cytokine (IL6 and RANTES) expression, thereby potentially enhancing HIV-1 pathogenesis. In preparation.

This thesis contains the following sections:

1. **Chapter 1: Literature Review.** This chapter gives a succinct overview of the relevant knowledge currently available in the literature focussing on GR function, molecular mechanisms of action and role of the progestins medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A) in cervical inflammation and HIV-1 pathogenesis.
2. **Chapter 2: Thesis Rationale, Aims and Hypotheses.** This chapter will briefly state the context, aims and hypotheses of this thesis.
3. **Chapter 3: Material & Methods.** This chapter provides detailed information on the protocols used to obtain the results presented in chapters 4 to 6.
4. **Chapter 4: Inflammatory gene regulation in human endocervical cells by the synthetic progestin MPA, unlike NET-A and P4, is mediated by the GR (Results).** This chapter contains results of the investigation into the differential

regulation of inflammatory genes by the progestogens (MPA, NET-A and P4) and the mechanism of action of the MPA-mediated regulation of inflammation. This is determined in the End1/E6E7 and HeLa cell lines and attempted in primary endocervical cells (VEN-100).

5. **Chapter 5: An investigation into the role of HIV-1 Vpr in GR-mediated inflammatory gene regulation in the presence of DEX, P4 and the synthetic progestins (Results).** This chapter contains the results of the investigation into the hypothesis that Vpr modulates the GR-regulated immune response in the presence of DEX and progestogens (MPA, NET-A and P4). This was investigated in the HeLa and TZM-bl cell lines.
6. **Chapter 6: An investigation into the role of GR and Vpr in the regulation of HIV-1 LTR transcription in the presence of DEX, P4 and the synthetic progestins (Results).** This chapter contains the results of a study investigating the regulation of HIV-1 LTR transcription in the HeLa and TZM-bl cell line. In particular the role of GR and Vpr in response to DEX and progestogens (MPA, NET-A, LNG and P4) was investigated.
7. **Chapter 7: Discussion & Conclusions.** In this chapter, results are discussed and conclusions are drawn from the combined results in chapters 4 to 6. Some perspectives about future research are also included.
8. **Addendum: Supplementary Data.** This addendum contains additional results from this study not presented in chapters 4 to 6.
9. The **Bibliography** contains a list of all the references used throughout the thesis in alphabetical order.
10. **Govender *et al.* 2014.** The published research article that contains most of the data from this thesis. The present author contributed to this research article, in terms of

intellectual planning, interpretation of the data, execution of 80% of the experiments and writing of the manuscript.

11. **Hapgood *et al.* 2014.** The present author made an intellectual and methodological contribution to this research article which relates to this thesis.

Note that all the experiments and all the cell work reported in results chapters 4, 5 and 6 and in the addendum were performed by the candidate.

Chapter 1: Literature Review

1.1 Inflammation

Inflammation is a physiological response as a consequence of tissue injury associated with various causes such as trauma, autoimmune reactions, and the detection of antigens or pathogens (Harada *et al.* 1994; Feghali & Wright 1997). This response consists of two phases; acute and chronic (Feghali & Wright 1997). Acute phase is characterized by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines (Feghali & Wright 1997). In the chronic phase, inflammation is characterized by the development of a specific humoral immune response, which is mediated by B-cells and involves the production of antibodies, and a cellular immune response, which involves the activation of macrophages, natural killer cells and T-cells, to the pathogen present at the site of injury (Rhoades & Bell 2012). During both acute and chronic inflammatory processes, a variety of soluble factors are involved in leukocyte recruitment through increased expression of cellular adhesion molecules and chemoattraction (Newton 2000; Busillo & Cidlowski 2013). Several cytokines play key roles in mediating acute inflammatory reactions, namely interleukin (IL)-1, TNF- α , IL-6, IL-11 and chemokines, IL-8, regulated-upon-activation-normal-T-cell-expressed-and-secreted (RANTES), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Feghali & Wright 1997). Chronic inflammation may develop following acute inflammation and may last for weeks or months, and in some instances for years. The cytokines known to mediate chronic inflammatory processes can be divided into those contributing to cellular inflammation such as IL-2, IL-12, interferons (IFNs), IFN- γ inducing factor (IGIF), and TNF- α and - β , and those participating in humoral inflammation such as IL-4, IL-10, IL-13, and transforming growth factor- β (TGF- β) (Feghali

& Wright 1997; Elenkov 2004; Akdis *et al.* 2004). T helper 1 (Th1) immunity provides protection against intracellular bacteria, protozoa, fungi, and several viruses, whereas T helper 2 (Th2) immunity provides protection against multicellular parasites, extracellular bacteria, some viruses, soluble toxins, and allergens (Elenkov & Chrousos 2002; Belardelli & Ferrantini 2002). The Th1 response supports the activities of macrophages and cytotoxic T-cells of the cellular immune system while the Th2 response promotes the actions B-cells of the humoral immune system (Rhoades & Bell 2012). It has been found that the Th1 and Th2 responses are mutually inhibitory, whereby IL-12 and IFN- γ inhibit Th2 cells' activities, while IL-4 and IL-10 inhibit Th1 responses (Mosmann & Sad 1996; Elenkov 2004).

Cytokines can be further classified into two groups' pro-inflammatory and anti-inflammatory. The regulation of pro-inflammatory cytokines, such as IL-6, TNF- α and interferon (IFN)- γ , and anti-inflammatory cytokines, such as IL-4 and IL-10, may provide critical insights into mechanisms underlying a variety of common human diseases (Wong *et al.* 2001; Elenkov & Chrousos 2002). Although the list of hormones and neurotransmitters that are able to modulate the production of pro/anti-inflammatory cytokine production is constantly enlarging, the best-characterized neuroendocrine factors that regulate the production of these cytokines include glucocorticoids (GCs), key stress hormones (Elenkov & Chrousos 2002).

1.2 Glucocorticoids in inflammation

Glucocorticoids (GCs) are the most potent and currently used treatment to combat allergic and chronic inflammatory diseases such as asthma, pulmonary diseases, dermatitis, rheumatoid arthritis and even some cancers (Baschant & Tuckermann 2010; Newton *et al.* 2010). The hypothalamic-pituitary-adrenal (HPA) axis controls the synthesis of GCs in the adrenal cortex (Yudt & Cidlowski 2002). Endogenous GCs, e.g. cortisol circulate in the body bound to corticosteroid-binding globulin and serum albumin (McKay & Cidlowski 1999).

Synthetic GCs such as prednisone and dexamethasone (DEX) are drugs that have been designed to resemble natural GCs (Newton 2000). However, they differ from natural GCs by their potency and metabolic clearance. Furthermore, unlike natural GCs, synthetic GCs do not bind corticosteroid-binding globulin and are thereby not susceptible to their regulation of available levels (Kadmiel & Cidlowski 2013). GCs can suppress inflammation by down-regulating the expression of pro-inflammatory cytokines such as IL-6 and TNF- α or by up-regulating cytokines such as IL-10, which in turn suppress the production of pro-inflammatory mediators (Barnes 1998; Verhoog *et al.* 2011). Evidence also indicates that GCs shift the cytokine response from Th1 to Th2 to suppress inflammation (Clerici *et al.* 1997). GCs mediate their action through binding of the glucocorticoid receptor (GR), which in turn regulates transcription of inflammatory genes to obtain the desired anti-inflammatory effects (Newton 2013).

1.3 Molecular mechanisms of action of the glucocorticoid receptor

1.3.1 Ligand-binding, GR activation and nuclear translocation

The glucocorticoid receptor (NR3C1, GR) belongs to the nuclear receptor superfamily and is a ligand-dependent transcription factor (Ratman *et al.* 2013; De Bosscher *et al.* 2013). The GR gene is comprised of ten exons spanning 110 kb, encoded on the fifth chromosome at region 5q31p (Encio & Detera-Wadleighs 1991). GR α is the classic receptor subtype which binds to GCs. It is mainly cytoplasmic and is most extensively studied. However, it is worth noting that an additional four isoforms exist that are generated via alternative splicing: GR β , GR γ , GR-A and GR-P (Oakley & Cidlowski 2011). The GR β isoform resides constitutively in the nucleus and acts as a natural dominant negative inhibitor of the GR α isoform (Kadmiel & Cidlowski 2013). GR β can also directly regulate genes that are not regulated by GR α (Kino *et al.* 2009). The other isoforms are less well-characterized and have been associated

with glucocorticoid insensitivity (Kadmiel & Cidlowski 2013). The GR α consists of three modular domains: an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (Busillo & Cidlowski 2013). In the absence of hormone, inactive GR is in a chaperone complex associated with heat shock protein 90 and 70 and several immunophilins such as FKBP51 in the cytoplasm (Zhou & Cidlowski 2005; Flammer & Rogatsky 2011). GCs are lipophilic and passively diffuse across the cell membrane (Zhou & Cidlowski 2005). Upon ligand binding the GR undergoes a conformational change and the GR translocates to the nucleus (Flammer & Rogatsky 2011).

1.3.2 Direct DNA binding or protein-protein interactions

Once in the nucleus, the active GR can either bind directly to the DNA through binding to glucocorticoid response elements (GREs) or bind to other transcription factors (De Bosscher 2010). The activated GR-ligand complexes can either dimerise to form homodimers or remain as monomers (Robertson *et al.* 2010). Dimerisation has been found to be ligand-specific and is required for transcriptional activation (transactivation), but not for transcriptional repression (transrepression) via tethering (Reichardt *et al.* 1998; Robertson *et al.* 2010). The activated GR dimers or monomers then translocates to the nucleus where the DBD of the active GR binds to GRE sequences in targeted genes and thereby activate transcription. The consensus sequence for GRE binding is the palindromic 15-bp sequence GGTACAnnnTGTTCT (where n is any nucleotide) (Barnes 1998). GC-responsive genes containing such simple-acting GREs include serine/threonine protein kinase (sgk1) (Schoneveld *et al.* 2004). In contrast, many genes contain glucocorticoid response units (GRU's), in which transcription not only depends on GR binding to the GRE, but also requires the binding of other transcription factors to adjacent sites e.g. phenylalanine hydroxylase gene (Schoneveld *et al.* 2004). The GR can also bind as a monomer to composite

GRE half sites. The GR has also been shown to bind to specific, widely prevalent inverted palindromic sequences called 'simple' negative GREs (nGREs) on target genes (Kadmiel & Cidlowski 2013). GR binds to nGREs which overlap response elements and subsequently prevents binding of the positively acting transcription factors to its recognition sequence and results in transrepression (Zhou & Cidlowski 2005). An example is the osteocalcin gene promoter, in which the nGRE overlaps with the TATA box and thereby prevents binding of the TFIID (Stromstedt *et al.* 1991). Another example is the prolactin gene promoter, in which the nGRE site overlaps with two other transcription factor binding sites, Oct-1 and Pbx (Subramaniam *et al.* 1998).

As mentioned earlier, in certain genes transcriptional regulation by the GR does not involve direct binding of the GR to DNA, but rather regulation via protein-protein interactions (Ratman *et al.* 2013). Although tethering is predominantly a transrepressive mechanism there have been reports of transactivation as a result of tethering (Kassel & Herrlich 2007). An example of tethering that results in transactivation is GR binding to Stat5 on the β -casein gene while an example of transrepression is GR binding to activator protein-1 (AP-1) or nuclear factor kappa B (NF- κ B) on the collagenase, IL6 and IL8 genes (Kassel & Herrlich 2007). Another example of protein-protein interaction is mutual antagonism of GR and NF- κ B/AP-1 whereby NF- κ B and AP-1 proteins and GR reciprocally modulate each other's activity (De Bosscher *et al.* 2003). Other models include GR-mediated co-factor competition, chromatin remodelling co-factors and modification of the basal transcriptional machinery (De Bosscher *et al.* 2003).

1.3.3 Transactivation

After DNA binding; the GR recruits the basal transcription machinery, chromatin remodelling complexes, co-activators, as well as a variety of other transcription factors which facilitate GR mediated transactivation (Chinenov *et al.* 2013). Many GC-responsive genes are upregulated by the direct binding of activated GR-ligand complexes to GREs (as illustrated in Figure 1.1). GCs generally transactivate genes that are involved in producing an anti-inflammatory response such as the glucocorticoid-induced leucine zipper (GILZ) and inhibitor of kappa B alpha (I κ B α) genes (Newton & Holden 2007; Reddy *et al.* 2009). GILZ codes for a leucine zipper protein and was first isolated as a DEX-responsive gene from thymocytes (D'Adamio *et al.* 1997). DEX-induced GILZ overexpression has been shown to inhibit both T-cell receptor (TCR)-induced IL-2/IL-2 receptor expression and apoptosis (Ayroldi *et al.* 2001; Berrebi *et al.* 2003). Furthermore, GILZ reduces NF- κ B activity; in particular, inhibits NF- κ B nuclear translocation and DNA binding due to a direct protein-protein interaction of GILZ with the NF- κ B subunits (Ayroldi *et al.* 2001). Transcriptional regulation of GILZ by GCs involves GR interaction with multiple GRE sequences (~6 sites) contained on the GILZ promoter (Ayroldi & Riccardi 2009). I κ B α codes for an inhibitory protein of NF- κ B that hold NF- κ B (p50/p65) inactive in the cytoplasm (Rupec *et al.* 1999). DEX-induced I κ B α upregulation has been shown to inhibit NF- κ B activation (Auphan *et al.* 1995). This inhibition is a result of the sequestration of NF- κ B dimers by I κ B α in the cytoplasm and thereby reduction in NF- κ B nuclear translocation (Auphan *et al.* 1995; Scheinman *et al.* 1995).

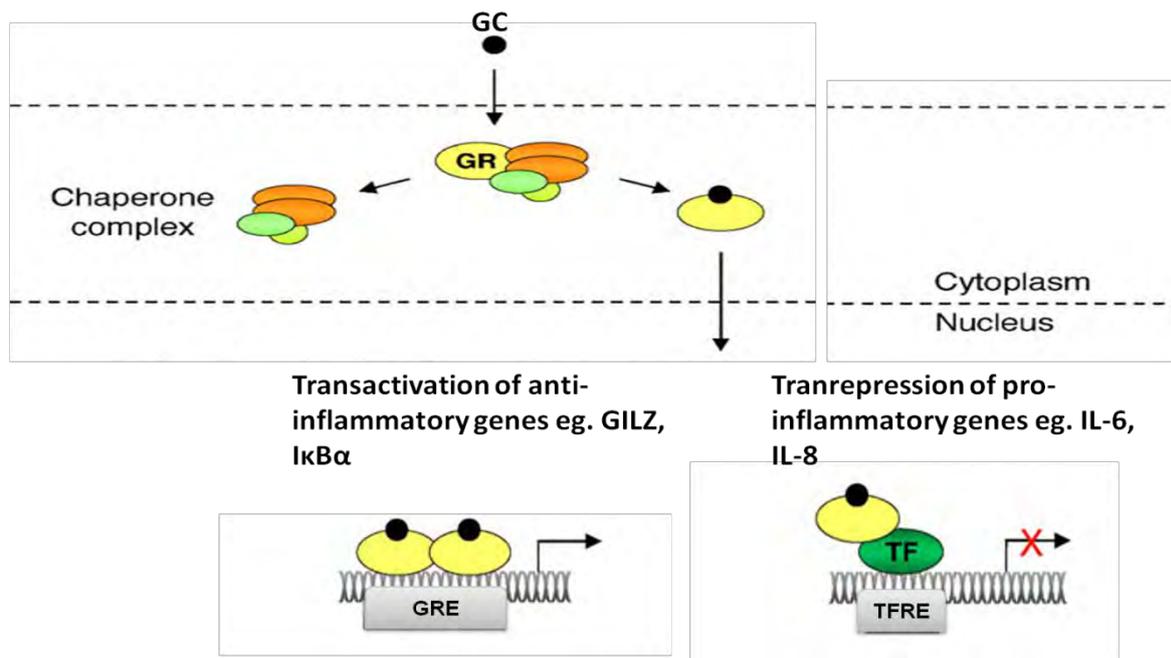


Figure 1.1: GR mechanism of action. Taken and modified from (Bogaert *et al.* 2010). Upon ligand binding, the GR undergoes a conformational change and translocates to the nucleus where it occupies GRE sites on anti-inflammatory genes to activate transcription or interacts with TFs bound to TFRE sites of pro-inflammatory genes to suppress transcription. Abbreviations: GC = glucocorticoid, GR = glucocorticoid receptor, GRE = glucocorticoid response element, TF = transcription factor; TFRE = transcription factor response element.

1.3.4 Transrepression

GR binds to nGREs or tethers to transcription factors and results in recruitment of co-repressors and chromatin remodelling complexes which facilitate chromatin condensation and transrepression (Chinenov *et al.* 2013). The transrepression of pro-inflammatory genes (such as IL-6 and IL-8) which contain AP-1 or NF- κ B binding sites occurs via the GR tethering to AP-1 or NF- κ B proteins, respectively (as illustrated in Figure 1.1).

1.4 Rapid non-genomic actions of GCs

In addition to genomic actions which are mediated by transactivation and transrepression, GCs can act via rapid non-genomic effects. Literature shows that rapid non-genomic GC actions include modulation of general cellular functions, brain and neurophysiological

functions as well as behavioural responses (Groeneweg *et al.* 2011). Evidence suggests that rapid non-genomic GCs effects are mediated by three different mechanisms. The first is direct interactions with biological membranes and this is a non-specific non-genomic effect (Buttgereit *et al.* 1999). Some non-specific non-genomic effects of GCs have been shown to be mediated by alteration of the physicochemical property of cell membranes (Song & Buttgereit 2006). It has been hypothesized that GCs intercalate at high concentrations in cellular membranes e.g. plasma and mitochondrial membranes which alter cell functions by influencing cation transport through the plasma membrane and by increasing the proton leak of the mitochondria (Song & Buttgereit 2006). The second is membrane-bound GR-mediated non-genomic effects. The presence of the membrane-bound GR in immune cells such as PBMCs provides evidence for the hypothesis that non-genomic GC signalling is involved in modulating immune function (Bartholome *et al.* 2004). Membrane-bound GR has also been shown to mediate rapid actions of GCs in mouse skeletal muscle fibres and L β T2 cells (Pérez *et al.* 2013; Wehmeyer *et al.* 2014). The third proposed mechanism is via cytosolic GR-mediated non-genomic effects. In addition to genomic effects, cytosolic GR has been shown to be involved in non-genomic actions via interactions with intracellular signalling proteins (Simoncini & Genazzani 2003). This is supported by studies that show crosstalk mechanisms between the classical GR and other signalling pathways (Qi *et al.* 2005; Kotitschke *et al.* 2009). For example, GCs have been reported to rapidly activate p38 and JNK in hippocampal cells (Qi *et al.* 2005). In addition, the GR has been shown to crosstalk with the protein kinase B (Akt) signalling pathway in A549 cells (Matthews *et al.* 2008).

1.5 The role of GR in HIV-1 pathogenesis

1.4.1 GR-mediated immune function in HIV-1 infection

Evidence shows that human immunodeficiency virus (HIV-1) disease progression is accompanied by a defective production of Th1 cytokines such as IL-2, IL-12 and IFN- γ , and an increased production of Th2 cytokines such as IL-4 and IL-10 (Clerici *et al.* 1994; Meyaard *et al.* 1996; Klein *et al.* 1997; Norbiato *et al.* 1997). As mentioned earlier, GCs acting via the GR suppress inflammation by shifting the immune response from Th1 to Th2, supporting a role for the GR in HIV-1 induced modulation of cytokine networks (Clerici *et al.* 1997; Hapgood & Tomasicchio 2010). The important role for the GR in HIV-1 pathogenesis is further supported by studies that show HIV-1 infection is accompanied by a chronic activation of the HPA system which either leads to GC hypersensitivity or results in elevated cortisol levels (Norbiato *et al.* 1997; Kino & Chrousos 2001; Kino *et al.* 2003). In HIV-1 infected patients, elevated levels of cortisol results in a compromised immune response which may promote disease progression (Norbiato *et al.* 1997).

There have also been reports of GC resistance in HIV-1 infected individuals (Norbiato *et al.* 1997). Although the mechanism of HIV-1 induced GC resistance is unclear, possible explanations have been proposed in literature. It has been suggested that GC resistance is due to lowered GR affinity for ligand as a result of the imbalance in cytokine production found in HIV-infected patients (Norbiato *et al.* 1997). This is supported by findings that show that the combination of IL-2 and IL-4 reduces the ligand binding affinity of the GR (Kam *et al.* 1993). Another possible explanation for the GC resistance is the relative increased expression of the GR β isoform to the GR α isoform (Zapanti *et al.* 2008). The GR α isoform is the main mediator of GC activity while the GR β isoform is known to inhibit GR α action (Bamberger *et al.* 1995). Thus, the increased proportion of GR β isoform leads to a decreased GC effect. It

has also been proposed that viral protein R (Vpr) may play a role in lowering GR affinity for GCs (Norbiato *et al.* 1997; Kogan & Rappaport 2011).

1.4.2 The role of GR in HIV-1 replication

In addition to a role for the GR in modulating host immune function in response to HIV infection, GCs have been shown to affect HIV replication by regulation of the HIV-1 LTR (Laurence *et al.* 1989; Kolesnitchenko & Snart 1992; Mitra *et al.* 1995; Russo *et al.* 1999; Kino *et al.* 2000). The identical copies of the HIV-1 LTR is located at the 5' and 3' end of the integrated provirus and contains the promoter and enhancer elements that regulate HIV-1 expression (Geeraert *et al.* 2008). In the presence of the HIV-1 transactivator (Tat) protein, the transcription of the HIV-1 LTR is activated, through its interaction with the transactivation response region (TAR) (Ammosova *et al.* 2006; Hapgood & Tomasicchio 2010). The HIV-1 LTR contains a variety of cis elements. Mutational analysis revealed that the SP-1, NF- κ B and AP-1 sites are required for viral replication (Ross *et al.* 1991; Ghosh 1992; Mitra *et al.* 1995; Van Lint *et al.* 1997). The HIV-1 LTR also contains three non-classical GRE elements: GRE I, GRE II and GRE III (Mitra *et al.* 1995). GRE I (AGAACAAnnnnGCTTGT) is an imperfect palindrome, while GRE II (TGTACT) and III (AGACCA) are half sites (Mitra *et al.* 1995). GR has been shown to bind to all three GRE sites (Ghosh 1992; Mitra *et al.* 1995), however, mutational analysis shows that GRE I and GRE II do not have a functional role in HIV-1 transcription but it appears that GRE III may play a role in the GC-regulated HIV-1 transcription (Mitra *et al.* 1995). Figure 1.2 illustrates the documented and potential transcriptional regulatory sequences in the HIV-1 LTR.

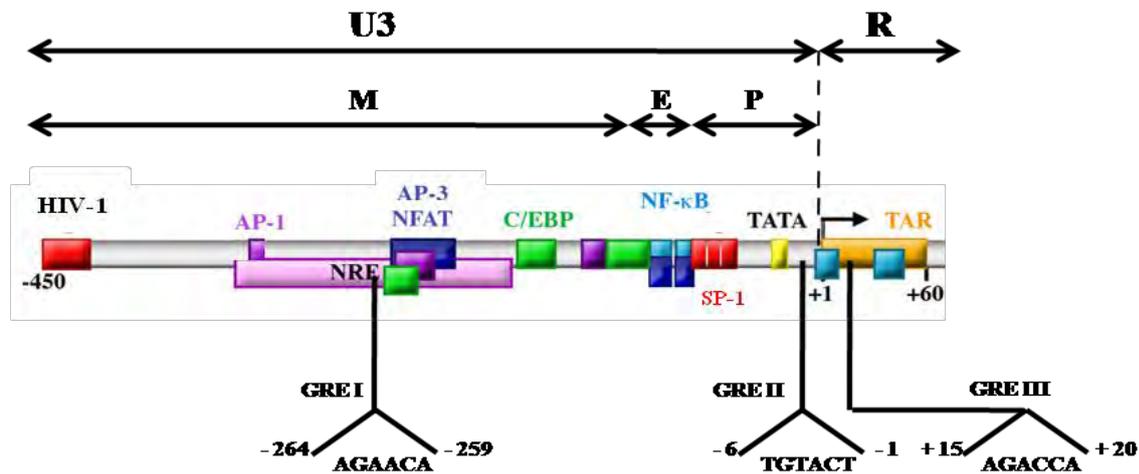


Figure 1.2: Structure of HIV-1 long terminal repeat. Taken and modified from (Mitra *et al.* 1995; Kilareski *et al.* 2009). The U3 region is divided into the modulatory (M), enhancer (E) and promoter regions (P). The promoter region contains the TATA box and SP factor binding sites while the enhancer contains the NF- κ B binding sites. The repeat (R) region contains a trans-acting responsive element (TAR) that forms an RNA stem loop structure upon transcription that binds to the viral protein Tat. A negative regulatory element (NRE) was identified that was subsequently shown to serve as both activator and repressor by binding NFAT proteins, AP-1 proteins and C/EBP factors. The sequences responsible for glucocorticoid receptor binding are highlighted and written in the order 5' to 3', GRE-I, -II and -III, respectively.

There are conflicting reports about regulation of the HIV-1 LTR by GCs. Some studies have shown *in vitro* that DEX upregulates HIV-1 LTR transcription in CEM-T4 (Human T lymphoblastoid) and 3T3 (mouse fibroblast) cell lines (Furth *et al.* 1990; Russo *et al.* 1999). However, the majority of studies show that DEX represses both overexpressed plasmid Tat-

activated and viral-activated HIV-1 LTR transcription in multiple human cell lines such as U937 (monocyte), H9 (embryonic stem cell), CD4⁺ Raji (B lymphocyte), H9V3 (chronic HIV-infection) and U1.1 (chronic HIV-infection) (Laurence *et al.* 1989; Kolesnitchenko & Snart 1992; Mitra *et al.* 1995; Kurata & Yamamoto 1999; Russo *et al.* 1999; Kino *et al.* 2000). Similarly, there are conflicting reports on the effect of GCs on viral replication as measured by HIV-1 p24 levels. Evidence shows that in peripheral blood mononuclear cells (PBMCs), GCs upregulate HIV-1 p24 levels while another report shows in CD4⁺ T cells that GCs reduce HIV-1 p24 levels (Ayyavoo, Mahalingam, *et al.* 1997; Chun *et al.* 1998; Wang *et al.* 1998). Although these studies suggest a role for the GR in viral replication, this has not been shown. Consequently, it remains to be determined whether GCs, acting via the GR, increase or decrease HIV-1 transcription in HIV-infected cells in a manner dependent on chromatin structure and cell-type.

1.6 The role of Vpr in HIV-1 pathogenesis

HIV-1 encodes retroviral Gag, Pol, and Env proteins along with six additional accessory proteins, Tat, Rev, Vpu, Vif, Nef, and Vpr (Kogan *et al.* 2012). Vpr is a 96 amino acid, 14 kDa protein and is highly conserved in both HIV-1 and simian immunodeficiency virus (SIV) (Forget *et al.* 1998; Morellet *et al.* 2003; Ayinde *et al.* 2010). Vpr protein is present in significant quantities in the serum of HIV-1 positive individuals (Levy *et al.* 1994) thereby indicating its importance in the virus life cycle. Interestingly, Vpr is found to occur unassociated with other proteins in serum and has the ability to passively diffuse across cell membranes (Tungaturthi *et al.* 2003). Vpr has multiple functions in viral pathogenesis, including host cell arrest in G2/M phase of the cell cycle, increasing the translocation of the HIV-1 pre-integration complex which is needed for reverse transcription and integration to the host cell nucleus and enables T cell and macrophage infection (Ayyavoo, Rafaeli, *et al.*

1997; Kino *et al.* 1999; Iijima *et al.* 2004; Muthumani *et al.* 2004). It is also suggested that Vpr's role in G2 cell cycle arrest creates a cellular environment in which the LTR is transcriptionally more active (Forget *et al.* 1998). Multiple studies have shown *in vitro*, in human cell lines and PBMCs using overexpressed Tat or HIV-1 pseudovirus or infectious molecular clones, that Vpr enhances Tat-/viral-activated LTR transcription (Felzien *et al.* 1998; Forget *et al.* 1998; Sawaya *et al.* 2000; Vanitharani *et al.* 2001; Kino *et al.* 2002; Cui *et al.* 2006). It has been suggested that this enhancement in viral transcription is due to cooperativity between Vpr, Tat, cyclin T1 and cyclin dependent kinase (CDK)-9 (Sawaya *et al.* 2000). In addition, Vpr has shown to bind directly to co-activators p300/CREB-binding protein (CBP) in enhancing Tat-mediated HIV-1 LTR transcription (Kino *et al.* 2002).

Evidence also suggests that Vpr could play a role in viral reactivation whereby Vpr can activate LTR transcription in the absence of Tat (Kino *et al.* 1999; Sawaya *et al.* 2000; Zhu *et al.* 2001; Varin *et al.* 2005). The study by Varin *et al.* 2005 has shown by mutational analysis in U937 and primary macrophages that Vpr activates transcription factors AP-1, c-Jun N-terminal kinase (JNK) and NF- κ B and thereby stimulates LTR transcription. Furthermore, it has been shown that purified serum Vpr activated virus expression from five latently infected cell lines (Levy *et al.* 1994). Serum Vpr also activated virus expression from resting PBMCs of HIV-infected individuals (Levy *et al.* 1994).

In addition to its role in viral transcription, it is suggested that Vpr has anti-inflammatory activity (Muthumani *et al.* 2004). Vpr has been shown in PBMCs to suppress pro-inflammatory cytokines such as IL-2, IL-12 and TNF- α similar to the synthetic GC, DEX (Ayyavoo, Mahboubi, *et al.* 1997; Mirani *et al.* 2002). It has been proposed that the Vpr-mediated anti-inflammatory effects are via the ability of the viral protein to suppress NF- κ B activity through effects on I κ B α expression (Ayyavoo, Mahboubi, *et al.* 1997; Kogan & Rappaport 2011). This is a similar mechanism used by the GR to inhibit NF- κ B (Auphan *et*

al. 1995). Another proposed mechanism is that Vpr acts as a co-activator of the GR and thereby promotes an anti-inflammatory response (Kino *et al.* 1999).

1.7 Evidence for crosstalk between the GR and Vpr in transcriptional regulation

Like host GR co-activator GR-interacting protein (GRIP)-1, Vpr contains a co-activator signature motif sequence LXXLL (at amino acids 64 to 68), which is necessary for interaction with the hormone receptor (Kino *et al.* 2002). It has been reported that Vpr co-activates DEX-dependent GR-mediated transactivation on GR responsive promoters' mouse mammary tumour virus (MMTV) and TAT-GRE (Kino *et al.* 1999; Sherman *et al.* 2000). In addition, GR/progesterone receptor (PR) antagonist (RU-486) repressed this Vpr-GR mediated transactivation. Furthermore, it has been shown in PBMCs that Vpr co-activates DEX-mediated upregulation of the endogenous gene, receptor of activated NF- κ B ligand (RANKL) (Fakruddin & Laurence 2005). As mentioned earlier, Vpr downregulates NF- κ B inducible cytokines, including IL-2, IL-12, TNF- α , and IL-4, and chemokines, MIP-1 α , MIP-1 β , and RANTES (Ayyavoo, Mahboubi, *et al.* 1997; Muthumani *et al.* 2000; Mirani *et al.* 2002) in a manner that suggests a role for the GR. This is supported by the Muthumani *et al.* study which shows that Vpr uses the GR pathway to recruit the NF- κ B co-activating protein, poly(ADP-ribose) polymerase- 1 (PARP-1) (Muthumani *et al.* 2006). The GR interaction with Vpr was found to be both necessary and sufficient to facilitate formation of a Vpr-GR-PARP-1 complex. Evidence also suggests that the recruitment of PARP-1 by the Vpr-GR complex prevents its nuclear localization, which is necessary for Vpr to suppress NF- κ B. An interaction between the GR and Vpr has been further demonstrated *in vitro* by co-immunoprecipitation experiments (Kino *et al.* 1999). Consistent with a role for GR in Vpr-mediated gene regulation it has been shown that GR antagonist, RU-486, reversed the Vpr-

mediated repression of several cytokine genes in PBMCs (Ayyavoo, Mahboubi, *et al.* 1997; Mirani *et al.* 2002).

Although RU-486 has shown to inhibit Vpr-activated LTR transcription (Soudenys & Wainberg 1997; Schafer *et al.* 2006) there is currently no direct evidence that a GR–Vpr interaction plays a role in HIV-LTR transcription via the non-classical GREs in the LTR (Hapgood & Tomasicchio 2010).

1.8 Progesterone and the progestins medroxyprogesterone acetate & norethisterone acetate

In addition to GCs, the GR can be activated by other steroid hormones such as the progestogens; progesterone (P4), medroxyprogesterone acetate (MPA) and norethisterone (NET) and its derivatives NET-acetate (NET-A) or NET-enanthate (NET-EN) (Hapgood *et al.* 2004; Koubovec *et al.* 2005). Progestogens can be divided into two types, natural and synthetic (Stanczyk *et al.* 2013). There is only one natural progestogen which is P4 (Figure 1.3 A) (Schindler *et al.* 2003). P4 is secreted primarily by the corpus luteum in the ovaries of females and plays a key role in the mammalian reproductive system (Graham & Clarke 1997; Africander, Verhoog, *et al.* 2011). For example it is important for the development and regular functioning of the mammary gland and uterus, for the control of ovulation, and it prepares the endometrium for implantation through cell proliferation (Madauss *et al.* 2007; Winneker *et al.* 2008). Furthermore, high levels of P4 are responsible for the lack of ovulation during pregnancy and this inhibitory effect of P4 is the basis for oral contraceptives (OCs) (Madauss *et al.* 2007). However, due to poor bioavailability (< 5%) and short half-life of P4, its use as a contraceptive is limited (Stanczyk *et al.* 2013). Consequently, synthetic progestogens (progestins) were designed to mimic the actions of P4, while having a better half-life and a more favourable bioavailability than the natural ligand. The progestin, MPA

(Figure 1.3 B), is structurally related to P4 and is a pregnane derivative while NET-A (NET is the active metabolite) (Figure 1.3 C) is an estrane derivative and structurally similar to testosterone (Stanczyk *et al.* 2013). Both MPA and NET-A are 1st generation progestins and are widely used in contraception and hormone replacement therapy (HRT) (Sitruk-Ware 2003).

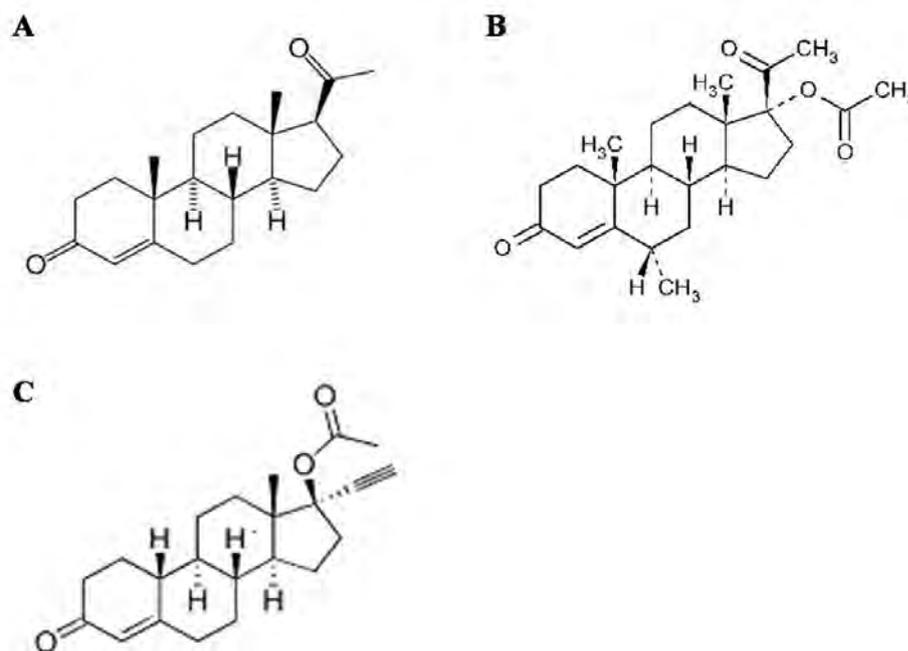


Figure 1.3: The chemical structures of (A) progesterone (P4), (B) medroxyprogesterone acetate (MPA) and (C) norethisterone acetate (NET-A) adapted from (Hapgood *et al.* 2004).

1.7.1. MPA and NET-A in contraception

MPA and NET-A are used as progestin-only contraceptives and are administered by injection (Hapgood *et al.* 2004). MPA, commercially available as Depo-Provera® or DMPA, is administered at a dose of 150 mg every 3 months (Hapgood 2013). The contraceptive effect of MPA stems primarily from its action at the pituitary and hypothalamic levels. Specifically, MPA prevents the mid-cycle surge of luteinizing hormone (LH) and follicle-stimulating

hormone (FSH), which is necessary for ovulation (Rivera *et al.* 1999; Erkkola & Landgren 2005). Thus suppression of ovulation is considered the main mechanism of action (Jain *et al.* 2004). In addition, MPA has an effect on cervical mucus whereby the mucus becomes thick and consequently makes sperm penetration unlikely (Rivera *et al.* 1999). Changes in the cervical mucus usually develop within 24 hours of injection but in some cases may take as long as 3 to 7 days (Rivera *et al.* 1999). MPA also transforms the endometrium making it thin and atrophic (Rivera *et al.* 1999). These changes stem from inhibition of ovarian function (Erkkola & Landgren 2005). NET-A is administered at a dose of 200 mg every 2 months. The mechanism of action of NET-A is the same as that of MPA but it is effective for a shorter period of time (Rivera *et al.* 1999). The contraceptive actions of MPA and NET-A are mediated by the PR and these actions are generally referred to as progestogenic effects (Hapgood *et al.* 2013). Although the progestins are effective in preventing pregnancy there are a range of side-effects (i.e. off-target effects) associated with their use such as irregular bleeding, amenorrhea, breast tenderness and migraines (Paul *et al.* 1997; Schrager 2002; Westhoff 2003). In addition, the use of progestins has been associated with more life-threatening diseases such as increased risk of breast cancer, cardiovascular disease and pulmonary emboli (Sitruk-Ware 2004; Ghatge *et al.* 2005).

1.7.2. MPA and NET-A off-targets effects are mediated via other steroid receptors

The off-target/non-progestogenic effects of MPA and NET-A are mediated by other steroid receptors (SRs) such as the androgen receptor (AR), mineralocorticoid receptor (MR), estrogen receptor (ER) and GR (Schindler *et al.* 2003; Hapgood *et al.* 2013). Both MPA and NET-A have been reported in cell lines to act like agonists for transactivation via the AR (Ghatge *et al.* 2005; Sonneveld *et al.* 2006; Sasagawa *et al.* 2008; Africander *et al.* 2014). This is in agreement with reports that indicate that the disruption of AR action by progestins

may increase the risk of developing breast cancer since the balance between estrogen signalling and androgen signalling plays a critical role in breast homeostasis (Birrell *et al.* 2007). Table 1.1 summarizes the progestogens biological activity via the SRs.

Table 1.1 Biological activity of the progestogens via the SRs

Progestogen	PR	GR	MR	AR	ER
	Progestogenic	Glucocorticoid	Anti-mineralocorticoid	Androgenic	Estrogenic
P4	+	+ -	+	+ -	-
MPA	++	+	-	+ -	-
NET/NET-A/NET-EN	++	-	-	+	+

Taken and modified from Schindler *et al.* 2003 and Hapgood *et al.* 2013. Key to hormonal activities: -, not effective; +, effective; ++, strongly effective.

Recent reports have confirmed that both MPA and NET-A display similar binding affinities for the MR (Africander *et al.* 2013), however, they display no MR agonist activity and weak MR antagonist activity (Sasagawa *et al.* 2008; Africander *et al.* 2013). It is suggested that they are unlikely to exert significant effects via the MR at doses used in hormonal therapy (Africander *et al.* 2013). This lack of anti-mineralocorticoid activity is associated with increased risk of cardiovascular disease and blood pressure in progestin usage (Young *et al.* 1994; Delyani 2000). Table 1.2 illustrates the relative binding affinities (RBAs) of the progestogens for the SRs.

It has been reported that MPA has a higher RBA (DEX reference agonist = 100%) compared to NET-A and P4 for the human GR as illustrated in Table 1.2 (Koubovec *et al.* 2005; Hapgood *et al.* 2013). Although RBA does not necessarily reflect biological activity, it has been shown in cell lines that MPA acts as a partial to full agonist for both transactivation and

transrepression via the GR while P4 acts a partial agonist and NET-A as an antagonist (Koubovec *et al.* 2004; Koubovec *et al.* 2005; Ronacher *et al.* 2009; Hapgood *et al.* 2013). This is consistent with MPA having a greater GC potency than P4 and NET-A for transactivation and transrepression (Koubovec *et al.* 2005). Furthermore, it has been reported that GR levels dictate the behaviour of MPA in transrepression (Zhao *et al.* 2003). When GR density is high MPA behaves as a full agonist whereas when GR density is low, MPA acts as an antagonist (Zhao *et al.* 2003). The GR agonist activity of MPA has major implications for regulation of immune function and inflammation (Hapgood *et al.* 2004).

Table 1.2 Relative binding affinities (RBAs) % of the progestogens for the SRs

Progestogen	PR	GR	MR	AR	ER
P4	100	5.6	9 - 1000	3 -80	0.5
MPA	65 - 298	79	0.1 - 160	36	< 0.02
NET/NET-A/NET-EN	134	0.88 - 1.6	0 – 2.7	55	0.15

Taken and modified from Koubovec *et al.* 2005, Africander *et al.* 2011 and Hapgood *et al.* 2013. The reference radiolabelled ligands and ligands used for 100% RBA were as follows: PR, P4; GR, DEX; MR, aldosterone (ALD); AR, testosterone; ER, estradiol (E2).

1.9 The female genital tract

The mucosal barrier formed by the epithelial cells of the female genital tract (FGT) forms the first line of defence against the entry of pathogens (Shey *et al.* 2013). STIs, including HIV-1, have to breach this barrier to establish infection (Ferreira *et al.* 2014). The FGT can be divided into two major areas the upper and lower FGT. The upper FGT can be subdivided into the sterile uterus and uterine/fallopian tube, while the lower FGT consists of the sterile endocervix and non-sterile vagina and ectocervix (Fichorova *et al.* 1997; Quayle 2002; Trifonova *et al.* 2014) as illustrated in Figure 1.4. The mainly sterile endocervix is separated

from the ectocervix by the transformation zone. The endocervix is comprised of columnar epithelial cells with numerous glands which produce mucus, which can trap microbial pathogens and prevent the ascent of bacteria (Fichorova *et al.* 1997; Wira *et al.* 2014). In addition, the columnar epithelial cells have tight junctions in between them which provide barrier protection (Hickey *et al.* 2011). In contrast, the ectocervix and vagina consist of stratified squamous epithelial cells which produce a hydrophilic layer of glycoprotein called the glycocalyx, which provides moisture (Quayle 2002). The commensals such as *Lactobacillus* spp. which colonize the mucosa play a significant role in vaginal defence by maintaining an acidic micro-environment (Quayle 2002; Borges *et al.* 2014).

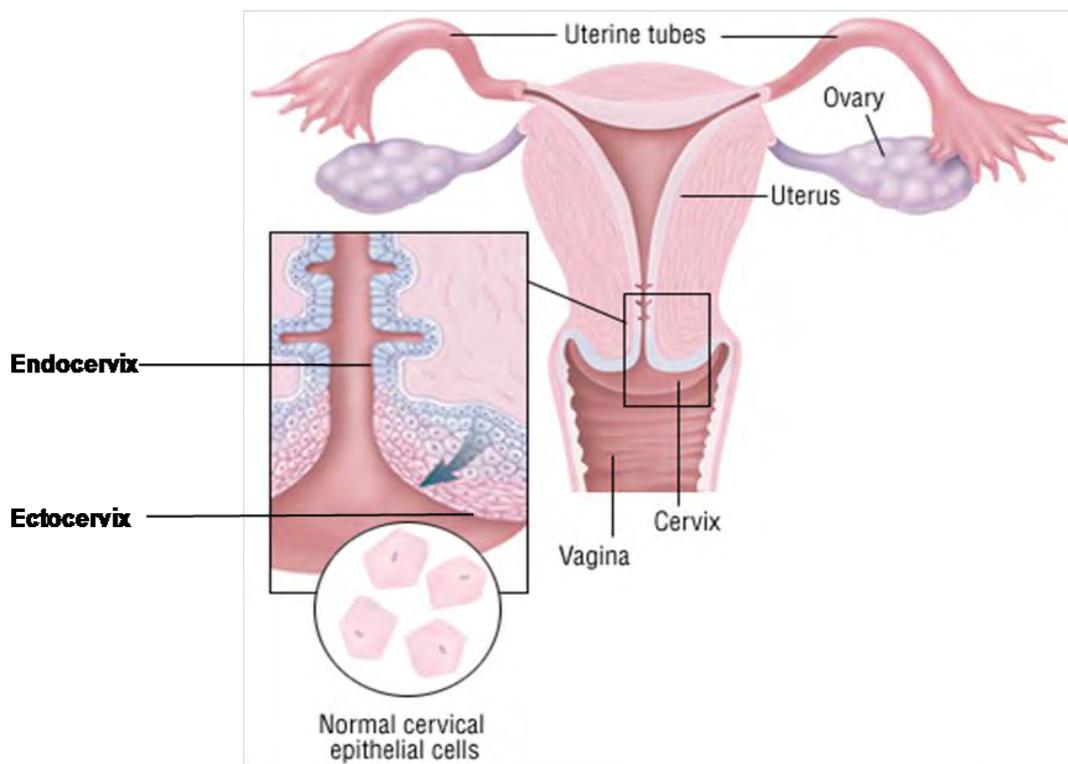


Figure 1.4: Schematic representation of the female genital tract. Taken and modified from All About Cervical Cancer 2012.

The FGT is capable of both innate and acquired immune responses which are highly regulated by cyclic changes in the sex hormones estradiol (E2) and P4 (Beagley & Gockel 2003). Innate FGT immunity involves mostly macrophages, neutrophils and dendritic cells which are important cells that phagocytose and subsequently kill pathogens through acidic and enzymatic digestion (Hickey *et al.* 2011; Shey *et al.* 2013; Wira *et al.* 2014). Innate immunity is also assisted by the epithelial cells which produce anti-microbial peptides such as the defensins (α and β) (Hickey *et al.* 2011). Acquired immunity entails pathogen-specific defence responses and involves the recruitment of CD4⁺ T-cells, CD8⁺ T-cells and B-cells by antigen-presenting dendritic cells (Hickey *et al.* 2011; Shanmugasundaram *et al.* 2014). Constitutive expression of cytokines and chemokines ensure the presence and activity of immune cells immediately on antigen presentation (Wira *et al.* 2005).

1.10 The role of MPA in inflammation, cervical mucosal immunity and HIV-1 pathogenesis

Lines of evidence have shown that MPA plays a role in inflammation and it has been suggested that the GR may be involved in mediating these actions (Hapgood *et al.* 2004; Hapgood 2013; Huijbregts *et al.* 2013). It has been shown in numerous *in vitro* (Bamberger *et al.* 1999; Mantovani *et al.* 2000; Kurebayashi *et al.* 2003; Elovitz & Wang 2004; Koubovec *et al.* 2004; Huijbregts *et al.* 2013) and animal *in vivo* (Trunova *et al.* 2006; Kleynhans *et al.* 2013) studies over the years that MPA has anti-inflammatory activity. For example, it has been reported in cell lines that MPA, unlike P4, represses IL-2, IL-6 and IL-8 synthetic promoter reporter genes and endogenous IL-2 and IL-6 protein expression (Bamberger *et al.* 1999; Koubovec *et al.* 2004). Furthermore, it was determined in the KTC-2 (thyroid cancer) cell line and PBMCs that MPA represses IL-6 mRNA and IL-6 protein, respectively (Bamberger *et al.* 1999; Kurebayashi *et al.* 2003). Reports also show that in PBMCs that

MPA suppressed phytohemagglutinin-induced IL-6, IL-1 β and TNF- α . It has also been reported that MPA suppresses cytokines IL-1 α , IL-12 p40, IL-10, IL-13, TNF- α and IFN- γ in human PBMCs and mouse models infected with *Mycobacterium tuberculosis* (Kleynhans *et al.* 2011; Kleynhans *et al.* 2013). Similarly, Hapgood *et al.* found in PBMCs that MPA suppresses pro-inflammatory mediators IL-6 and IL-8 (Hapgood *et al.* 2014). In addition, MPA suppresses cellular immune responses in simian-human immunodeficiency virus (SHIV)-infected rhesus macaques (Trunova *et al.* 2006). Consistent with this, a recent study has found that in both PBMC from non-infected and HIV-1 infected individuals, MPA significantly represses the levels of most cytokine and chemokine proteins, such as TNF- α , IFN- γ and IL-12 (Huijbregts *et al.* 2013). Furthermore, MPA has been shown to increase apoptosis of T-cells, which is enhanced after HIV-1 infection, potentially decreasing the ability of T-cells to mount an anti-viral defence (Tomasichio *et al.* 2013).

In addition to cellular immunity, evidence also shows that MPA plays a role in regulating cervical mucosal immunity and thereby influences susceptibility and disease predisposition to many genital tract infections (Mestecky *et al.* 2009; Kaushic, Roth, *et al.* 2011). As mentioned earlier, the mucosal environment of the FGT is comprised of the vaginal, ectocervical and endocervical regions and serves as the entry point for STI's (Fichorova *et al.* 1997; Rodriguez-Garcia *et al.* 2013). These regions display different immunological profiles as they constitutively express a distinct array of cytokines (IL-1, IL-6 and TNF- α) and chemokines (IL-8 and RANTES) with the endocervical cells being more active in cytokine secretion than the vaginal and ectocervical cells (Fichorova & Anderson 1999; Fichorova *et al.* 2001; Rodriguez-Garcia *et al.* 2013). Reports show that MPA treatment of mice was found to increase their susceptibility to genital herpes simplex virus type 2 (HSV-2) infection (Kaushic *et al.* 2003). Consistent with this, MPA-usage has been found to be associated with increased acquisition of cervical chlamydial and gonococcal infections (Baeten *et al.* 2001;

Morrison *et al.* 2004). Clinical reports also show that the use of MPA in HIV-1 seropositive patients is associated with an increased risk of genital ulcer disease (Lavreys, Baeten, Kreiss, *et al.* 2004). Evidence also suggests that increased shedding of HIV-1 infected cells from the cervix and vagina is associated with MPA usage (Mostad *et al.* 1997). This is consistent with findings that show a modest but significant increase in cervical shedding of HIV-1 DNA detected with MPA usage (Wang *et al.* 2004).

The risk associated with HIV-1 acquisition and MPA usage is controversial. However, it has been reported that there is an increase in risk of both HIV-1 acquisition and transmission with the injectable hormonal contraceptive (Martin *et al.* 1998; Lavreys, Baeten, Martin, *et al.* 2004; Baeten *et al.* 2007; Heffron *et al.* 2012). In contrast, some findings show no association between MPA-usage and HIV-1 acquisition (Morrison *et al.* 2007; Myer *et al.* 2007; Morrison *et al.* 2012). Notably, although the Morrison *et al.* study found no significant increase in the risk of HIV-1 acquisition they do mention that the effect estimate of their study does not rule out a moderate increase in HIV risk associated with DMPA use found in other studies (Morrison *et al.* 2012). There are several proposed mechanisms for increased HIV-1 susceptibility with MPA (Murphy *et al.* 2014). As reviewed in Murphy *et al.* possible mechanisms include thinning or disruption of the cervicovaginal epithelial barrier and/or alterations in the vaginal microbiome (Murphy *et al.* 2014). Some reports also suggest that MPA increases susceptibility by suppressing pro-inflammatory mediators and thereby lowering host resistance to HIV-1 and invading pathogens (Hapgood 2013; Huijbregts *et al.* 2013) while others associate MPA-usage with an increase in pro-inflammatory markers and thereby an increase in the recruitment of HIV-1 target cells (Morrison, Fichorova, *et al.* 2014). For example, the Gillgrass *et al.* study found longer treatment (15 days) with MPA failed to protect mice from subsequent HSV-2 challenge due to a decrease in both innate and adaptive immune responses (Gillgrass *et al.* 2003). This is consistent with the Huijbregts *et*

al. study that investigated the effects of MPA on vaginal mucosal mononuclear cells (VMMCs) isolated from human vaginal tissue and showed immunosuppressive effects for MPA on IFN- γ (Huijbregts *et al.* 2013). This group also showed that there are lower IFN- γ levels in cervico-vaginal lavage (CVL) samples from individuals on MPA (Huijbregts *et al.* 2013). In contrast to this, a more recent study by the Morrison group has shown that MPA-usage is associated with higher pro-inflammatory cytokines and chemokines such as RANTES in CVL samples (Morrison, Fichorova, *et al.* 2014).

In summary, although there have been conflicting reports on whether MPA increases HIV-1 acquisition or not, the general consensus is that MPA does play a role in regulating cervical mucosal immunity and this increases susceptibility to genital tract infections such as HSV-2 and possibly HIV-1. In addition, the mechanism of the MPA-mediated regulation of cervical mucosal immunity is unclear.

Chapter 2: Thesis rationale, Hypotheses & Aims

2.1 Thesis rationale

The usage of injectable contraceptives is very high in some areas of South Africa, such as at the Kwazulu-Natal site for the CAPRISA microbicide trial, where about 82% of the women investigated were on injectable progestin-only contraceptives (DMPA and NET-EN), as compared to 15% on oral contraceptives (Abdool Karim *et al.* 2010). Most, but not all, high quality observational clinical studies report that injectable DMPA usage, increases risk of HIV-1 acquisition (Heffron *et al.* 2012; Wand & Ramjee 2012; Crook *et al.* 2014; Morrison, Chen, *et al.* 2014; Polis *et al.* 2014). However, whether or not injectable contraceptives increase susceptibility to heterosexual transmission of HIV in developing countries is still controversial (Morrison *et al.* 2012; McCoy *et al.* 2013; Jones 2014; Polis *et al.* 2014; Rees 2014; Westhoff & Winikoff 2014). A recent systematic review of epidemiological evidence showed most studies suggested no significantly increased HIV risk with NET-EN. However, increased HIV risk with NET-EN in some studies tended to be larger than for DMPA, though 95% confidence intervals overlapped substantially (Polis *et al.* 2014). However, the World Health Organisation (WHO) 2014 guidance statement does not recommend discontinuation of DMPA usage but they do recommend that women on DMPA should use condoms (WHO 2014). Following this report there has been raging debate on whether randomized clinical trials are needed to conclusively determine whether DMPA increases the risk of HIV-1 acquisition or whether sufficient evidence already exists on the increased HIV risk with DMPA (Jones 2014; Rees 2014; Westhoff & Winikoff 2014).

The mucosa of the lower FGT which consists of the vaginal, ectocervical and endocervical compartments is the first line of defence against pathogens (Fichorova *et al.* 1997). Although the epithelial cells of the FGT do not get productively infected by HIV-1, they express a wide

variety of immune mediators aiding in both innate and adaptive immunity (Kaushic *et al.* 2010; Kaushic *et al.* 2011 b; Rodriguez-Garcia *et al.* 2013). There have been conflicting reports on whether MPA increases or decreases inflammation in the FGT (Huijbregts *et al.* 2013; Morrison, Fichorova, *et al.* 2014). A question which remains unanswered is whether doses of MPA used in injectable contraception regulate inflammation of cervical mucosal immunity and whether this increases host susceptibility to HIV-1 and STIs. In addition, the mechanism of MPA-mediated inflammatory regulation in the lower FGT is unclear. It is crucial to understand the molecular mechanisms of hormonal contraceptives such as MPA since it is a factor that influences inflammation and disease susceptibility of the lower FGT.

The off-target anti-inflammatory effects of MPA have been proposed to be mediated via the GR (Hapgood *et al.* 2004; Koubovec *et al.* 2004; Koubovec *et al.* 2005). The study by Koubovec *et al.* was the first to show by dose-response analysis of GRE- and NF- κ B/AP-1 regulated reporter genes that MPA, unlike NET-A, acts as a partial agonist for the GR in the human lung cancer (A549) cell line (Koubovec *et al.* 2005). However, it is uncertain whether the effects of MPA are mediated by the GR in any of the three compartments of the lower FGT. Although a recent study suggested that MPA-mediated anti-inflammatory effects in VMHCs are via the GR by antagonist experiments using RU-486, a GR and PR antagonist (Huijbregts *et al.* 2013), there have been no studies showing proof of direct GR mechanism of action by MPA in the FGT. In addition, there have been no studies showing the precise mechanism of GR-mediated gene regulation in response to MPA in the FGT.

The role of MPA in modulation of HIV-1 pathogenesis has been proposed to involve not only the GR but also HIV-1 Vpr (Hapgood & Tomasicchio 2010). It has been previously reported that the virion associated HIV-1 protein, Vpr, is immunosuppressive and possibly interacts with the GR (Ayyavoo, Mahboubi, *et al.* 1997; Mirani *et al.* 2002). Evidence suggests that Vpr may act as a co-activator of the GR in the regulation of reporter genes in the presence of

DEX (Kino *et al.* 1999; Sherman *et al.* 2000). There are limited data on whether Vpr and GR interact to regulate endogenous inflammatory genes in the presence of DEX (Fakruddin & Laurence 2005). Additionally, since the GR has been shown to be activated by MPA and P4, it is possible that Vpr may also modulate progestin-mediated inflammatory gene regulation.

The off-target effects of MPA mediated by the GR may not only be important in immune function regulation but also for viral gene expression in the FGT in response to HIV-1. In addition to its role in inflammatory gene regulation, the GR has been reported to be involved in the regulation of HIV-1 LTR transcription in the presence of DEX (Kino *et al.* 2000; Sawaya *et al.* 2000). However, the direct role of the GR in this DEX-mediated modulation has not been shown. In addition, it has been shown that P4 represses Tat-activated HIV-1 LTR transcription (Lee *et al.* 1997). However, there are no data showing the effect of synthetic progestins on HIV-1 LTR regulation. Vpr has previously been shown to enhance Tat-activated HIV-1 LTR transcription (Kino *et al.* 2002). Considering Vpr may act as co-activator of the GR in the regulation of inflammatory genes, it is proposed that Vpr may play a role in GR-mediated LTR transcription in the presence of DEX and the progestogens.

2.2 Hypotheses, Aims and Strategies

From the literature it is clear that the progestins MPA and NET-A differ from each other and to that of the natural hormone P4 in their off-target biological activity via SRs other than the PR. In particular MPA appears to be immunosuppressive, unlike NET-A and P4. However, little is known about the molecular mechanism of action of MPA and NET-A in the cervical mucosa. To this end it was hypothesized that **MPA and NET-A would differentially regulate inflammatory gene expression in the cervical mucosa**. In addition, it was hypothesized that the **MPA-mediated gene regulation occurs via the GR**. Thus in the present study, the regulation of inflammatory genes GILZ, I κ B α , IL-6, IL-8 and RANTES by

MPA, NET-A and P4 was investigated in cell models for cervical mucosa. Dose response analysis, time course experiments, SR screening, GR knockdown, *de novo* protein synthesis inhibition and chromatin immunoprecipitation (ChIP) assays were performed to assess mechanism of action. The host genes (GILZ, I κ B α , IL-6, IL-8 and RANTES) investigated in this thesis were chosen based on the key roles they play in mediating inflammatory actions and because they have been previously shown to be regulated in a GR-dependent manner (Auphan *et al.* 1995; Newton & Holden 2007; Hadley *et al.* 2011; Verhoog *et al.* 2011) whereby GILZ and I κ B α are examples of GR transactivation model genes and IL-6, IL-8 and RANTES are examples of transrepression model genes.

The epithelial cells of the endocervix constitutively express IL-6, IL-8, and RANTES genes (Fichorova & Anderson 1999), with the endocervical cells being more active in cytokine secretion than the ectocervical cells (Fichorova *et al.* 2001). Thus, the End1/E6E7 endocervical cell line was chosen as the model to investigate inflammatory gene regulation. In addition, this cell line displays similar morphological and immunocytochemical properties to those of primary endocervical epithelial cells (Fichorova *et al.* 1997). The experiments in this thesis were performed in the absence of induction of the cytokine/chemokine genes with a pro-inflammatory ligand since these genes are constitutively expressed in cervical epithelial cells (Fichorova & Anderson 1999). Ligand concentrations for DEX (100 nM), P4 (10 μ M), MPA (1 μ M) and NET-A (10 μ M) were calculated to result in GR saturation based on the K_D 's and were used for some experiments, unless otherwise indicated (Kontula *et al.* 1983; Ronacher *et al.* 2009).

Literature also suggests that the GR may play a key role in regulating immune function and HIV-1 pathogenesis via interaction with Vpr. It was therefore hypothesized that **Vpr acts a co-activator of the GR and results in the modulation of GR-regulated inflammatory genes in the presence of DEX, P4 and the synthetic progestins.** Thus in the present study,

the effect of Vpr on the regulation of GR-regulated reporter gene and endogenous inflammatory gene expression in the presence of the ligands was investigated. The Vpr study was done in the HeLa cervical epithelial and TZM-bl (modified HeLa cell line that expresses CD4, CXCR4 and CCR5 and contains a stably integrated HIV-1 LTR promoter) cell lines. Two different strategies were used to deliver Vpr into the cervical epithelial cells. The first was the use of a HA-tagged Vpr expression vector and the second was the use of HIV-1 pseudovirus to deliver physiological amounts of Vpr protein and is thus a more physiological delivery method.

It was also hypothesized that the **GR and/or Vpr regulate HIV-LTR transcription in the presence of DEX, P4 and the synthetic progestins**. This hypothesis was investigated by examining the role of Vpr in the regulation of the LTR in the presence of the ligands. In addition, the role of GR in LTR transcription in the presence of the ligands was investigated by GR knockdown. LTR luciferase reporter gene assays were done in the HeLa and TZM-bl cell lines to assess regulation of LTR transcription. The TZM-bl cells contain a stably integrated HIV-1 LTR-luc gene while for the HeLa cells; an HIV-1 LTR-luc expression vector was used.

The current study chose cell line models to represent cervical epithelial cells. The author is well aware of the limitations of cell line models; however, choice of model is very question dependent and cell lines can be a useful tool to perform biochemical experiments to answer questions about direct effects and mechanism of action. This was the case in this study, where questions regarding the role of the GR, Vpr and progestogens on host inflammatory and viral gene regulation were investigated.

Chapter 3: Material and Methods

3.1 Compounds and antibodies

The following primary antibodies were obtained from Santa Cruz Biotechnology, USA; GR (H-300; sc-8992), PR (C-20; sc-539), AR (441; sc-7305), ER α (MC-20; sc-542) and MR (MCR, H-300; sc-11412) and GAPDH (0411; sc-47724). The HA (12CA5) antibody was obtained from Roche Applied Science, South Africa. The flotillin-1 (610820) antibody was purchased from BD Transduction Laboratories (USA). The following secondary antibodies were obtained from Santa Cruz Biotechnology inc., USA; anti-mouse: sc-2005 and anti-rabbit: sc-2313. Ampicillin was obtained from Sigma-Aldrich, South Africa. The ligands dexamethasone (DEX), 6 α -Methyl-17 α -hydroxyprogesterone acetate (MPA), progesterone (P4), norethisterone acetate (NET-A), norethisterone (NET), promegestone (R5020), aldosterone (ALD), mibolerone (MIB) and estradiol (E2) were obtained from Sigma-Aldrich, South Africa. The hormone ligands were reconstituted in 100% EtOH as recommended in the specification sheets provided by Sigma-Aldrich, South Africa. It is also worth noting that hormone incubations were performed by using 0.1 % v/v reconstituted hormone or vehicle control. Tumor Necrosis Factor- α (TNF- α) was purchased from Merck chemicals, South Africa. Cycloheximide (CHX) was purchased from Sigma-Aldrich, South Africa.

3.2 Plasmids

The pTAT.GRE-E1b-luc (TAT-GRE) luciferase reporter gene plasmid is controlled by the inducible E1b promoter and contains two glucocorticoid response elements (GRE) from the rat tyrosine aminotransferase (TAT) gene, and was a gift from Dr. G. Jenster (Erasmus University of Rotterdam, Rotterdam, Netherlands) and has been described before (Sui *et al.* 1999). The pcDNA3 (empty vector) plasmid contains a CMV promoter in the pcDNA vector with no

inserted downstream DNA sequence and was obtained from Invitrogen, UK. The SR plasmid pcDNA3-hGR (GR) constitutively expresses the full length and untagged human GR α isoform cloned into the pcDNA3 vector and was a kind gift from Prof. D.W. Ray (University of Manchester, UK) and previously described (Ray *et al.* 1999). The pMT-PR-B (PR) plasmid constitutively expresses the full length and untagged human PR-B isoform cloned into the pMT vector and was obtained from Prof. S. Okret (Karolinka Institute, Sweden). The pRS-hMR (MR) plasmid constitutively expresses the full length and untagged human MR cloned into the pRS vector and was obtained from Prof. R.M. Evans (University of California, USA) and has been described previously (Arriza *et al.* 1987). The pSV-hAR (AR) plasmid was a gift from Prof. F. Claessens (Catholic University of Leuven, Belgium) and constitutively expresses the full length and untagged human AR cloned into the pSV vector and previously described (Brinkmann *et al.* 1999). The pSG5-hER (ER) plasmid constitutively expresses the full length and untagged human ER cloned into the pSG5 vector and was obtained from Prof. F. Gannon (European Molecular Biology Laboratory, Germany) and previously described (Zhou & Chen 2001). The ERE-luc luciferase reporter gene plasmid contains the estrogen response element (ERE) and was also obtained from Prof. S. Okret (Karolinka Institute, Sweden). The pCMV4-3HA-Vpr (HA-Vpr) plasmid expresses a HA tagged variant of Vpr protein obtained from the HIV-1 type B NL4-3 clone and is under control of a constitutive CMV promoter in the CMV4 vector plasmid. It was obtained from Dr. W. Greene (University of California, San Francisco) and has been described previously (Sherman *et al.* 2000). The pSVtat72 (Tat) plasmid expresses Tat protein (residues 1-72) and is controlled by the SV40 early promoter (Frankel & Pabo 1988). The pBlue3'LTR-luc (HIV-1 LTR-luc) luciferase reporter gene plasmid expresses LTR for HIV-1 subtypes A, C, D, E, F, and G (Jeeninga *et al.* 2000). Both the Tat and HIV-1 LTR-luc plasmids were obtained from NIH AIDS Research and Reference Reagent Program (Germantown, MD, USA). The p1168hu-IL-6P-luc (IL-6 full length

reporter) reporter gene plasmid expresses the full length human IL-6 promoter while the p(IL-6 κ B)3-50hu.IL-6P-luc (IL-6 minimal reporter) reporter gene plasmid is a derivative of the IL-6 full length reporter plasmid and only contains three motifs of the NF κ B binding sites. Both the IL-6 full length reporter and IL-6 minimal reporter plasmids were obtained from Prof. G. Haegeman (University of Ghent, Belgium) (Plaisance *et al.* 1997). The following pseudovirus constructs pDU151A (encoding the HIV-1 envelope (*env*) which displays R5 tropism) (Williamson *et al.* 2003), pSg3E⁻ (containing the HIV-1 genomic sequences with a mutated *env* gene) (Andjelic *et al.* 2008), pLET-LAI (encoding the HIV-1 *env* which displays X4 tropism), pNL4-3.E⁻ (containing the HIV-1 genomic sequences with a mutated *env* gene) and pNL4-3.E^R (containing the genomic sequences with a mutated *env* and *vpr* gene) were obtained from the NIH AIDS Research and Reference Reagent program.

3.3 Plasmid transformation and preparation

Plasmids were prepared by transformation into competent *Escherichia coli* DH5 α bacterial cells. This was done as described in Sambrook *et al.* but briefly, to 100 μ l of competent cells 50 ng plasmid DNA was added and mixed, without vortexing (Sambrook *et al.* 1989). The mixture was then incubated on ice for 20 mins, heat shocked for 45 secs at 42°C and placed back on ice for 5 mins. Thereafter 1 ml SOC medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 20 mM glucose] was added and then placed at 37°C for 60 mins, shaking. Then, 200 μ l of the mixture was plated out on a LB-AMP selection plate [1% (w/v) tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agarose containing 50 μ g/ml ampicillin]. The remainder was centrifuged, the supernatant was discarded, while the pellet was resuspended in 200 μ l SOC and plated on a selection plate. The selection plates were incubated overnight at 37°C. A day culture was prepared by inoculating a bacterial colony in 5 ml LB medium [1% (w/v) tryptone, 0.5% yeast extract and

1% NaCl] with ampicillin (100 ng/μl), and placed at 37°C. After approximately 8 hrs, 1 ml of the day culture was inoculated in 100 ml LB with ampicillin (100 ng/μl) and placed at 37°C overnight for preparation of plasmid DNA. The DNA was purified using the Promega® Midiprep system catalogue no. A2492 (Promega, USA) following manufacturer's protocol. The purified DNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies).

3.4 Restriction enzyme digest

Restriction enzyme digests of plasmid DNA were performed to ensure the correct plasmid was purified and that the plasmid was in the proper conformation (i.e. supercoiled). A standard 50 μl reaction consisting of 1 unit enzyme, 5 μl (10X) enzyme buffers, 300 ng DNA and distilled water was set up for each digest. The control (undigested) reaction for each plasmid contained only 300 ng DNA and distilled water. After a reaction time of 1 hr at 37°C, the RE digest samples were electrophorated on a 10 μg/ml ethidium bromide (EtBr) stained 0.8% agarose gel in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8) at 90 V for 45 mins and visualized under short wavelength ultraviolet light using a Syngene, G:Box (Vacutec).

3.5 Cell culture

Human epithelial cervical cancer cells (HeLa), monkey kidney fibroblast cells (COS-1) and human embryonic kidney cells (HEK293T) were purchased from the American Type Culture Collection (ATCC, USA). TZM-bl (HeLa cells modified to contain a stably integrated HIV-1 LTR-luc and express CD4, CXCR4 and CCR5) cells were obtained from the NIH AIDS Research and Reference Reagent program. The above mentioned cell lines were cultured in 75 cm² flasks (Greiner Bio-one International, Austria) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 10% (v/v) fetal calf

serum (FCS) (Highveld Biological, South Africa) 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen, UK).

Human endocervical cells immortalized with human papillomavirus 16 E6/E7 (End1/E6E7) (Fichorova *et al.* 1997) were obtained from Dr Fichorova, Brigham & Women's Hospital, Boston, USA. The End1/E6E7 cells were cultured in 175 cm² flasks (Greiner Bio-one International, Austria) in keratinocyte serum-free medium (ker-sfm) (Sigma-Aldrich, South Africa) supplemented with the provided keratinocyte growth supplement, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen, UK).

All cells were maintained at 37°C in a 5% CO₂ incubator. Cells were passaged with 0.25% trypsin/0.1% EDTA in PBS (Highveld Biological, South Africa). Trypsinization was terminated with neutralization medium [DMEM (Sigma-Aldrich, South Africa), 10% (v/v) calf serum (Highveld Biological, South Africa), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen, UK)]. The cell lines were regularly tested for mycoplasma infection by means of Hoechst staining (Freshney 1987), and only mycoplasma-negative cells were used in experiments.

3.6 Reporter assays

3.6.1 SRE promoter-reporter assay

HeLa cells or End1/E6E7 cells were seeded into 24-well plates (Greiner Bio-one, Cellstar) at a density of 7.5×10^4 cells/well or 17.5×10^4 cells/well, respectively and incubated for 24 hrs.

For the Vpr overexpression assays, the HeLa cells were transiently transfected with 82 ng/well TAT-GRE and 125 ng/well HA-Vpr or empty vector. **For the Vpr dose assays**, the HeLa cells were transiently transfected with an increasing dose (25 ng, 125 ng and 300 ng)/well of HA-Vpr. **For the assays with the overexpressed SRs**, both cell lines were transfected with 82 ng/well TAT-GRE or 250 ng/well ERE-luc and 125 ng/well of either GR,

PR, AR, ER or MR expression vectors. For all assays the cells were transiently transfected using FuGENE™ 6 (Roche Applied Science, South Africa) according to manufacturer's instructions and incubated for 24 hrs. On day three, cells were washed once with PBS and were treated with the respective compounds or vehicle (EtOH) in serum free DMEM medium for 24 hours. The cells were harvested by washing twice with PBS and lysed in 50 µl 1X Reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity in the lysates was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). The luciferase values obtained were normalized to protein content per well as determined by standard Bradford assay (Bradford 1976). It is important to note that prior to performing the reporter assays above and below, transfection conditions were optimized for both cell density and DNA amount. Cells were seeded to obtain an optimal confluency of 60% - 70% for transfection. DNA concentration was optimized for each reporter plasmid by adding increasing amounts of plasmid DNA (15 ng, 47 ng, 82 ng, 125 ng and 250 ng)/well and assessing for optimal reporter activity.

3.6.2 HIV-1 LTR promoter-reporter assay

HeLa cells were seeded into 24-well plates (Greiner Bio-one, Cellstar) at a density of 7.5×10^4 cells/well. The next day, the cells were transiently transfected with 47 ng/well HIV-1 LTR-luc, 125 ng/well Tat and 125 ng/well Vpr or empty vector. The cells were transfected using FuGENE™ 6 (Roche Applied Science, South Africa) according to manufacturer's instructions and incubated for 24 hrs. On day three, the cells were washed once with PBS and were treated with the respective compounds or vehicle (EtOH) in serum free DMEM medium for 24 hours. The cells were harvested by washing twice with PBS and lysed in 50µl 1X Reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity in the lysates was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus

microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). The luciferase values obtained were normalized to protein content per well as determined by standard Bradford assay (Bradford 1976).

3.6.3 IL-6 full length and minimal promoter-reporter assays

HeLa cells were seeded into 24-well plates (Greiner Bio-one, Cellstar) at a density of 7.5×10^4 cells/well. The next day, the cells were transiently transfected with 250 ng/well IL-6 full length reporter plasmid or IL-6 minimal reporter plasmid. The cells were transfected using FuGENE™ 6 (Roche Applied Science, South Africa) according to manufacturer's instructions and incubated for 24 hrs. On day three, cells were washed once with PBS and were treated with the respective compounds or vehicle (EtOH) in serum free DMEM medium for 24 hours. The cells were harvested by washing twice with PBS and lysed in 50 μ l 1X Reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity in the lysates was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). The luciferase values obtained were normalized to protein content per well as determined by standard Bradford assay (Bradford 1976).

3.7 Western blotting

3.7.1 Preparation of protein control samples

For the SR controls, COS-1 cells were seeded into 12-well plates (Greiner Bio-one, Cellstar) at a density of 25×10^4 cells/well. The next day the cells were transfected with 1 μ g/well of GR, AR, PR or empty vector and 2 μ g/well of MR or ER α using FuGENE™ 6 (Roche Applied Science, South Africa). After 24 hrs, the cells were washed once with PBS and lysed with 50 μ l 2X SDS sample buffer (diluted from 5X SDS sample buffer: 100 mM Tris-Cl pH

6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% β -mercaptoethanol and 0.1% (w/v) bromophenol-blue) and boiled for 10 min at 100°C.

3.7.2 Preparation of samples from the reporter assays

For the reporter experiments, after the transactivation and Bradford assays were done, 7.5 μ l of 5X SDS sample buffer was added to the 30 μ l HeLa cell lysates and boiled for 10 min at 100°C.

3.7.3 Preparation of samples for other applications

HeLa and End1/E6E7 cells were plated into 12-well plates at different amounts of cells/well, as indicated in the figure legends and treated with test compound or vehicle (EtOH), as indicated in the figure legends. After ligand stimulation the cells were washed twice with PBS and harvested in 50 μ l 2X SDS sample buffer and boiled 10 min at 100°C.

3.7.4 Western blot analysis and quantification

Equal amounts of sample were loaded and resolved on a 6%, 8% or 10% SDS polyacrylamide gel (250 mM Tris-Cl pH 8, 0.1 (w/v) SDS, 0.1% (w/v) APS and 6%, 8% or 10% (v/v) acrylamide) in running buffer (25 mM Tris-Cl pH 8; 250 mM glycine and 0.1% (w/v) SDS). The protein molecular weights were estimated using the PAGERuler™ Prestained protein ladder SM0671 (Fermentas Life Sciences). The separated proteins were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham, South Africa) in transfer buffer (25 mM Tris, 250 mM glycine and 20% (v/v) methanol) using the Mini Protean III blotting system (Bio-Rad, South Africa) for 1 hr at 0.18 A. Membranes were blocked in 4% ECL blocking buffer [4% (w/v) ECL blocking powder (Amersham, South Africa) and 1X TBST (0.1% (v/v) Tween in 1X TBS)] for 1 hr at room temperature, followed by incubation with primary antibody in 4% ECL blocking buffer overnight at 4°C. Membranes were then washed twice with 1X TBST for 10 mins and 5 mins, followed by incubation with the

appropriate secondary antibody in 5% fat-free milk powder buffer [5% (w/v) fat-free milk powder and 1X TBST] for 1 hr at room temperature. Thereafter the membranes were washed twice with 1X TBST for 10 mins and 5 mins and exposed to X-ray film. For antibody dilution see Table 3.1 below. The proteins were visualized using Pierce® ECL western blotting substrate (Thermo Scientific, South Africa), according to the manufacturer's protocol, and Hyperfilm MP high performance autoradiography film (Amersham, South Africa). Bands on the X-ray film were scanned and quantification was done using AlphaEaseFC FluorChem 5500 (Alpha Innotech).

Table 3.1: Antibody concentrations used in Western blot analysis

Primary Antibody	Dilution
GR (H-300)*	1:4000
PR (C-20)*	1:1000
AR (441)**	1:1000
ER α (MC-20)**	1:1000
MR (MCR, H-300)*	1:1500
HA (12CA5)**	1:2000
GAPDH **	1:20 000

Secondary antibodies are indicated by * and ** for anti-rabbit (1:10 000) and anti-mouse (1:2000), respectively.

3.8 RNA isolation and cDNA synthesis

HeLa or End1/E6E7 cells were seeded into 12-well plates at a density of 15×10^4 cells/well and 35×10^4 cells/well, respectively for 24 hrs and treated with test compound or vehicle (EtOH) for 4 hrs or 24 hrs. For the Vpr overexpression studies, prior to treatment with test compounds, the HeLa cells were transiently transfected with 1 μ g HA-Vpr or an increasing dose (50 ng, 250 ng, 600 ng and 1 μ g)/well HA-Vpr using FuGENE™ 6 (Roche Applied

Science, South Africa) according to manufacturer's instructions and incubated for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested. Total RNA was isolated from cells using TRI Reagent® (Sigma-Aldrich, South Africa) according to the manufacturer's instructions. The isolated RNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). The integrity of the RNA (presence of intact 18S and 28S ribosomal bands) was confirmed by denaturing agarose gel electrophoresis. Briefly, 15 µl sample loading buffer [12% (v/v) DEPC water, 5% (v/v) bromophenol blue solution, 7% (v/v) glycerol, 10% (v/v) 10X MOPS buffer (0.2 M MOPS in DEPC water, 0.05 M CH₃COONa and 0.01 M EDTA), 17% (v/v) 12.3 M formaldehyde and 49% (v/v) formamide] with 20 µg/ml EtBr was added to 0.5 µg of total RNA of each sample, then electrophorated on a 1% agarose gel [1% (w/v) agarose, 70% (v/v) DEPC water, 10% (v/v) 10X MOPS buffer and 20% (v/v) formaldehyde] at 65 V for 40 mins. Total RNA (500 ng) was reverse transcribed using the anchored oligo-dT method provided in the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, South Africa) according to the manufacturer's instructions.

3.9 Real-time quantitative reverse transcription PCR (qRT-PCR)

Equal volumes of synthesised cDNA were used for real-time qRT-PCR using the Sensi-Mix SYBR Green I system (Celtic Diagnostics, South Africa) and the Rotor-gene, RG-3000A (Corbett Research). For the inflammatory gene expression analysis; IκBα, IL-6, IL-8, and RANTES gene expression were measured using mRNA specific primer sets (Table 3.2) at a final concentration of 0.25 µM. GILZ gene expression were measured using QuantiTect Primer Assay (catalogue no. QT00091035) a validated primer set purchased from Qiagen, South Africa at a final concentration of 1X assay solution. GAPDH (Table 3.2) which served as the 'housekeeping gene' was used at a final concentration of 0.1 µM. Briefly, the 20 µl

PCR reaction mix contained 10 μ l Sensi-Mix SYBR Green I system (Celtic Diagnostics, South Africa), 1 μ l cDNA, 1 μ l sense primer, 1 μ l anti-sense primer and 7 μ l PCR grade water. Melting curve analysis and gel electrophoresis was performed to confirm the generated amplicon in each sample. Standard curves were used to determine the efficiency of each primer set and relative transcript levels were calculated by the method described by Pfaffl and was normalized to relative GAPDH transcript levels (Pfaffl 2001). GAPDH has been shown by others in the laboratory to be a suitable housekeeping gene in these cells since it is not regulated by the hormones tested.

Table 3.2: Primer sequences of inflammatory genes investigated

Gene	Primer Sequence (5' - 3')	Strand	Annealing Temp. (°C)	Reference
GILZ	Qiagen Proprietary Information	forward	60	Qiagen, South Africa
	Qiagen Proprietary Information	reverse		
IkB α	ACTCGTTCCTGCACTTGGCC	forward	60	Emmerich <i>et al.</i> 2011
	TGCTCACAGGCAAGGTGTAG	reverse		
IL-6	TCTCCACAAGCGCCTTCG	forward	60	Wolf <i>et al.</i> 2002
	CTCAGGGCTGAGATGCCG	reverse		
IL-8	TGCCAAGGAGTGCTAAAG	forward	60	Wolf <i>et al.</i> 2002
	TGCCAAGGAGTGCTAAAG	reverse		
RANTES	TACCATGAAGGTCTCCGC	forward	60	Wolf <i>et al.</i> 2002
	GACAAAGACGACTGCTGG	reverse		
GAPDH	TGAACGGGAAGCTCACTGG	forward	55	Ishibashi <i>et al.</i> 2003
	TCCACCACCCTGTTGCTGTA	reverse		

For the SR mRNA screen; GR, PR-AB, AR, ER α and MR specific primer sets (Table 3.3) were used at a final concentration of 0.25 μ M. 20 μ l PCR reaction mixes were set up (as described above) followed by gel electrophoresis to confirm the generated amplicon in each sample. 10 ng SR plasmid DNA was used as PCR positive controls.

Table 3.3: Primer sequences of the SRs

Gene	Primer Sequence (5'-3')	Strand	Annealing Temp. (°C)	Product size (bp)	Reference
GR	TGCTGTGTTTTGCTCCTGATCTG	forward	53	299	Tomasicchio <i>et al.</i> 2013
	TGTCAGTTGATAAAACCGCTGCC	reverse			
PR-AB	GTGCTCAAGGAGGGCCTGCCG	forward	60	214	
	TGTGCTGCCCTTCCATTGCC	reverse			
AR	CAGGAAAGCGACTTCACCGCACC	forward	60	209	
	ATCAGGCAGGTCTTCTGGGGTGG	reverse			
ER α	TCGACGCCAGGGTGGCAGAG	forward	60	218	
	TGGTGCACTGGTTGGTGGCTGG	reverse			
MR	GAGCAGTGGAAGGGCAACAC	forward	60	182	
	TGGCTGCTCCTCGTGAATCC	reverse			

3.10 Small interference RNA (siRNA) transfections

End1/E6E7 cells were seeded in 12-well plates at a density of 35×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transfected with 10 nM validated GR HS_NR3C1_5 (GR5) (catalogue no. SI02654757) (Qiagen, South Africa) siRNA directed

against the human GR or validated non-silencing scrambled sequence control (NSC) siRNA (catalogue no. 1027310) (Qiagen, South Africa) using HiPerfect transfection reagent (Qiagen) as per the manufacturer's instructions. Briefly, specific or NSC siRNA was diluted in pre-warmed Optimem medium with GlutaMAX™ (Gibco-BRL Life Technologies) to which 3.5 µl transfection reagent was added. The transfection mixture was incubated at room temperature for 10 min and then added drop-wise to the cells to a final concentration of 10 nM. Cells were incubated for 48 hrs before being treated with 100 nM test compounds for 24 hrs. RNA was then harvested and mRNA levels were analysed by qRT-PCR, as described above. Cells which were transfected in parallel were analysed by Western blotting as described above to verify the protein knockdown.

3.11 Luminex

Supernatants were collected from the siRNA experiments prior to cell harvest. Thereafter, human cytokine protein levels were measured using a 27-plex assay kit according to manufacturer's protocol (Biorad, South Africa).

3.12 MSD® singleplex cytokine assay

Supernatants were collected from the dose response experiments prior to cell harvest. Samples were frozen at -80°C and shipped to the Fichorova laboratory (Brigham & Women's Hospital, Boston, USA). Thereafter, cytokine protein levels were measured using singleplex assay kits for human IL-6, IL-8 and RANTES according to manufacturer's protocol (Meso Scale Discovery (MSD®), USA).

3.13 Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as described in Verhoog *et al.* with modifications (Verhoog *et al.* 2011). HeLa cells were plated at 3×10^6 cells/dish in 15 cm dishes and grown for 24 hrs in full

DMEM. Cells were then washed twice with PBS and incubated in phenol-free DMEM (Sigma-Aldrich, South Africa) for another 24 hrs. Thereafter, the cells were washed twice with PBS and incubated with serum-free, phenol-free DMEM for 2 hrs, before treatment with 100 nM DEX, MPA, P4 and NET-A for 1 hr. Cells were crosslinked for 10 min at 37°C by addition of formaldehyde to a final concentration of 1%. Crosslinking was stopped by addition of glycine to a final concentration of 0.1 mM for 5 min, shaking at room temperature. Cells were scraped in PBS, pelleted by centrifugation at 1200 x g for 10 min and resuspended in 300 µl nuclear lysis buffer (1% SDS, 50 mM Tris-HCL, pH 8.0, 10 mM EDTA, 1X Complete Mini Protease Inhibitor Cocktail (Roche Applied Science, South Africa)). Chromatin was sheared by sonication (Misonix Sonicator 3000) with a microtip (20 s on//40 s off //10 cycles) before centrifugation at 15 000 x g for 10 min. For immunoprecipitation 100 µg chromatin was diluted with 900 µl IP dilution buffer (0.01% SDS, 20 mM Tris-HCL, pH 8.0, 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA, 1X Complete Mini Protease Inhibitor Cocktail). This solution was pre-cleared with 20 µl pre-blocked protein A/G agarose beads (sc-2003, Santa Cruz Biotechnology, USA) for 1 hr, rotating at 4°C. Beads were spun down and 2 µg anti-GR (H300) (Santa Cruz Biotechnology, USA) or 2 µg anti-goat (Santa Cruz Biotechnology, USA) for IgG control was added to the supernatant, which was incubated on a rotator at 4°C overnight. The following day, 40 µl pre-blocked protein A/G agarose beads were added, and tubes were incubated on a rotator for 6 hrs at 4°C. Beads were washed sequentially with 1 ml each of wash buffer I [0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8, and 150 mM NaCl], wash buffer II [(0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8, and 500 mM NaCl] and wash buffer III [(1% (v/v) NP-40, 1% (v/v) sodium deoxycholate, 500 mM LiCl, 1 mM EDTA, 10 mM Tris-Cl pH 8]. Beads were then washed three times with 1 ml TE buffer [10 mM Tris pH 8, 0.1 mM EDTA]. Proteins were eluted from the beads by

addition of 300 μ l elution buffer (1% SDS, 100 nM NaHCO₃) for 30 min at room temperature on a rotator. NaCl was added to a final concentration of 300 nM. Tubes were then incubated at 65°C overnight to reverse crosslinks. The following day EDTA (final concentration 15 nM), Tris-HCl pH 6.5 (final concentration 125 nM) and proteinase K (final concentration 60 ng/ μ l) (Roche Applied Science, South Africa) were added and samples were then incubated at 45°C for 1 hr. DNA was purified using the QIAquick® PCR purification kit (Qiagen, USA) according to manufacturer's instructions. Real-time qRT-PCR was performed on a Corbett Rotorgene, using the Sensi-Mix SYBR Green I system (Celtic Diagnostics, South Africa). ChIP primers used were for the GRE region of the GILZ promoter, AP-1 region of the IL-6 promoter and NF- κ B region of the IL-8 promoter (Table 3.4). Each real-time qRT-PCR reaction contained the following: template DNA 2 μ l for input or 4 μ l for immunoprecipitated samples, 1 μ l forward primer (0.5 μ M final concentration), 1 μ l reverse primer (0.5 μ M final concentration), 10 μ l Sensi-Mix SYBR Green I system (Celtic Diagnostics, South Africa) and PCR grade water to make a final volume of 20 μ l. Melting curve analysis and gel electrophoresis was performed to confirm the generated amplicon in each sample. Relative protein recruitment was determined using real-time qRT-PCR and calculated by the method described by Pfaffl *et al.* 2001 and normalized to input, which was set to one.

Table 3.4: Sequences the ChIP primer sets

Gene	Primer Sequence (5'- 3')	Strand	Annealing Temp. (°C)	Product size (bp)	Reference
GILZ	AGTGAATGTTCTTGATGACCC ATAAGTATAG	forward	45	118	Hadley <i>et al.</i> 2011
	GGACATTCTGTAACTTTAAG ACACAACCTC	reverse			
IL-6	TCTACAACAGCCGCTCACAG AGCGTTCCAGTTAATTTGTAT TTGT	forward reverse	45	162	Govender <i>et al.</i> 2014
IL-8	GGGCCATCAGTTGCAAAT TTCCTCCG GTG GTTTCTTC	forward reverse	53	186	Garside <i>et al.</i> 2004

3.14 Preparation and titration of HIV-1 pseudovirus

Three different HIV-1 pseudoviruses were prepared to investigate different questions. In order to investigate the role of GR in LTR transcription, HIV-1 wildtype (wt) pseudovirus was prepared using pDU151A *env* and pSg3E⁻ packaging plasmids. In order to investigate the role of Vpr in inflammatory gene and LTR regulation HIV-1 wt and HIV-1 with a mutated *vpr* gene (HIV-1 Δ Vpr) were prepared. This HIV-1 wt pseudovirus was prepared using pLET-LAI *env* and pNL4-3.E⁻ packaging plasmids and the HIV-1 Δ Vpr pseudovirus was prepared using pLET-LAI *env* and pNL4-3.E⁻R⁻ packaging plasmids. All three pseudoviruses were prepared as follows; HEK293T cells were seeded into 6-well plates at a density of 80×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently co-transfected

with an *env* expressing plasmid and corresponding packaging plasmid using Xtreme-GENE 9 DNA transfection reagent (Roche Applied Science, South Africa) according to manufacturer's instructions. Cells were incubated for 3 days at 37°C. Thereafter the supernatants were collected, centrifuged at 1200 rpm for 5 min and passed through a 0.22 µm filter and charcoal stripped (cs) FCS (Highveld Biological, South Africa) was added to a final concentration of 40%. The viral stocks were aliquotted and stored at -80 °C until use. The titre of the pseudotyped viruses was determined using the Reed-Muench method in TZM-bl cells to obtain TCID₅₀ expressed as log₁₀ values (Reed & Muench 1938; Janas & Li 2010). Thereafter, virus stocks were calculated from the TCID₅₀ values and expressed as infectious units/ml (IU/ml).

3.15 HIV-1 pseudovirus infection assays

TZM-bl cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 hrs. Cells were infected with either HIV-1 wt or HIV-1 ΔVpr, as indicated in the figure legends, and incubated for 72 hrs. Pseudovirus was added to obtain a multiplicity of infection (MOI) (0.0001, 0.0005 or 0.001) also as indicated in the figure legends. To calculate the volume of virus used at a specific MOI, the following equation was used; $MOI = [(virus\ stock\ IU/ml) \times (volume\ of\ virus\ used)] / (number\ of\ cells\ in\ infection)$ (Janas & Li 2010). Thereafter the virus medium was removed and the cells were washed twice with PBS to remove extracellular virus. Then, the cells were treated with test compounds in serum free DMEM medium for 24 hrs. The cells were then harvested with Bright-Glo™ (Promega, Madison, WI, USA) according to manufacturer's specifications. Luciferase activity was measured using a Modulus microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). Luciferase activity was normalized to cell viability as determined using the Thiazolyl Blue Tetrazolium Bromide (MTT) cell determination kit (catalogue no. CGD1, Sigma-

Aldrich, South Africa) according to manufacturer's instructions. Figure 3.1 shows a brief overview of the pseudovirus strategy.

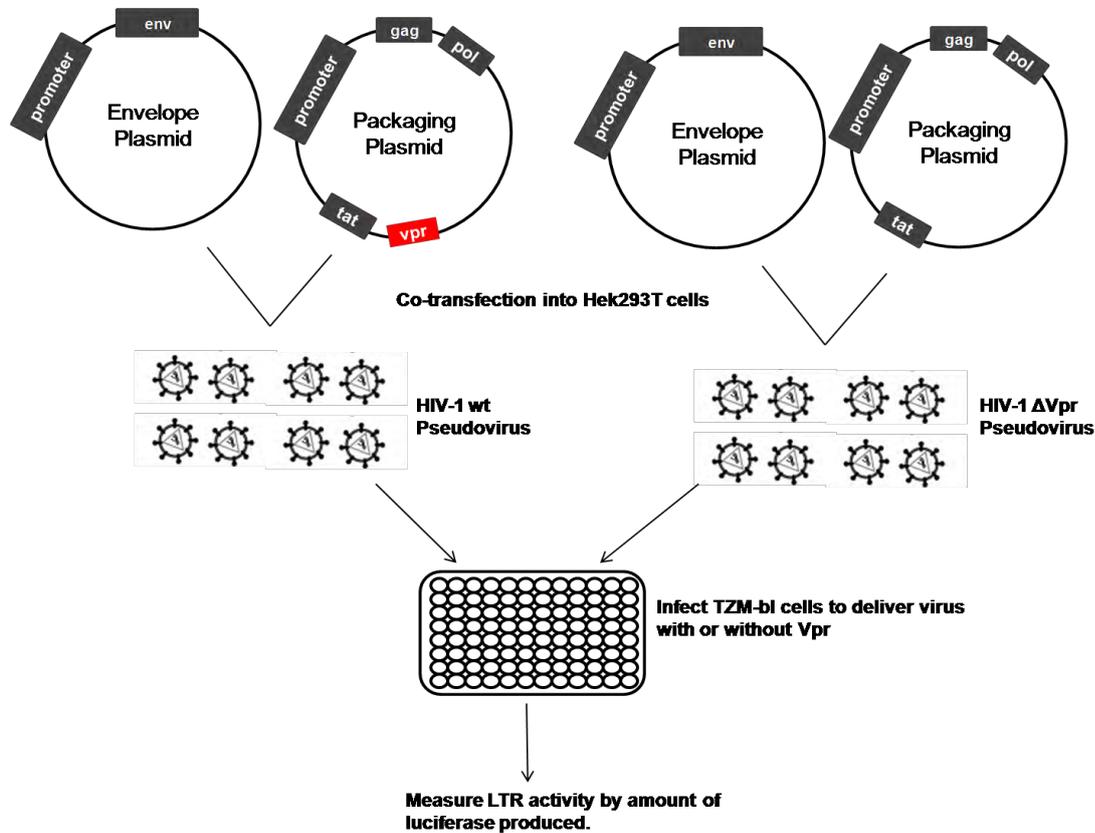


Figure 3.1 Brief overview of the HIV-1 Pseudovirus Strategy. Envelope plasmid represents pDU151A or pLET-LAI and packaging plasmid represents pSg3E⁻, pNL43.E⁻ or pNL4-3.E⁻R⁻.

3.16 Primary endocervical cells

Primary endocervical cells (VEN-100) were bought from MatTek Corporation (USA). Upon arrival, the cells were incubated overnight in VEN-100-MM medium (MatTek Corporation, USA) at 37°C in a 5% CO₂ incubator. The following day cell viability was determined using the Thiazolyl Blue Tetrazolium Bromide (MTT) cell determination kit (catalogue no. CGD1, Sigma-Aldrich, South Africa) according to the manufacturer's instructions. At this time, some cells were washed with PBS and either lysed with a N-[Tris(hydroxymethyl)-methyl]-

3-aminopropanesulfonic acid (TAPS) buffer (0.1 M TAPS, pH 9.5) on ice to perform western blotting as described above or with TRIzol® (for RNA isolation, cDNA synthesis and qRT-PCR as described above). Having established the viability of the cells, the majority of the VEN-100s were incubated in VEN-100-ASY-HCF hydrocortisone free assay medium (MatTek Corporation, USA) and 100 nM test compound for 48 hours, before performing an additional MTT assay.

3.17 Statistical analysis

Results were analysed with GraphPad PRISM™ (version 5) software from GraphPad Software Inc., using One-way ANOVA, with a Dunnett's post-test when comparing all treatments to a single control, a Bonferroni's or Newman-Keul's post-test when comparing all treatments to each other and a post-test for linear trend. In addition, P values for comparison of 2 treatments were obtained by using the t-test (paired and unpaired) as indicated in the figure legends. Statistical significance of difference is denoted by *, ** or ***, to indicate $P < 0.05$, $P < 0.001$ and $P < 0.0001$, respectively.

Chapter 4: Inflammatory gene regulation in human endocervical cells by the synthetic progestin MPA, unlike NET-A and P4, is mediated by the GR (Results).

Aims

The main aim of this present study was to investigate the hypothesis that MPA, P4 and NET-A differentially regulate inflammatory gene expression in the cervical mucosa and that the MPA-mediated gene regulation occurs via the GR. More specifically the goals were to (i) determine whether the synthetic progestins and P4 differentially regulate inflammatory gene expression in the End1/E6E7 human endocervical cell line (ii) determine whether the differential regulation of the genes by the synthetic progestins is mediated via the GR (iii) further investigate the GR mechanism of action and (iv) confirm the differential regulation of the inflammatory genes by the synthetic progestins in human primary cervical cells.

Results

4.1 DEX- and MPA-mediated regulation of the inflammatory genes is dose- and time-dependent

In order to both determine whether the synthetic progestins and P4 differentially regulate inflammatory gene expression in the cervical mucosa and to determine whether this regulation is in a dose- and/or time-dependent manner, experiments were performed in the End1/E6E7 cell line. This was done by treating the cells with an increasing dose (1 nM, 10 nM, 100 nM and 1 μ M) of DEX, MPA, P4 and NET-A for 4 hrs and 24 hrs, respectively. Thereafter, gene expression was measured by qRT-PCR. Figure 4.1 A and B shows that DEX- and MPA-dose dependently induced GILZ mRNA expression, while P4 and NET-A

appear to have no effect on GILZ gene expression at any of the concentrations or time-points. It also appears that the maximal response for MPA and DEX regulation of GILZ mRNA levels does not change between 4 and 24 hrs. DEX and MPA, unlike P4 and NET-A, repress IL-6 mRNA levels dose-dependently at both 4 hrs and 24 hrs (Figure 4.1 C and D). The DEX-mediated repression of IL-6 mRNA at 24 hrs is more potent than the repression at 4 hrs whereby DEX has an EC₅₀ (potency) of ~11 nM at 4hrs and ~3 nM at 24 hrs. In addition, MPA appears to have a greater maximal repression of IL-6 mRNA levels at 24 hrs than 4hrs. It also appears that NET-A (100 nM and 1 μM) upregulates IL-6 mRNA while 10 nM NET-A represses IL-6 mRNA at 24 hrs; however this is not statistically significant. Figure 4.1 E and D shows that DEX and MPA dose-dependently repress IL-8 mRNA levels; however both the DEX- and MPA-mediated repression are more potent at 24 hrs. At 4 hrs, P4 appears to upregulate and NET-A appears to dose-dependently upregulate IL8 mRNA levels while at 24 hrs they both appear to repress IL-8 mRNA (Figure 4.1 E and F). The regulation of RANTES at 4 hrs appears to be inconclusive and no repression of RANTES is apparent (Figure 4.1 G). At 24 hrs, both DEX and MPA dose-dependently repressed RANTES mRNA (Figure 4.1 H). Although not statistically significant, both P4 and NET-A show a trend for repression of RANTES mRNA levels (Figure 4.1 H). The MPA- and DEX-mediated dose and time dependent regulation of these genes are more apparent on the dose response plots in Figure 4.2. MPA appears to have a potency of ~24 nM for transactivation of GILZ and a potency of ~21, 4 and 5 nM for repression of IL-6, IL-8 and RANTES mRNA, respectively, at 24 hrs.

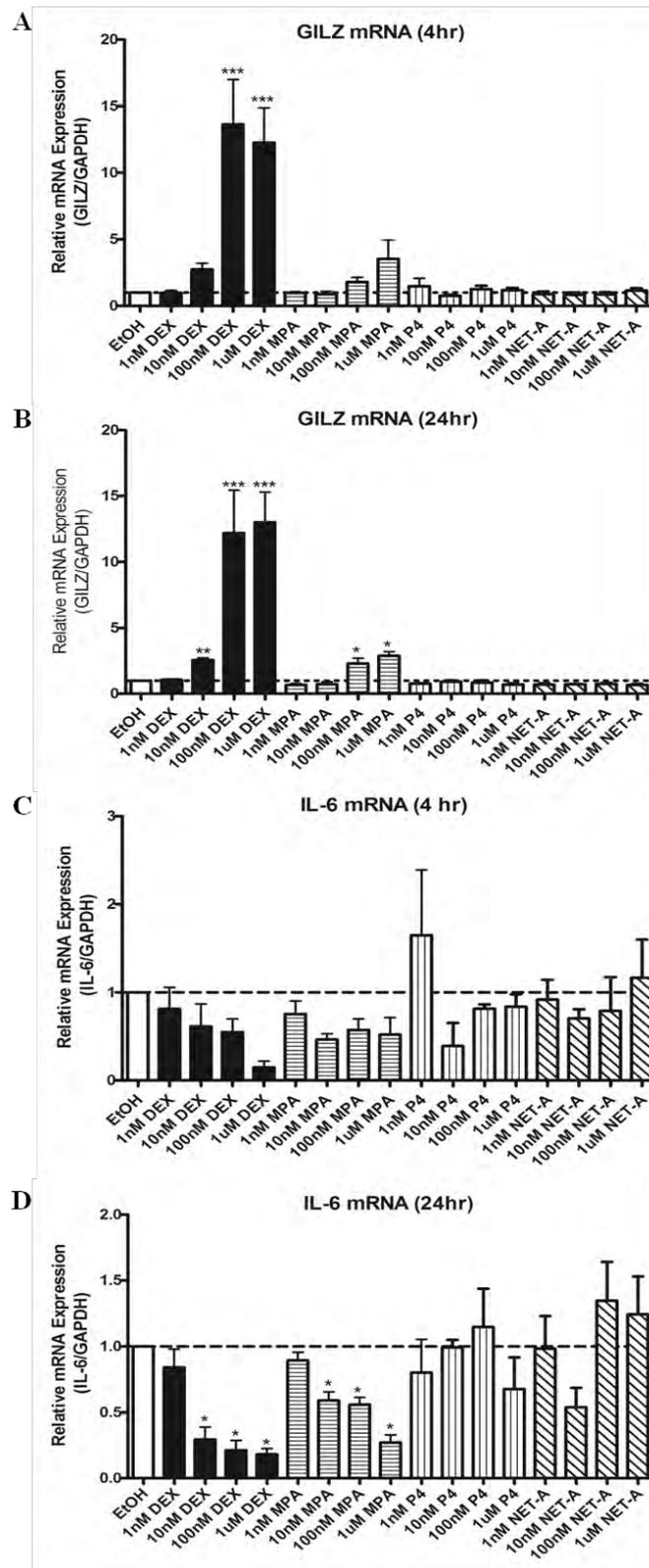


Figure 4.1: DEX- and MPA-mediated transactivation and transrepression is dose- and time dependent. (continued on next page)

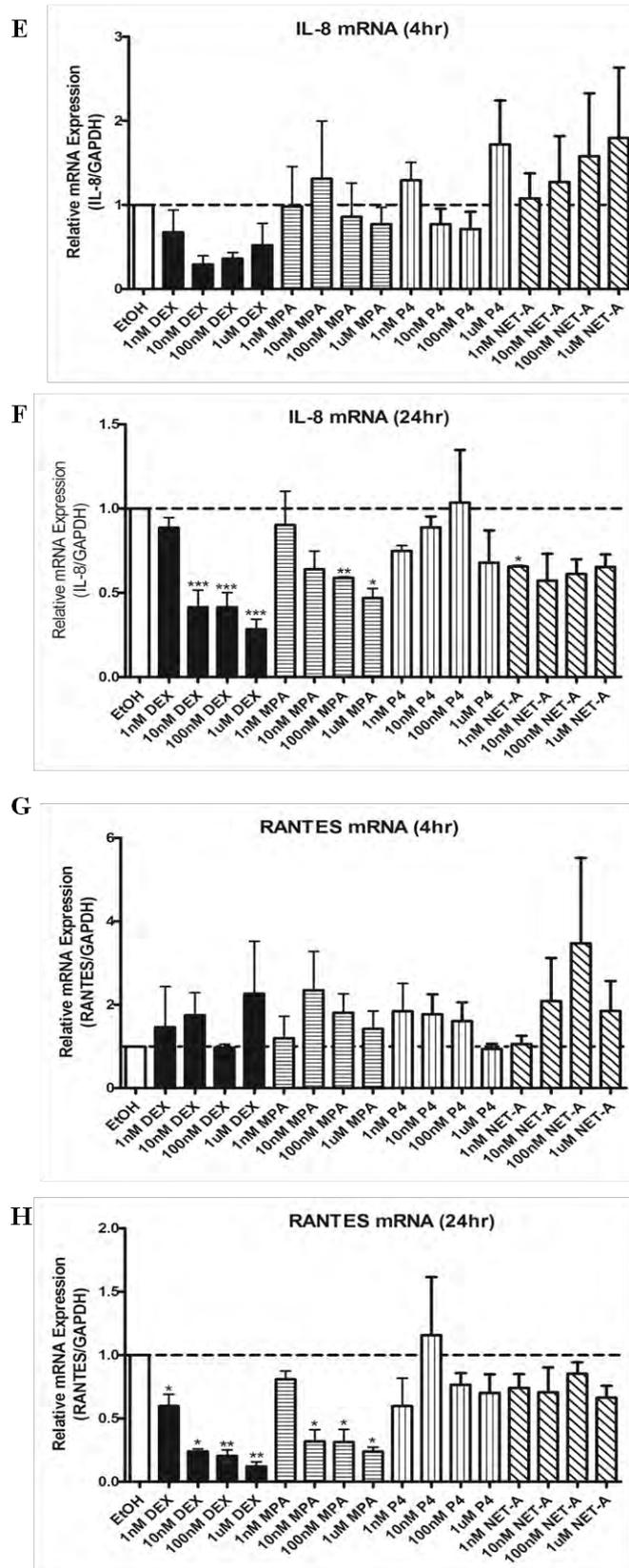


Figure 4.1: DEX- and MPA-mediated transactivation and transrepression is dose- and time dependent. (figure legend to follow on next page)

End1/E6E7 cells were seeded into 12-well plates at a density of 35×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were treated with increasing amounts (1 nM, 10 nM, 100 nM and 1 μ M) of DEX, MPA, P4, NET-A or EtOH (0.1% v/v, vehicle) for 4 hrs and 24 hrs, respectively. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative GILZ (**A, B**), IL-6 (**C, D**), IL-8 (**E, F**) and RANTES (**G, H**) gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalized to basal activity (EtOH) set to 1 to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA with either Dunnett post-test or paired t-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

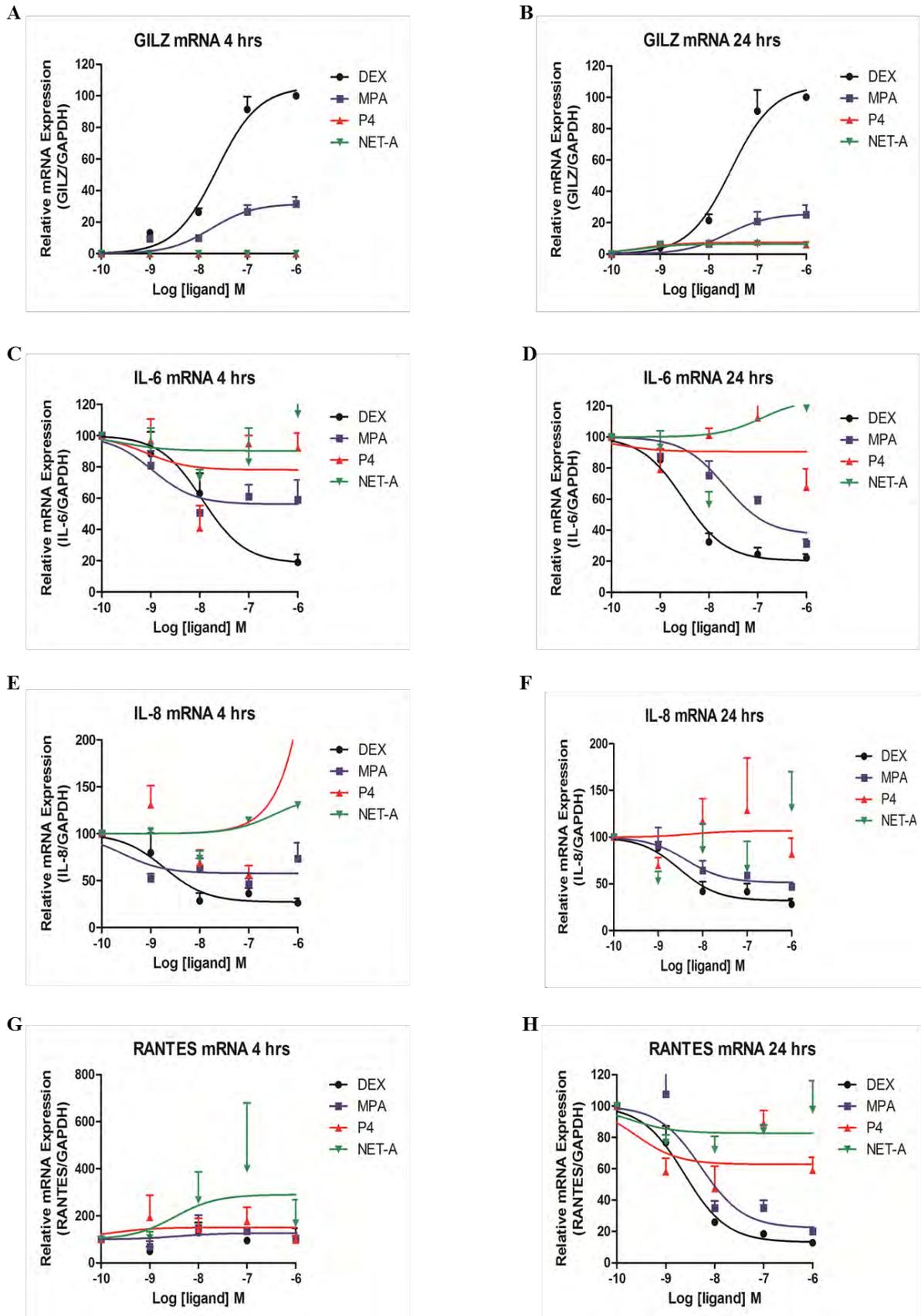
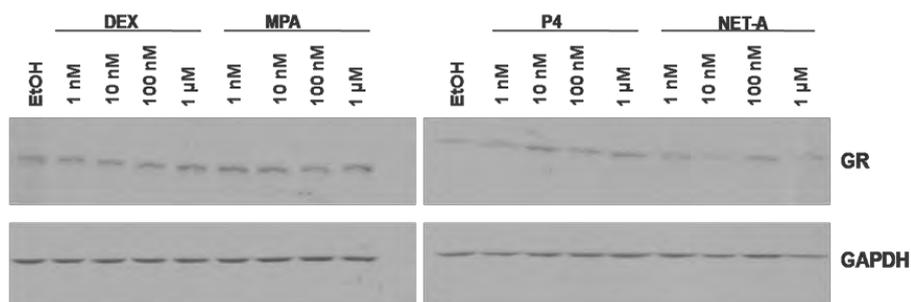


Figure 4.2: DEX- and MPA-mediated regulation of inflammatory genes is more potent at 24 hrs than 4 hrs. (figure legend to follow on next page)

End1/E6E7 cells were seeded into 12-well plates at a density of 35×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were treated with increasing amounts (1 nM, 10 nM, 100 nM and 1 μ M) of DEX, MPA, P4, NET-A or EtOH (0.1% v/v, vehicle) for 4 hrs and 24 hrs, respectively. Thereafter, the cells were washed twice with PBS and harvested for either total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative (A, B) GILZ, (C, D) IL-6, (E, F) IL-8 and (G, H) RANTES gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative GILZ gene expression was normalized to 1 μ M DEX set to 100% in order to obtain % partial agonist activity. Relative IL-6, IL-8 and RANTES expression was normalized to basal activity (EtOH) set to 100 in order to obtain % repression.

The Western blots in Figure 4.3 A and B show the effect of the ligands on the expression of endogenous GR protein levels at 4 hrs and 24 hrs, respectively. At 4 hrs, the ligands appear to have no effect on GR protein levels (Figure 4.3 A), while at 24 hrs, only DEX (10 nM, 100 nM and 1 μ M) and MPA (1 nM) reduced GR protein levels (Figure 4.3 B).

A



B

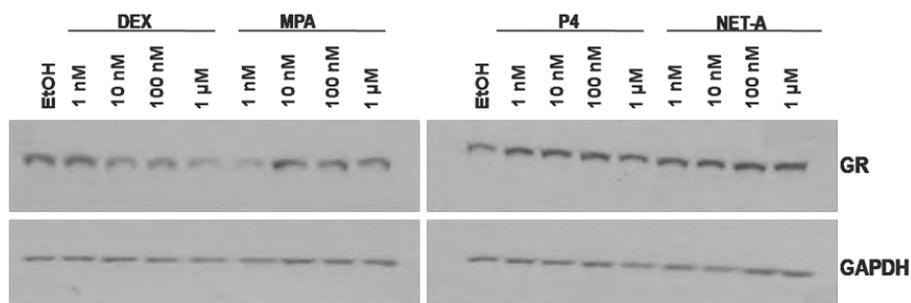


Figure 4.3: Effect of progestins on GR protein expression. (figure legend continued on next page)

End1/E6E7 cells were seeded into 12-well plates at a density of 35×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were treated with increasing amounts (1 nM, 10 nM, 100 nM and 1 μ M) of DEX, MPA, P4, NET-A or EtOH (0.1% v/v, vehicle) for 4 hrs (**A**) and 24 hrs (**B**), respectively. Thereafter, the cells were washed twice with PBS and harvested with 5X SDS sample buffer. Equal amounts of whole cell lysates were loaded on SDS page gels and analyzed by Western blotting with antibodies specific for GR and GAPDH (loading control). Single representative blots are shown for each time point.

In order to determine whether the differential gene regulation by the ligands is mimicked at the protein level, cytokine protein levels were measured. This was done by collecting the supernatants from the 24 hr experiments prior to cell harvest. Thereafter the cytokine secretions were measured by MSD® singleplex cytokine assays. Figure 4.4 A and B shows that DEX dose-dependently repressed both IL-6 and IL-8 protein levels at 24 hrs. The trend of the MPA dose appears to repress IL-6 protein levels; however this trend is not statistically significant (Figure 4.4 A). MPA does not appear to repress IL-8 protein at 24 hrs (Figure 4.4 B). Although not statistically significant, P4 appears to repress IL-6 protein levels but not IL-8 protein at 24 hrs. Interestingly, NET-A dose-dependently upregulates both IL-6 and IL-8 protein levels at 24 hrs (Figure 4.4 A and B). Although the regulation of RANTES protein by the ligands at 24 hrs is not statistically significant, there appears to be a trend of downregulation with DEX and MPA, while the trend with P4 appears to be inconclusive (Figure 4.4 C). Also, there appears to be a trend of upregulation with the NET-A. Due to the large data-sets, statistical significance could not be determined for some of the mRNA and protein results. Taken together, results indicate that MPA, unlike NET-A and P4, represses mRNA levels and possibly protein levels of the inflammatory genes. In addition, the regulation of these inflammatory genes by MPA is dose- and time-dependent and it is similar to the GR agonist, DEX. Furthermore, NET-A dose-dependently upregulates the mRNA and protein levels of the cytokines and this may also be time-dependent.

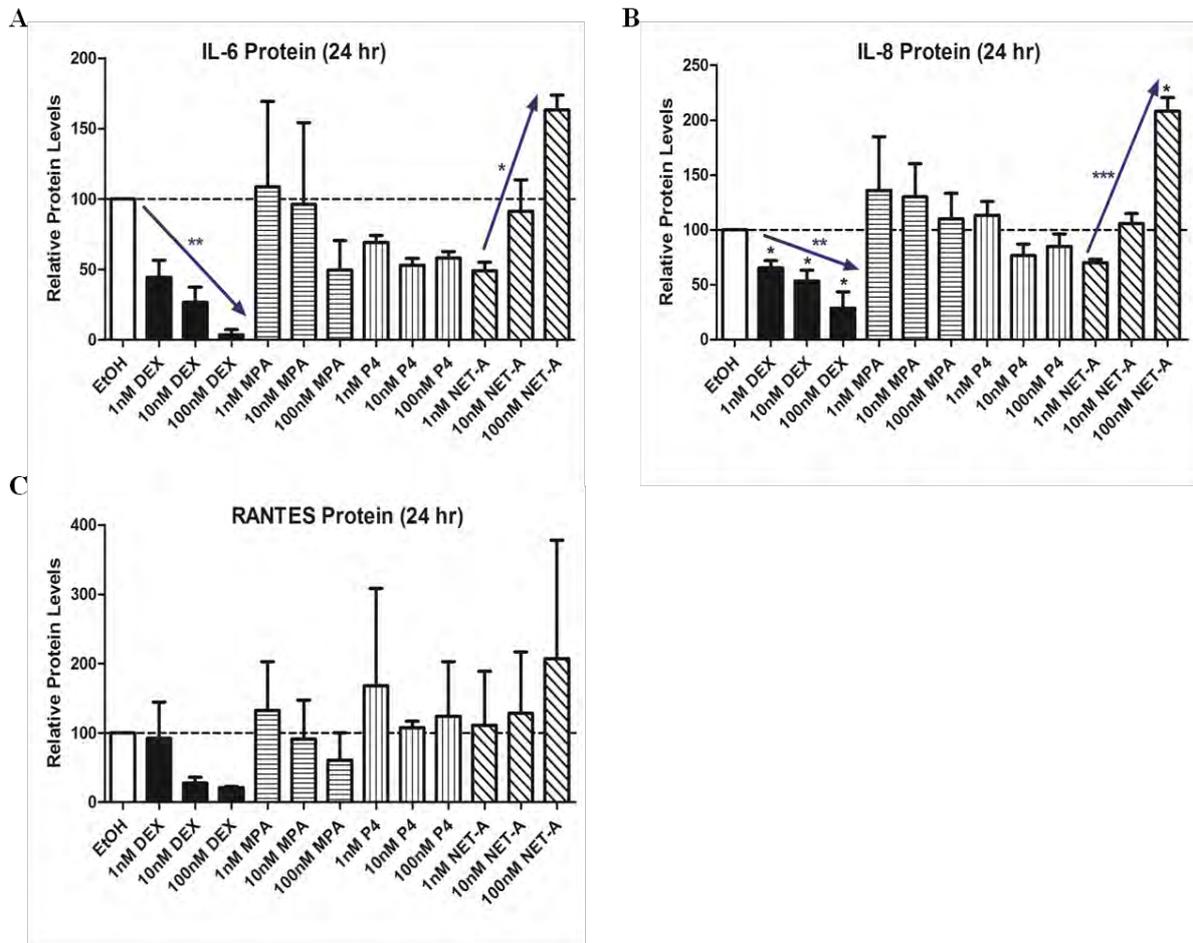


Figure 4.4: DEX dose-dependently represses IL-6, IL-8 and RANTES protein levels. End1/E6E7 cells were seeded into 12-well plates at a density of 35×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were treated with increasing amounts (1 nM, 10 nM, 100 nM and 1 μ M) of DEX, MPA, P4, NET-A or EtOH (0.1% v/v, vehicle) for 24 hrs. Then, supernatants were collected prior to cell harvest and cytokine protein levels for (A) IL-6, (B) IL-8 and (C) RANTES were determined by MSD® singleplex cytokine assays. Relative protein expression was normalized to basal activity (EtOH) set to 100% to obtain relative fold repression. Graphs represent pooled results of at least two independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA followed by a linear trend post-test and a paired t-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

4.2 DEX and MPA regulation of the inflammatory genes is mediated via the GR

Given the differential SR selectivity of P4 and the synthetic progestins, it was investigated whether the GR, AR, PR, MR or ER α are expressed in the End1/E6E7 endocervical cell line. The End1/E6E7 cells were assessed for SR mRNA content by qRT-PCR using primer pairs designed specifically for each receptor followed by gel electrophoresis. The endocervical cells were also assessed for endogenous SR protein content by Western blotting. Western blotting controls were made by transiently transfecting COS-1 cells with the different SR expression vectors or empty vector to produce positive and negative controls, respectively. Figure 4.5 A indicates that the End1/E6E7 cells contain only GR mRNA and Figure 4.5 B shows that the End1/E6E7 cells express GR protein only. Although it appears that the End1/E6E7 cells contain MR protein (Figure 4.5 B) it is highly unlikely that this is true since the End1/E6E7 cells do not express MR mRNA. On further examination, it is evident that this is a non-specific band which also appears in the negative control.

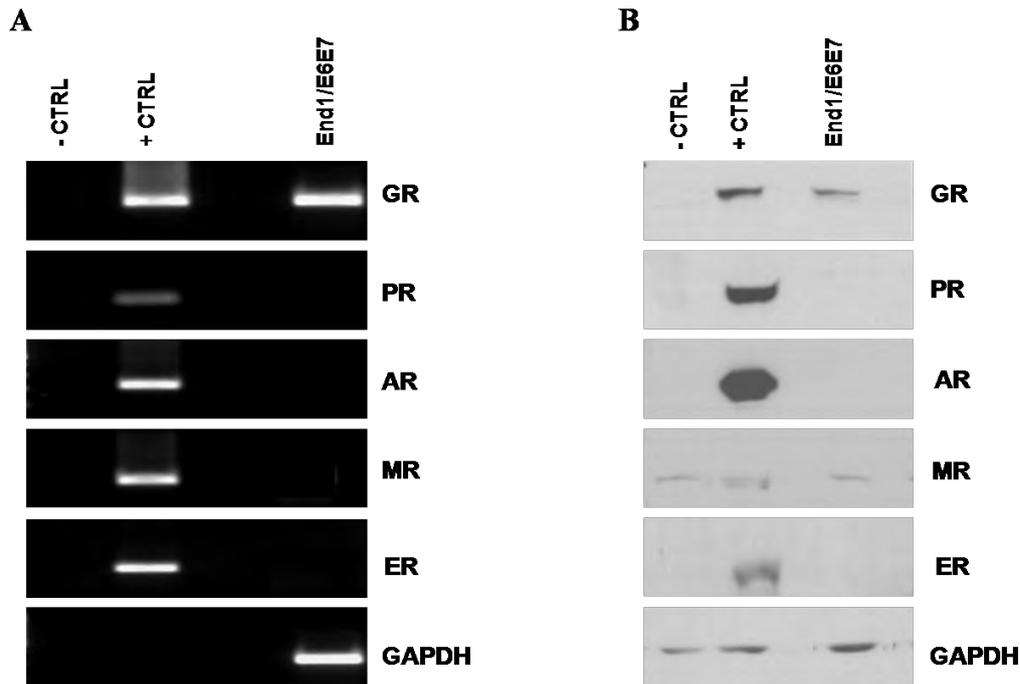


Figure 4.5: End1/E6E7 cells only express detectable GR mRNA and protein. (A) End1/E6E7 cells were seeded into 12-well plates at a density of 35×10^4 cells/well and incubated for 24 hrs. Thereafter, cells were washed once with PBS and harvested with TRIzol. Total RNA was isolated and 500 ng RNA was reverse-transcribed. Steroid receptor gene expression was measured by qRT-PCR with receptor specific and GAPDH (loading control) primers, followed by gel electrophoresis to confirm the PCR products. (B) End1/E6E7 cells were seeded into 12-well plates and incubated for 24 hrs. Thereafter, cells were washed once with PBS and were lysed with 2X SDS sample buffer. COS-1 cells were seeded into a 12-well plate. After 24 hrs, the cells were transiently transfected with 1 μ g/well pcDNA3 (empty vector) which served as negative control (-CTRL) or with 1 μ g/well steroid receptor expression vectors (pcDNA3-hGR, pMT-PR-B, pSV-hAR, pRS-hMR and pSG5-hER) which served as positive controls (+CTRL). After 24 hrs, the COS-1 cells were washed once and lysed with 2X SDS sample buffer. Equal volumes of lysate were analysed by Western blotting with steroid receptor specific antibodies and GAPDH specific antibody as loading control.

In order to confirm that the GR is involved in the regulation of the inflammatory genes in response to the synthetic progestin MPA, GR knockdown experiments were performed in the

End1/E6E7 cell line. This was carried out by transfecting 10 nM GR or NSC siRNA for 48 hrs. Then the cells were treated with 100 nM DEX, MPA, P4 and NET-A for 24 hrs. Thereafter, gene expression was measured by qRT-PCR. As expected DEX and MPA upregulated GILZ mRNA, while P4, NET-A and NET do not (Figure 4.6 A). Notably, NET was included in this experiment as a control to exclude the possibility that the acetate form (NET-A) would regulate the genes differently. Figure 4.6 A also shows that both the DEX- and MPA-induced upregulation of GILZ is diminished when GR is knocked down. Figure 4.6 B shows that DEX induces $\text{I}\kappa\text{B}\alpha$ gene expression and this induction is repressed when GR is knocked down. Here the MPA induction of $\text{I}\kappa\text{B}\alpha$ is not significant; therefore a loss of induction is not apparent with the knockdown. Once more, P4, NET-A and NET do not affect the expression of $\text{I}\kappa\text{B}\alpha$. To verify the successful knock down of GR protein in the cells, cell lysates were prepared and Western blotting was performed (Figure 4.6 C). The average percentage knock down achieved was ~40% (Figure 4.6 D). This was the best GR protein knockdown achievable in this cell line while preserving cell viability.

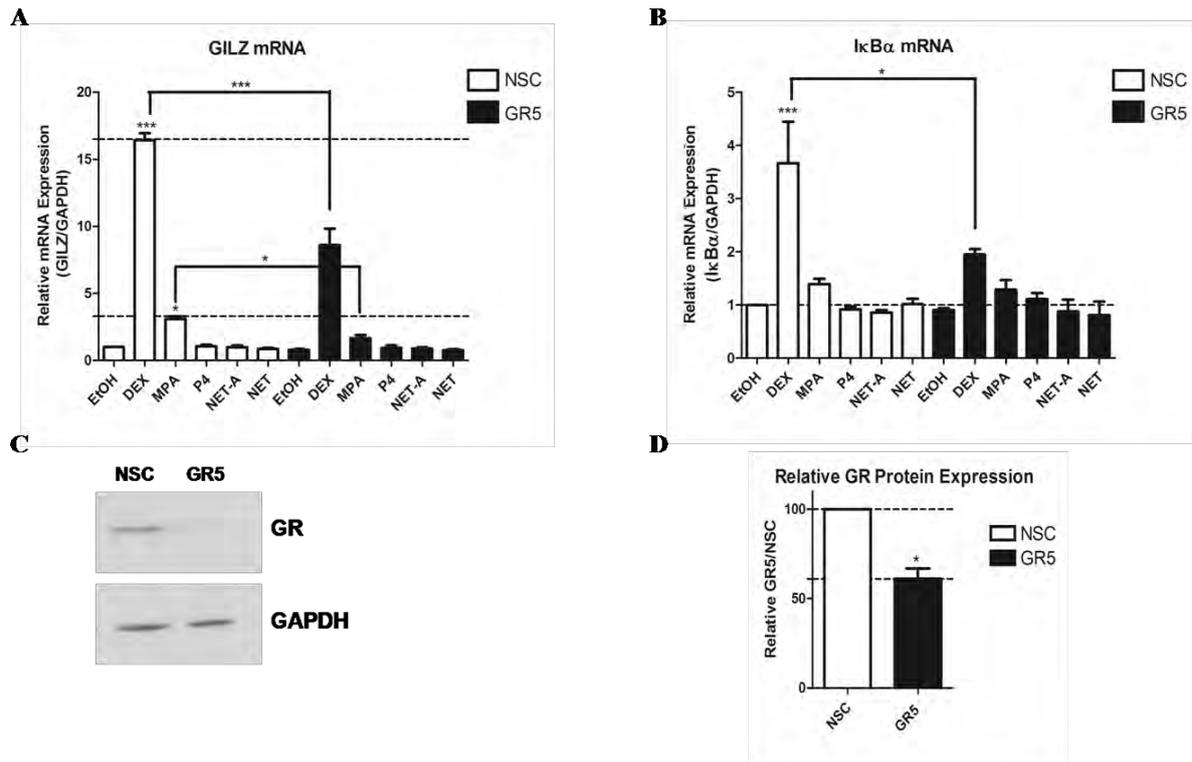


Figure 4.6: MPA- and DEX-mediated induction of anti-inflammatory genes are mediated by the GR. End1/E6E7 cells were seeded into 12-well plates at a density of 40×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transfected with 10 nM GR siRNA (GR5) or non-silencing control (NSC) siRNA for 48 hrs (A-D) and then treated with 100 nM DEX, MPA, P4, NET-A, NET or EtOH (0.1% v/v, vehicle) for 24 hrs. Then, the cells were washed twice with PBS and harvested for total RNA with TRIzol and 500 ng RNA was reverse-transcribed. Relative (A) GILZ and (B) IκBα gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Relative gene expressions were normalized to basal activity (EtOH) in order to obtain relative fold expression. Graphs in (A) and (B) represent pooled results of at least three independent experiments and are plotted as mean \pm SEM, while for verification of GR knockdown a representative blot is shown in (C). Cells were harvested with 2X SDS sample buffer and equal volumes of lysate were analysed by Western blotting with an antibody specific for GR and GAPDH (loading control). (D) Western blots of at least three independent experiments were quantified to determine the relative GR protein expression and is plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with a Dunnett post-test, followed by a student's t-test to compare specific conditions to each other. Statistical significance is denoted by * or *** to indicate $P < 0.05$ or $P < 0.0001$, respectively.

Again, as expected DEX and MPA repress IL-6 mRNA expression (Figure 4.7 A), while P4 and NET-A appear to have no effect on IL-6 mRNA expression (Figure 4.7 A). Although not statistically significant, NET does appear to induce IL-6 mRNA. However, this induction is not reflected in the IL-6 protein expression (Figure 4.7 A and B, respectively). The DEX- and MPA-mediated repression of IL-6 mRNA expression is lifted when GR is knocked down. In addition, DEX-mediated repression is also seen on IL-6 protein levels (Figure 4.7 B). Interestingly, it appears that MPA and P4 repress IL-6 protein levels and the repression is lifted in the knockdown, however these results were not statistically significant. A similar result is seen in the regulation of IL-8 mRNA expression by DEX and MPA (Figure 4.7 C). Figure 4.7 D shows that DEX appears to repress IL-8 protein levels and the knockdown lifts basal IL-8, thereby lifting the DEX repression. RANTES mRNA level are also repressed by DEX and MPA, but not by P4 and NET-A, in a GR-dependent manner (Figure 4.7 E). Although not statistically significant, NET appears to upregulate RANTES mRNA levels (Figure 4.7 E). It appears that the RANTES protein levels (Figure 4.7 F) are not regulated by any of the ligands, however this could possibly be due to its instability in the medium. Again, the lack of statistical significance for some of the mRNA and protein results could be due to the large number of variables in the data-sets. Taken together, these results confirm the differential regulation of the inflammatory genes by P4 and the synthetic progestins, MPA and NET-A occurs on both the mRNA and protein level. In addition, it shows that both the DEX- and MPA-mediated regulation of the inflammatory genes is via the GR.

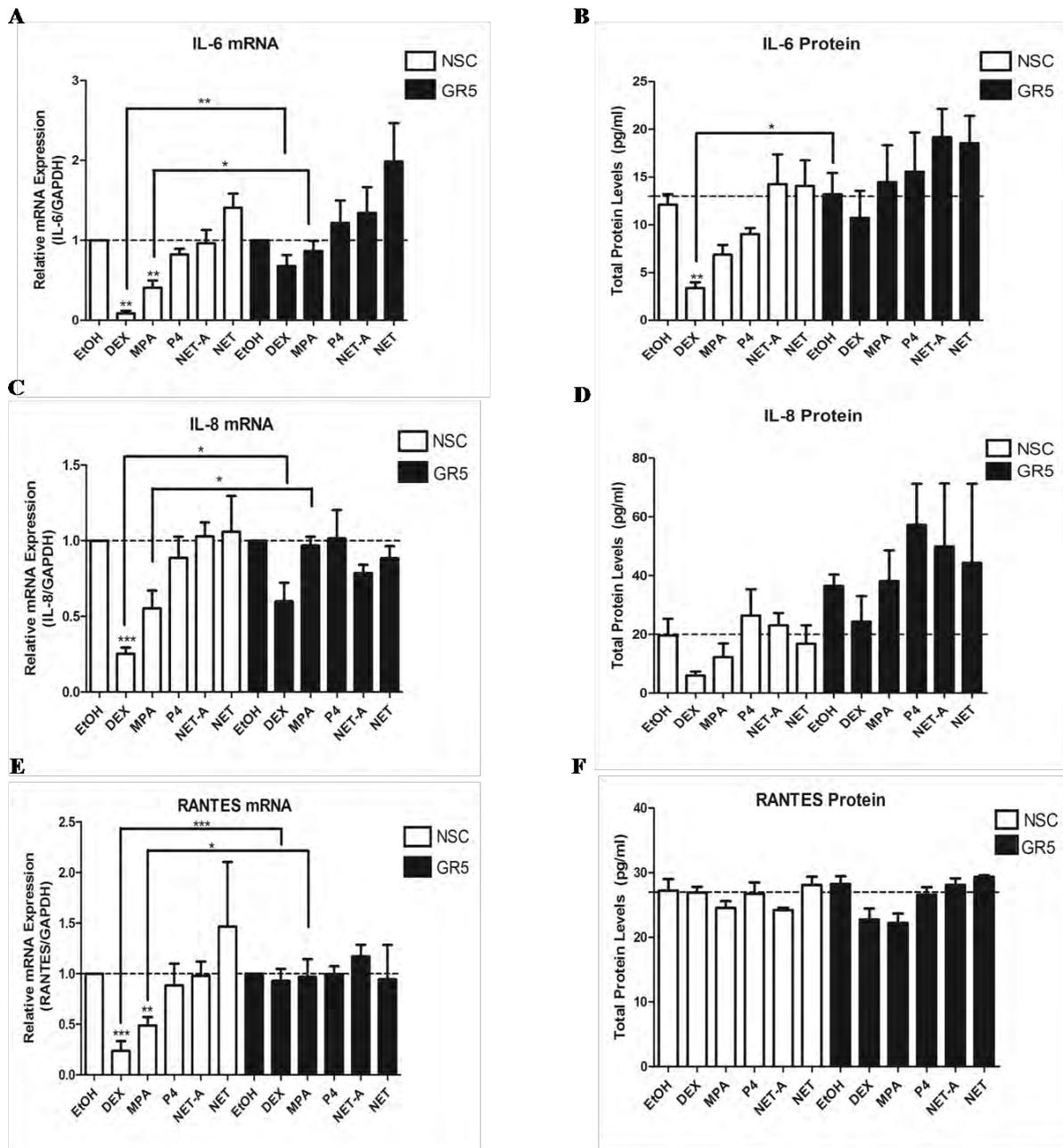


Figure 4.7: MPA- and DEX-mediated repression of pro-inflammatory cytokine genes are mediated via the GR. End1/E6E7 cells were seeded into 12-well plates at a density of 40×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transfected with 10 nM GR siRNA (GR5) or non-silencing (NSC) siRNA for 48 hrs (A-F) and then treated with 100 nM DEX, MPA, P4, NET-A, NET or EtOH (0.1 % v/v, vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative (A) IL6 (C) IL8 and (E) RANTES gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (EtOH) in order to obtain fold

expression. The corresponding cytokine protein levels were determined by Luminex of supernatants collected prior to cell harvest, **(B)** IL6 protein levels, **(D)** IL8 protein levels and **(F)** RANTES protein levels. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with either a Dunnett post-test or paired t-test. Statistical significance is denoted by * or ** to indicate $P < 0.05$ or $P < 0.001$, respectively.

4.3 Inhibition of protein synthesis supports a mechanism of partially direct regulation by the GR of the inflammatory genes

In order to investigate the mechanism of gene regulation, cycloheximide (CHX; *de novo* protein synthesis inhibitor) experiments were performed in the End1/E6E7 cells to determine whether the GR-mediated regulation of the cytokine genes require new protein synthesis. This was done by pre-treating the cells with 1 μ g/ml CHX for 30 min and then treating with 100 nM DEX, MPA, P4 and NET-A for 24 hrs. Figure 4.8 A shows the expected induction of GILZ mRNA in response to DEX and MPA. The addition of CHX appears to partially reverse the DEX induction of GILZ mRNA and fully reverses the MPA induction. The effect of the ligands on I κ B α mRNA expression is not reversed by CHX (Figure 4.8 B). Figure 4.8 C shows that DEX- and MPA-mediated repression of IL-6 mRNA is not influenced by pre-treatment with CHX. Similarly, in Figure 4.8 D, IL-8 mRNA is repressed by DEX and this repression is not reversed by CHX. However, for this gene the results for MPA were inconclusive. In contrast, the DEX-mediated repression of RANTES mRNA is partially reversed by CHX (Figure 4.8 E). However, the MPA-mediated repression of RANTES mRNA is not reversed. To confirm that the CHX inhibited *de novo* protein synthesis, End1/E6E7 cells were pre-treated with 1 μ g/ml CHX and then treated with 100 nM DEX for 24 hrs, thereafter cell lysates were prepared and western blotting was performed (Figure 4.8

F). It is worth noting (to prevent confusion), that in this thesis I κ B α was used for a dual purpose. In addition to GILZ, the I κ B α gene was used as a GR transactivation model while I κ B α protein levels were used as a positive control for the CHX experiments. I κ B α protein levels were measured since this protein has been shown to have a high turnover (Kogan *et al.* 2012). In addition, DEX induces I κ B α protein levels and if new protein synthesis is inhibited by CHX this DEX-induction will be lost and thereby is an indicator of new protein synthesis inhibition. As seen in Figure 4.8 G, the 2-fold DEX induction is lost with the addition of CHX. These results suggest that upregulation of GILZ mRNA levels is only partially dependent on transactivation by the GR and it is also in part dependent on synthesis of another protein. I κ B α mRNA upregulation appears to be independent of new protein synthesis and thereby suggests that the induction of the gene in these cells are predominantly dependent on transactivation of the GR. Given that the GR-mediated repression of IL-6, IL-8 and RANTES is either not reversed or only partially reversed by CHX; these results indicate that the GR-mediated regulation may partially require new protein synthesis for some of these pro-inflammatory genes. In summary, results indicate that under conditions where CHX is shown to inhibit new protein synthesis, all the anti-inflammatory and pro-inflammatory genes investigated are at least in part regulated by direct effects of DEX without a requirement for new protein synthesis, and where this could be established, similar trends are observed for MPA.

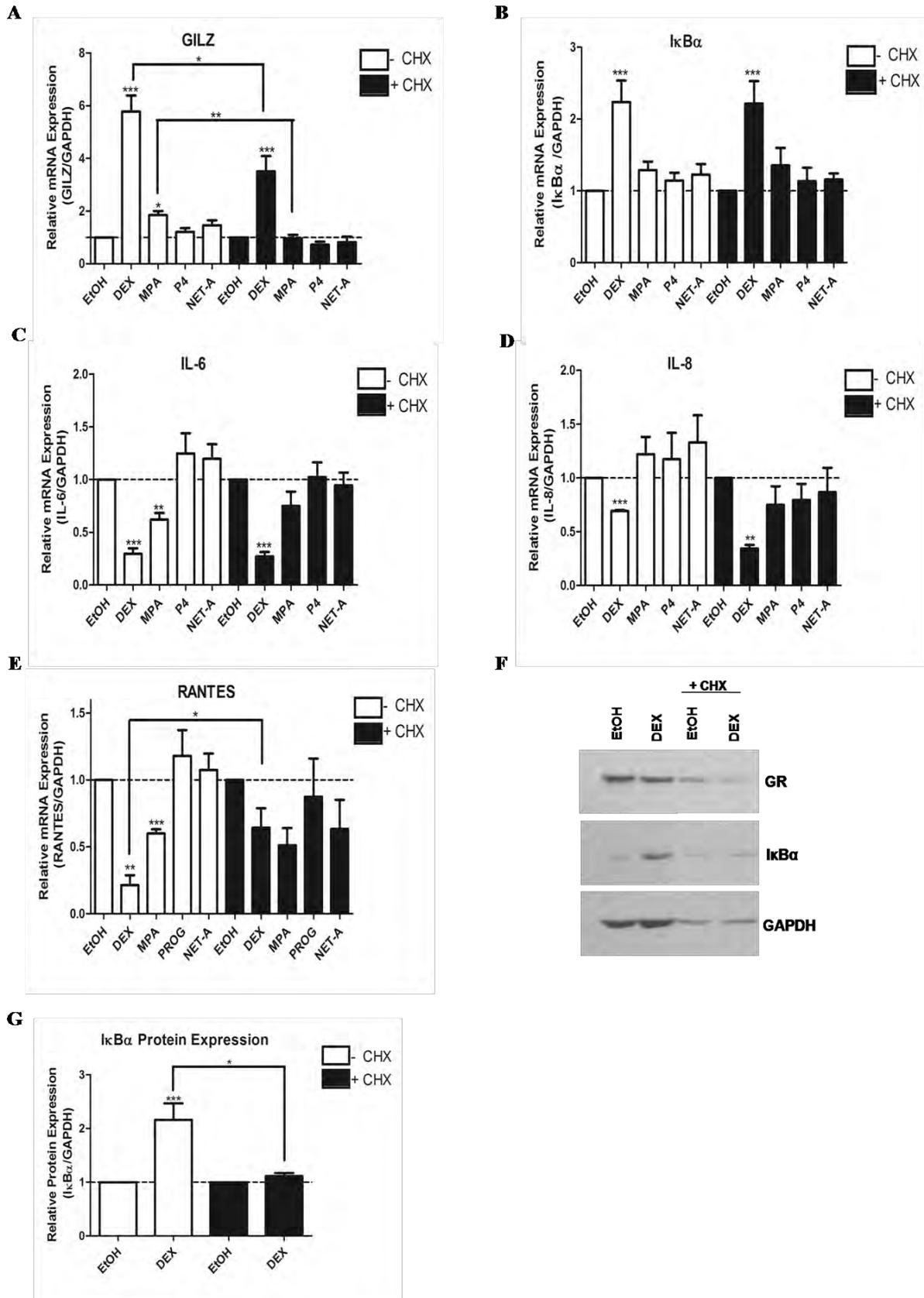


Figure 4.8: The GR at least in part directly regulates mRNA levels of the inflammatory genes. (figure legend to follow on next page)

End1/E6E7 cells were seeded into 12-well plates at a density of 35×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were pre-treated with 1 $\mu\text{g/ml}$ cycloheximide (CHX) then treated with 100 nM DEX, MPA, P4, NET-A or EtOH (0.1% v/v, vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative (A) GILZ (B) I κ B α , (C) IL-6, (D) IL-8 and (E) RANTES gene expressions was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (EtOH) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. To verify that the CHX inhibited *de novo* protein synthesis. End1/E6E7 cells were seeded into 12-well plates and incubated for 24 hrs. Thereafter, the cells were pre-treated with 1 $\mu\text{g/ml}$ cycloheximide (CHX) then treated with 100 nM DEX or EtOH (0.1% v/v, vehicle) for 24 hrs. (F) Cells were harvested with 2X SDS sample buffer and equal volumes of lysate were analysed by Western blotting with an antibody specific for I κ B α and GAPDH (loading control). (G) Western blots of four independent experiments were quantified to determine the relative GR protein expression. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA with either a Dunnett post-test or a paired t-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

4.4 DEX and MPA recruit GR to the promoters of the IL-6 and IL-8 gene

Having shown that GR directly regulates expression of the inflammatory genes, a further investigation into the mechanism of transcriptional regulation of these genes via the GR was performed. Attempts to perform ChIP assays in the End1/E6E7 cells using the same conditions as described below were unsuccessful. This may be due to high background and low sensitivity for ChIP signals in these cells. Concurrent experiments with synthetic progestins were done in the HeLa cell line to investigate the hypothesis that HIV-1 Vpr acts a GR co-activator (as described in Chapter 5) and it was established that the HeLa cells contain functional GR. It was therefore decided to perform the ChIP assays in the HeLa cell line.

HeLa cells were stimulated for 1 hr with 100 nM DEX, MPA, P4 and NET-A, and ChIP assays were performed using an anti-GR antibody for immunoprecipitation. In addition, gene expression experiments at a 4 hr time point with 100 nM of ligands were done in the HeLa cells and it was determined that the effects of the ligands on gene expression of GILZ, IL-6 and IL-8 were similar to that obtained in the End1/E6E7 cell line (Addendum Figure 1). Figure 4.9 A, shows that stimulation with DEX, but not MPA resulted in the recruitment of the GR to the GILZ promoter. Furthermore, DEX and MPA stimulation resulted in the recruitment of the GR to the IL-6 and IL-8 promoters (Figure 4.9 B and C). Interestingly, it appears that the level of GR recruitment in response to the ligands is gene-specific. Figure 4.9 B shows that stimulation with DEX and MPA recruit similar amount of GR to the IL-6 promoter, whereas Figure 4.9 C shows that stimulation with DEX recruits more GR to the IL-8 promoter than MPA. In summary these results strongly suggest that both DEX and MPA suppress inflammation in the cervical cells by a mechanism of activating and thereby recruiting the GR to promoters of these genes and consequently inducing transcription of the anti-inflammatory gene GILZ, while repressing transcription of the pro-inflammatory genes IL-6 and IL-8.

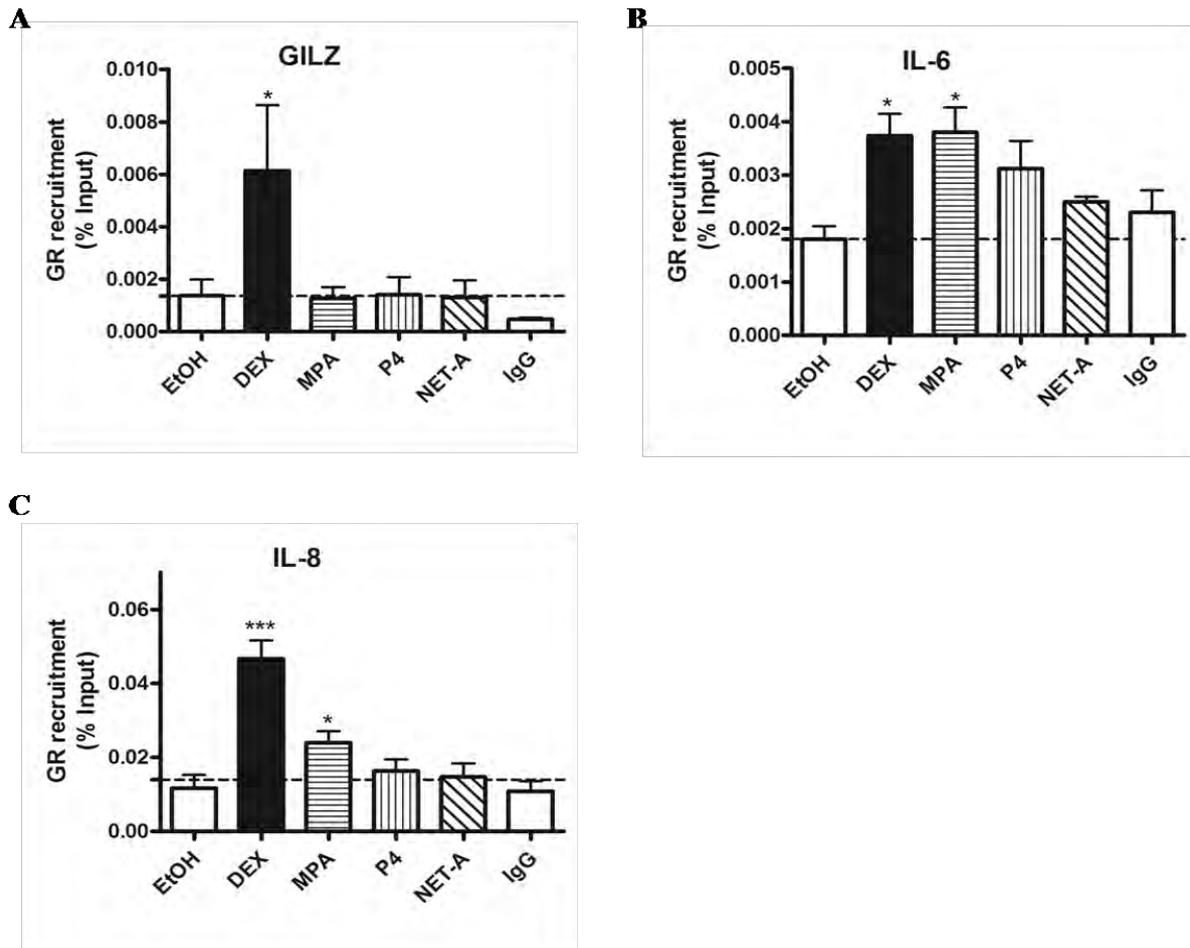


Figure 4.9: DEX and MPA recruit GR to the IL-6 and IL-8 cytokine gene promoters. HeLa cells were seeded into 10 cm dishes at a density of 300×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were serum starved for 2 hrs and then treated with 100 nM DEX, MPA, P4, NET-A or EtOH (0.1% v/v, vehicle) for 1 hr. ChIP was carried out using an anti-GR antibody to immunoprecipitate endogenous GR and an anti-IgG antibody as a negative control. qRT-PCR was performed on input and immunoprecipitated DNA with primers specific for endogenous (A) GILZ, (B) IL-6 and (C) IL-8 promoters. GR recruitment was measured relative to input. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA with Dunnett post-test. Statistical significance is denoted by * or *** to indicate $P < 0.05$ or $P < 0.0001$, respectively.

4.5 Effect of synthetic progestins on inflammatory gene regulation in a primary endocervical model (VEN-100)

Having shown that the progestogens differentially regulate the immune response genes in two cervical cell lines, gene regulation in a more physiologically relevant model was next investigated. The model used consisted of a bio-engineered multilayer of a primary endocervical cells (VEN-100) purchased from MatTek Corporation (Ashland, MA, USA). Upon arrival of the cells, they were incubated for 24 hrs and then treated with 1 nM, 10 nM and 100 nM DEX or MPA for 48 hrs. Thereafter, gene expression was measured by qRT-PCR. In Figure 4.10 A it appears that neither DEX nor MPA induced GILZ gene expression in the VEN-100. In order to determine viability of the cells a MTT based cell growth determination test was done on both day of treatment and day of harvest. Figure 4.10 B shows that the cells were viable on day of treatment. However, on day of harvest the viability of the cells had significantly decreased (Figure 4.10 B).

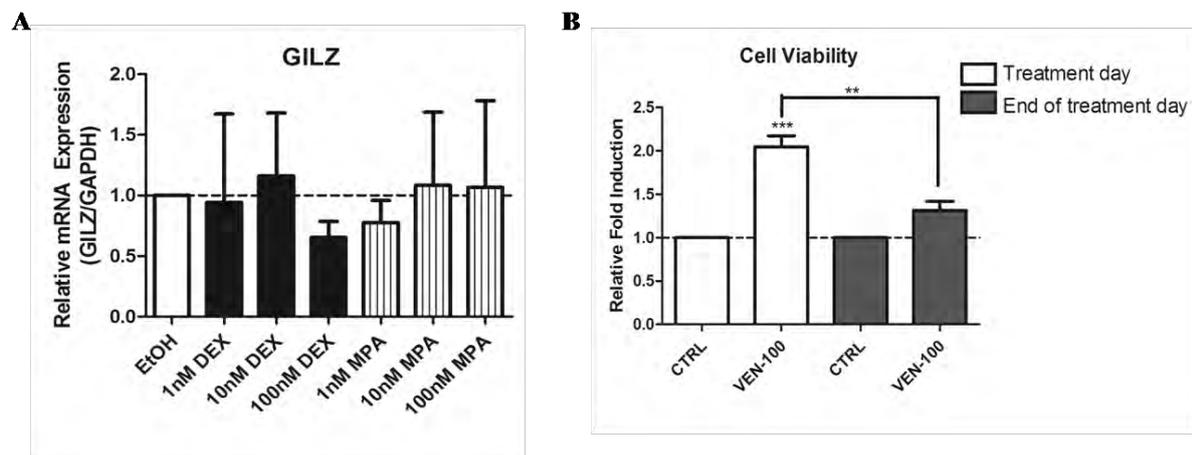


Figure 4.10: The primary cells were unresponsive and non-viable on day of harvest (A) VEN-100 cells were incubated for 24 hrs. Thereafter, the cells were either treated with an increasing dose (1 nM, 10 nM and 100 nM) of DEX, MPA or with EtOH (0.1% v/v, vehicle) for 48 hrs. Then, the cells were washed twice with PBS and harvested for total RNA with

TRIzol® and 500 ng RNA was reverse-transcribed. Relative GILZ gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (EtOH) in order to obtain relative fold expression. Graph represent results of at least three independent experiment performed in duplicate and are plotted as mean +/- SEM. **(B)** VEN-100 cells were either incubated for 24 hrs (treatment day) or 72 hrs (end of treatment day). Then, a cell-growth determination (MTT based) assay was performed. Absorbance readings were measured at wavelength at 570 nm. Cell culture media served as the CTRL (control). CTRL for each day was set to 1 to obtain relative fold induction of cell viability. Graph represent results of at least three independent experiments and are plotted as mean +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA with Dunnett post-test and an unpaired t-test. Statistical significance is denoted by * or *** to indicate $P < 0.05$ or $P < 0.0001$, respectively.

Concurrent to the dose experiment, the effect of the synthetic progestins in the presence of the GR/PR agonist (RU-486) was also investigated. In Figure 4.11 A, it appears that all the ligands induce expression of GILZ mRNA, but this was not statistically significant. The treatment with RU-486 does not appear to affect the ligand-mediated induction of GILZ; however this was not significant (Figure 4.11 A). NET-A appears to upregulate IL-6 gene expression (Figure 4.11 B). However, DEX, MPA and P4 do not appear to regulate IL-6. For IL-6, the treatment with RU-486 appears to upregulate basal gene expression. It appears that the combination of ligands with RU-486 also upregulates gene expression (Figure 4.11 B). Again, it is not surprising that the cells did not respond as expected since the viability of the cells did decrease significantly by the time they were harvested for analysis. Therefore, unfortunately, not much can be suggested from these results.

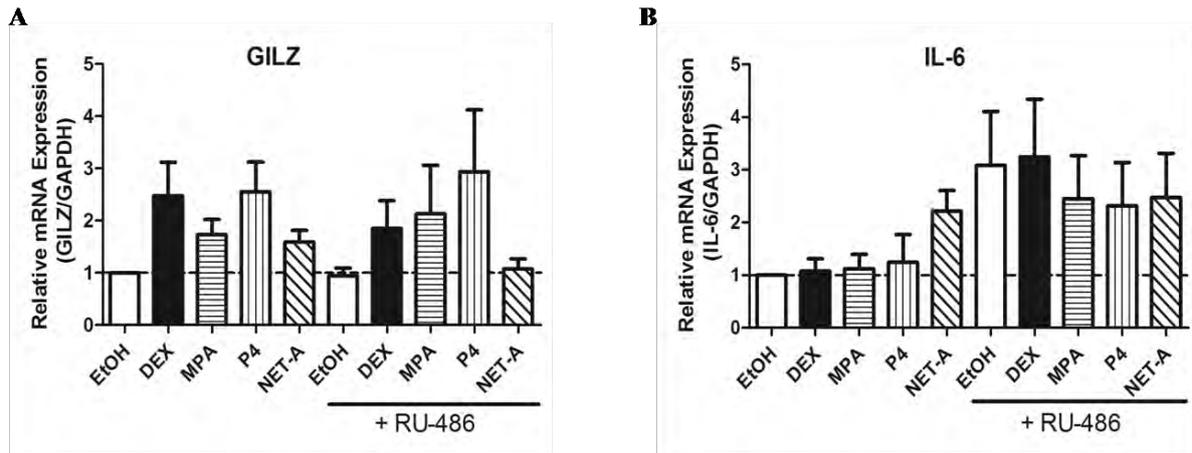


Figure 4.11: The primary cells appear to respond non-specifically to ligand treatment. VEN-100 cells were incubated for 24 hrs. Thereafter, the cells were treated with or without 1 μ M RU-486 and 100 nM DEX, MPA, P4, NET-A or EtOH (0.1% v/v, vehicle). Then, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative **(A)** GILZ and **(B)** IL-6 gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (EtOH) in order to obtain relative fold expression. Graphs represent results of one independent experiment performed in duplicate and are plotted as mean \pm SEM.

4.6 Primary endocervical cells (VEN-100) express endogenous GR, MR and ER mRNA and GR protein

Although the gene regulation experiments in the primary model were not conclusive, the SR content of the VEN-100 model was examined since the cells were still viable at this time-point. Upon arrival of the cells, they were harvested for total RNA and screened for SR mRNA by qRT-PCR using primer pairs designed specifically for each receptor followed by gel electrophoresis. In addition, the cells were harvested for assessment by Western blotting. For the Western blotting analysis, COS-1 cells were transiently transfected with GR or empty vector to produce positive and negative controls, respectively. Figure 4.12 A shows, for the

first time, that the VEN-100 primary cells contain endogenous GR, MR and ER α mRNA. In addition, these primary cells contain endogenous GR protein (Figure 4.12 B).

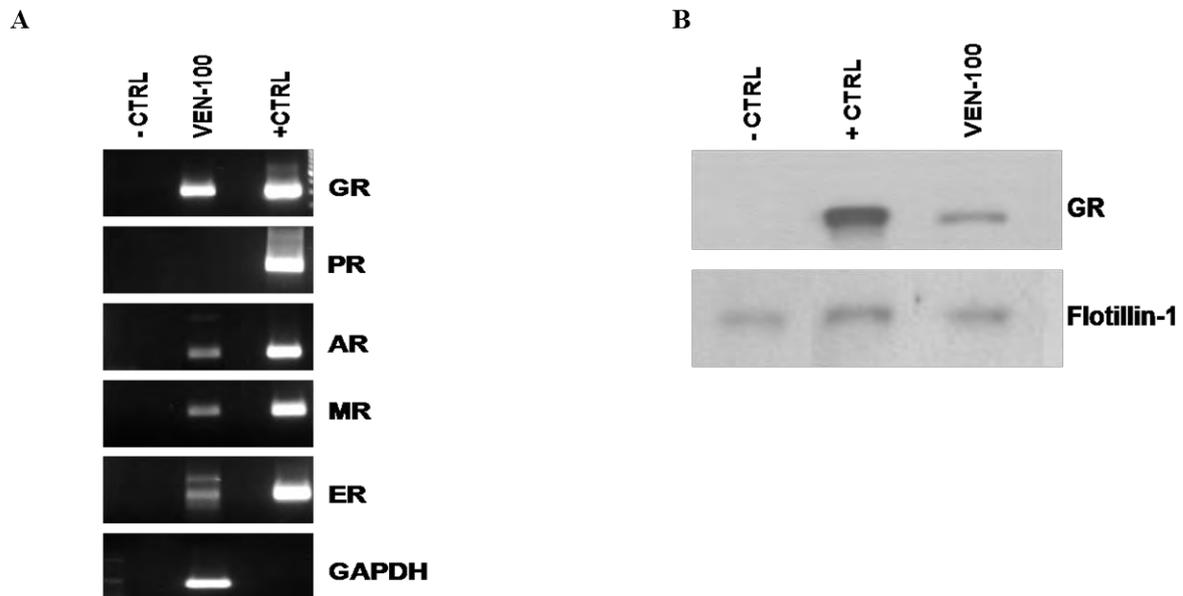


Figure 4.12: VEN-100 primary cells contain GR, AR, MR and ER mRNA and GR protein. (A) Upon arrival the VEN-100 cells were incubated for 24 hrs. Thereafter, cells were washed once with PBS and harvested with TRIzol®. Total RNA was isolated and 500 ng RNA was reverse-transcribed. Steroid receptor gene expression was measured by qRT-PCR with receptor specific and GAPDH (loading control) primers, followed by gel electrophoresis to confirm the PCR products. (B) For the controls, COS-1 cells seeded into a 12-well plate. After 24 hrs, the cells were transiently transfected with 1 μ g/well pcDNA3 (empty vector) which served as negative control (-CTRL) or with 1 μ g/well GR expression vector which served as positive control (+CTRL). 24 hrs later, the COS-1 cells were washed once and lysed with 2X SDS sample buffer. VEN-100 cells were incubated for 24 hrs. Thereafter, cells were washed once with PBS and were lysed with 2X SDS sample buffer. Equal volumes of lysate were analysed by Western blotting with antibodies against anti-GR specific antibody and Flotillin-1 (loading control).

Chapter 5: An investigation into the role of HIV-1 Vpr in GR-mediated inflammatory gene regulation in the presence of DEX, P4 and the synthetic progestins (Results).

Aims

The aim of this chapter was to investigate the hypothesis that HIV-1 Vpr acts as a co-activator of the GR and results in the modulation of GR-regulated inflammatory genes in the presence of DEX and the synthetic progestins. In order to investigate this hypothesis the main goals were to (i) determine whether the P4 and the synthetic progestins differentially regulate GR-regulated reporter and inflammatory endogenous genes in the HeLa cell line as found for the End1/E6E7 cell line, (ii) determine whether Vpr modulates GR-regulated reporter and inflammatory endogenous genes in the presence of DEX and the progestogens.

Results

5.1 The GR is the predominant SR protein expressed in the cervical cell lines

As mentioned in the previous chapter, the synthetic progestins and P4 display differential SR selectivity. In order to determine whether the ligands differentially regulate GR-regulated genes in the HeLa cells similar to that of the End1/E6E7 cells and to determine whether Vpr plays a role in GR-mediated gene regulation in the HeLa and TZM-bl cell lines, the SR content of both the cell lines had to be first examined. Cell lysates were prepared and the SR mRNA and protein levels were detected by qRT-PCR and Western blotting, respectively. Figure 5.1 A indicates that both HeLa and TZM-bl cells contain GR, AR and MR mRNA. However, it is shown in Figure 5.1 B that the HeLa cells express only GR protein while the TZM-bl cells express GR and AR protein. It appears that the HeLa and TZM-bl cells contain

MR protein (Figure 5.1 B) however this is a non-specific band that also appears in the negative control. Still, it is possible that the HeLa cells do contain small amount of the AR and MR protein that is beyond the detection level of the Western blots.

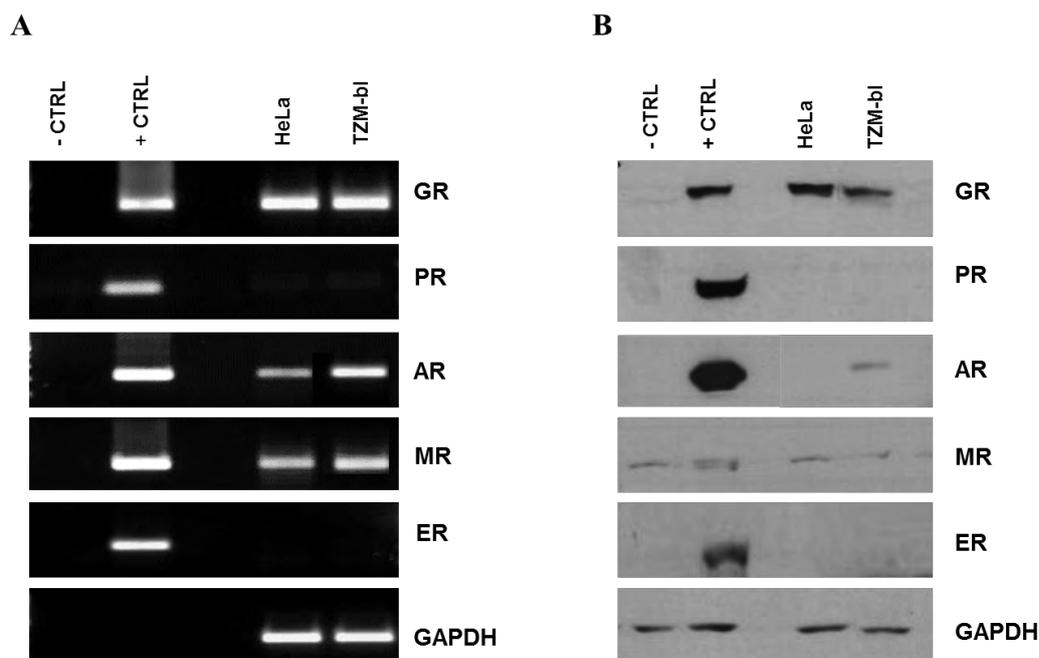


Figure 5.1: HeLa cells only express detectable GR protein, while TZM-bl cells express GR and AR protein. (A) HeLa and TZM-bl cells were seeded into 12-well plates at a density of 15×10^4 and 10×10^4 cells/well, respectively and incubated for 24 hrs. Thereafter, cells were washed once with PBS and harvested with TRIzol. Total RNA was isolated and 500 ng RNA was reverse-transcribed. Steroid receptor gene expression was measured by qRT-PCR with receptor specific and GAPDH (loading control) primers, followed by gel electrophoresis to confirm the PCR products. (B) HeLa and TZM-bl cell lines were seeded into 12-well plates and incubated for 24 hrs. Thereafter, cells were washed once with PBS and were lysed with 2X SDS sample buffer. COS-1 cells were seeded into a 12-well plate. After 24 hrs, the cells were transiently transfected with 1 μ g/well pcDNA3 (empty vector) served as negative control (-CTRL) or with 1 μ g/well steroid receptor expression vectors (pcDNA3-hGR, pMT-PR-B, pSV-hAR, pRS-hMR and pSG5-hER) served as positive controls (+CTRL). After 24 hrs, the COS-1 cells were washed once and lysed with 2 X SDS

sample buffer. Equal volumes of lysate were analysed by Western blotting with antibodies against steroid receptor specific antibodies and GAPDH specific antibody as loading control.

It was next determined if the endogenously expressed GR, AR and MR in the HeLa cells are functional. Luciferase reporter assays were performed using overexpressed SRs which served as positive controls for a transactivation response via the respective receptor-specific agonists. Figure 5.2 A shows that when HeLa cells were stimulated with the GR agonist, DEX, a significant transactivation response occurs and the addition of exogenous GR produced a greater DEX-induced response. However, when the HeLa cells were stimulated with AR and MR specific agonists (MIB and ALD, respectively) no significant response was detected (Figure 5.2 B and C). As expected, when the cells were transfected with either AR or MR and then stimulated with MIB and ALD, transactivation occurred (Figure 5.2 B and C). These results indicate that in the HeLa cells only endogenous GR is functional. In order to confirm that the expressed SRs in the HeLa cells are functional, endogenous transactivation (GILZ) and transrepression (IL-6 and IL-8) models were used. HeLa cells were stimulated with SR specific agonists (DEX, MIB and ALD) and gene expression was measured by qRT-PCR. Figure 5.2 D indicates that when HeLa cells were stimulated with the SR agonists, only DEX-induced a significant transactivation of GILZ which is consistent with the endogenous GR being active. In addition, only DEX significantly repressed IL-6 gene expression (Figure 5.2 E). Although not statistically significant, it appears that ALD may upregulate IL-6 expression (Figure 5.2 E). In Figure 5.2 F, although it appears that DEX may repress IL-8, statistical significance was not established. ALD significantly induces IL-8 mRNA expression and although it appears that MIB also induces IL-8 mRNA expression, this induction is not statistically significant (Figure 5.2 F). These results are consistent with the reporter-promoter assay results and show that endogenous GR is functional. In addition, it

shows that only the GR-specific agonist could induce the transactivation of GILZ mRNA and transrepression of IL-6 mRNA and possibly IL-8 mRNA. Furthermore, the significant induction of IL-8 mRNA by the MR agonist indicates that the MR is also functional.

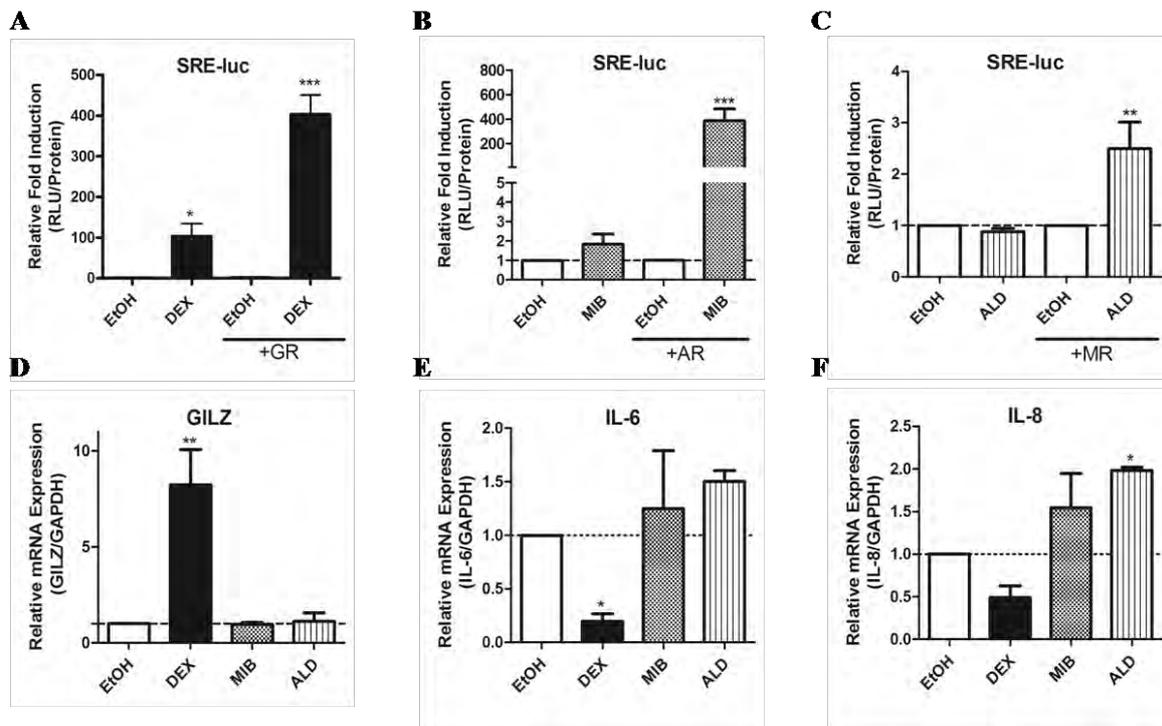


Figure 5.2: Receptor-specific agonists induced changes in mRNA levels indicate that the GR is functional in the HeLa cells. HeLa cells (A-C) were seeded into 24-well plates at a density of 7.5×10^4 cells/well and incubated for 24 hrs. Thereafter, cells were transiently transfected for 24 hrs with 82 ng/well TAT-GRE, 125 ng/well pcDNA-3 (empty vector) and either 125 ng/well of (A) pcDNA3-hGR, (B) pSV-hAR or (C) pRS-hMR. Then, the cells were treated with 100 nM DEX, 100 nM MIB, 10 nM ALD or EtOH (0.1% v/v; vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and lysed with 1X reporter lysis buffer and the luciferase activity was measured. Luciferase activity was normalized to protein content per well as determined by Bradford assay. In addition, the ligand stimulated responses were normalized with the basal activity (EtOH) in order to obtain a relative fold induction. HeLa cells (D-F) were seeded into 12-well plates at a density of 15×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were treated for 4 hrs with 100 nM DEX, 100 nM MIB, 10 nM ALD or EtOH (0.1% v/v; vehicle). Then, the cells were washed once with PBS and harvested with TRIzol. Total RNA was isolated and 500 ng RNA was reverse-

transcribed. Relative **(D)** GILZ **(E)** IL-6 and **(F)** IL-8 gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (EtOH) in order to obtain fold expression. Graphs **(A-C)** represent pooled results of at least three independent experiments, performed in triplicate and are plotted as mean \pm SEM. Graphs **(D-F)** represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with Dunnett post-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

5.2 MPA, unlike NET-A and P4, acts like a GR partial agonist for transactivation of a GRE reporter gene in the HeLa cell line

Having established that the HeLa cells contain functional endogenous GR protein, it was next investigated whether the synthetic progestins would differentially regulate GR-regulated GRE-and IL-6 reporter genes in the HeLa cell line. HeLa cells were transiently transfected with either TAT-GRE, IL-6-luc minimal promoter or IL-6-luc full length promoter reporter constructs. For the TAT-GRE reporter assay, the cells were treated with saturating concentrations of progestins MPA, P4 and NET-A and the GR agonist DEX for 24 hrs. For the IL-6-luc minimal and IL-6-luc full length reporter assays, the cells were treated with saturating concentrations of progestins DEX, MPA, P4 and NET-A but in the presence and absence of TNF- α . Thereafter, luciferase activity was measured. As expected, DEX induced the highest transcriptional response of the TAT-GRE reporter (~80 fold) followed by partial agonist MPA (~13 fold) while P4 and NET-A produced a response similar to basal (Figure 5.3 A). However, unexpectedly, DEX did not repress the transcriptional activity of basal or TNF- α -induced IL-6-luc minimal and IL-6-luc full length promoter reporters (Addendum Figure 2). In addition, the ligands did not produce a typical response on the IL-6-luc minimal

promoter or IL-6-luc full length reporter genes (Addendum Figure 2). It was therefore decided that these IL-6-luc reporter genes were not an ideal transrepression model in this cell line, while the GR transactivation model experiments indicate that P4 and the synthetic progestins MPA and NET-A differentially regulate the expression of the GRE-reporter gene.

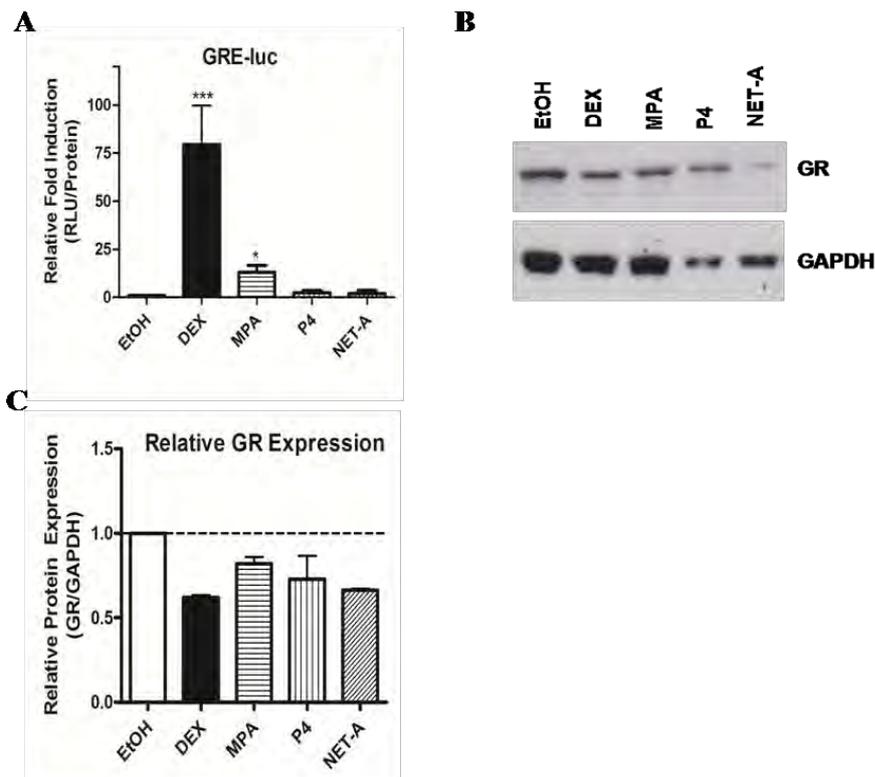


Figure 5.3: MPA, unlike P4 or NET-A, induces transactivation of a GRE-reporter gene.

(A) HeLa cells were seeded into 24-well plates at a density of 7.5×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with 82 ng/well TAT-GRE. After 24 hrs, the cells were treated with 100 nM DEX, 1 μ M MPA, 10 μ M P4, 10 μ M NET-A or EtOH (0.1% v/v; vehicle) for 24 hrs. Then, the cells were washed twice with PBS and lysed with 1X reporter lysis buffer and the luciferase activity was measured. Luciferase activity was normalized to protein content per well as determined by Bradford assay. In addition, the ligand stimulated responses were normalized with the basal activity (EtOH) in order to obtain a fold induction. **Effect of progestins on GR protein expression.** (B) After transactivation assays the remaining HeLa cell lysates were prepared for Western blotting

analysis by adding 5X SDS sample buffer. Then equal amounts of sample were loaded on SDS page gels and analyzed by Western blotting with antibodies specific for GR as well as GAPDH (loading control). The Western blot shown is a single representative blot. **(C)** GR protein levels were quantified and normalized to GAPDH protein levels; additionally the ligand stimulated responses were normalized to basal activity (EtOH). Graph **(A)** represents pooled results of at least two independent experiments, performed in triplicate, and are plotted as means \pm SEM. Relative protein expression graph **(C)** represents pooled results of at least two independent experiments and are plotted as means \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with Dunnett post-test and a paired t-test. Statistical significance is denoted by * or *** to indicate $P < 0.05$ or $P < 0.0001$, respectively.

Having shown that the progestogens differentially regulate a GR transactivation reporter model gene in the HeLa cells, Western blotting was next used to determine whether the ligands would differentially regulate GR protein levels. This was done by re-suspending the cell lysates after the transactivation assays with 5X SDS sample buffer and assessment of the samples by western blotting. From Figure 5.3 B it appears that all the ligands reduce the GR levels as compare to vehicle (EtOH). However, stimulation with P4 and NET-A may have an effect on cell numbers/cell death as seen by the loading control GAPDH (Figure 5.3 B). After quantification and normalization (Figure 5.3 C) it appears that DEX and NET-A reduces GR protein levels, which is an indication of GR protein turnover, more than P4 and MPA. It is worth noting that the concentration (10 μ M) of P4 and NET-A used may be toxic to the cells as indicated by the lower levels of GAPDH protein in comparison to the EtOH (control) lane shown in Figure 5.3 B. It is suggested that the lower levels of GAPDH could be due to cell death because prior to harvest the cells were viewed under the microscope and there were fewer cells in P4 and NET-A conditions compared to the control. In addition, equal volumes of harvested protein were loaded on the SDS page gel

5.3 Vpr co-activates the DEX- and MPA-mediated transactivation of a GRE reporter gene

Having established that MPA, unlike P4 or NET-A, acts a partial agonist for the GR in the HeLa cell line, the hypothesis that Vpr acts a co-activator of the GR in the presence of DEX and the progestogens could now be investigated. HeLa cells were transiently transfected with TAT-GRE and HA-Vpr or empty expression vectors prior to treatment with saturated concentrations of ligands for 24 hrs. In the absence of Vpr, only DEX (~40 fold) and MPA (~8.3 fold) induce transactivation (Figure 5.4 A), which is consistent with the trend of the previous result (Figure 5.3 A). When Vpr protein was expressed, there was no change in basal activity (EtOH) but there was a significant increase in both the DEX-mediated transactivation (with a fold increase from ~40 to ~321) and MPA-mediated transactivation (with a fold increase from ~8.3 to ~67). Figure 5.4 B shows the expression of Vpr protein (~20 kDa) in the cells, while Figure 5.4 C shows that Vpr did not affect the levels of endogenous GR.

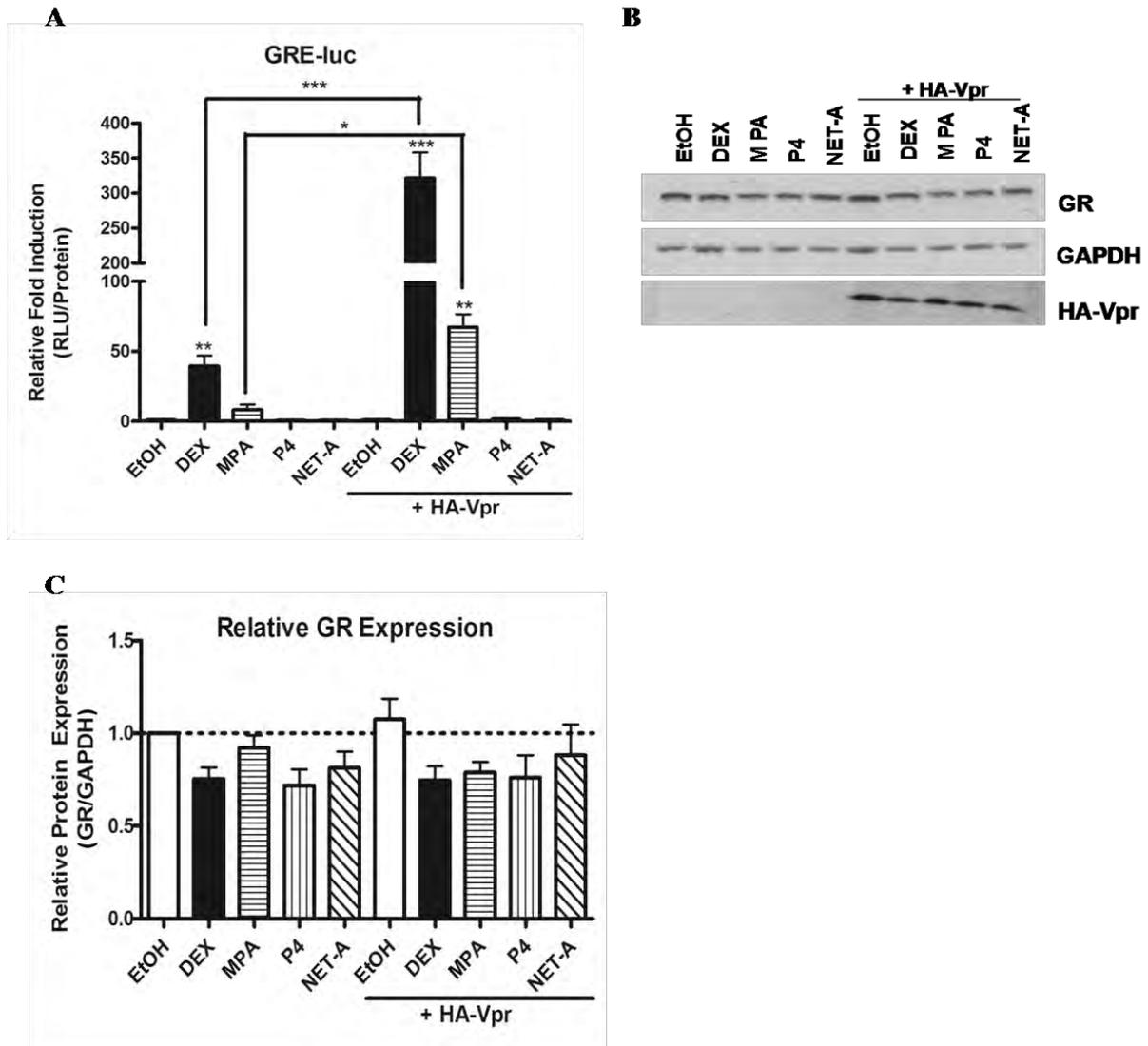


Figure 5.4: Vpr enhances DEX- and MPA- mediated transactivation of a GRE-reporter gene. (A) HeLa cells were seeded into a 24-well plate at a density of 7.5×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with 82 ng/well TAT-GRE and 125 ng/well HA-Vpr or empty vector. After 24 hrs, the cells were treated with 100 nM DEX, 1 μ M MPA, 10 μ M P4, 10 μ M NET-A or EtOH (0.1% v/v; vehicle) for 24 hrs. The cells were then washed twice with PBS and lysed with 1X reporter lysis buffer and the luciferase activity was measured. Luciferase activity was normalized to protein content per well as determined by Bradford assay. In addition, the ligand stimulated responses were normalized with the -Vpr basal activity (EtOH) in order to obtain a fold induction. **Relative GR protein expression.** (B) After transactivation assays the remaining HeLa cell lysates were prepared for analysis by Western blotting by adding 5X SDS sample buffer. Then equal amounts of sample were loaded on SDS page gels and analyzed by Western blotting. The

blots were cut in strips and probed with antibodies specific for GR, HA- and GAPDH (loading control). The Western blot shown is a single representative blot. (C) GR protein levels were quantified and normalized to GAPDH protein levels; additionally the ligand stimulated responses were normalized to basal activity (EtOH). Graph (A) represents pooled results of at least two independent experiments, performed in triplicate, and are plotted as means \pm SEM. Relative protein expression graph (C) represents pooled results of at least three independent experiments and are plotted as means \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with a Newman-Keuls Multiple Comparison Test and a paired t-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

5.4 Vpr does not affect the DEX- or MPA-mediated transactivation and transrepression of the endogenous inflammatory genes

Having shown on a GRE-reporter gene that MPA acts like a GR agonist for transactivation and subsequently Vpr co-activates the DEX- and MPA-mediated transactivation, it was next confirmed if this occurs on endogenous GR-regulated inflammatory genes; GILZ, I κ B α , IL-6, IL-8 and RANTES. HeLa cells were either transiently transfected with HA-Vpr or empty expression vectors and then treated with saturating concentrations of DEX, MPA, P4 and NET-A. Thereafter, mRNA expression was measured by qRT-PCR. The experiments to investigate the effects of the synthetic progestins on the regulation of the endogenous genes were done in parallel with the experiments to investigate the effect of Vpr on the modulation of the endogenous genes in the presence of the ligands. Thus, the results in the absence of Vpr will be reported first. Both DEX and MPA induced transactivation of GILZ and I κ B α mRNA (Figure 5.5 A and B, respectively), however, P4 and NET-A had no effect on GILZ and I κ B α transcription.

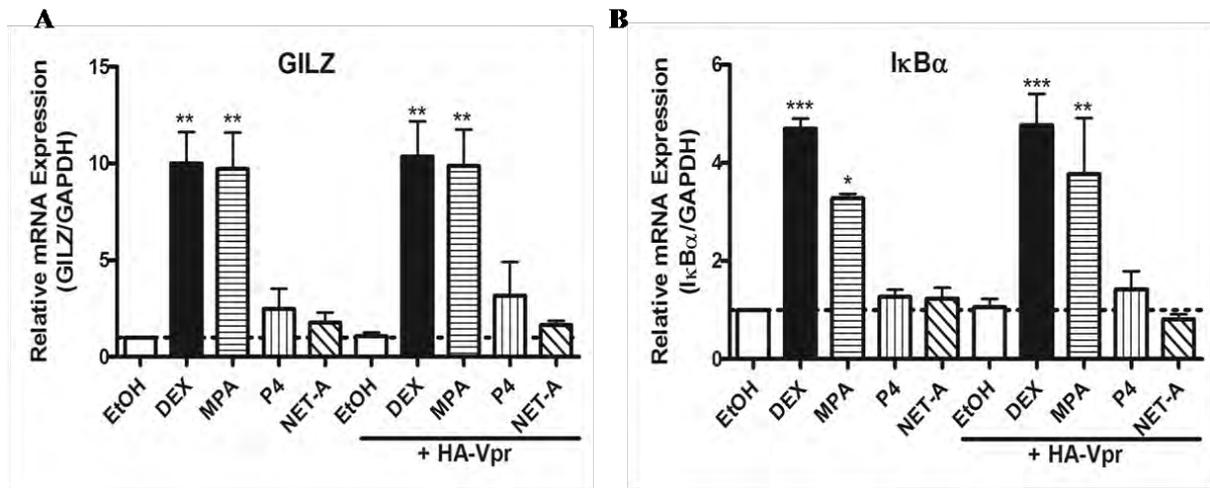


Figure 5.5: Vpr does not affect DEX- and MPA-mediated transactivation of GILZ and IκBα. HeLa cells were seeded into a 12-well plate at a density of 15×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with 1 μ g/well HA-Vpr or empty vector. After 24 hrs, the cells were treated with 100 nM DEX, 1 μ M MPA, 10 μ M P4, 10 μ M NET-A or EtOH (0.1% v/v; vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative **(A)** GILZ and **(B)** IκBα gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalized to –Vpr basal activity (EtOH) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with Dunnett post-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

Figure 5.6 A shows that MPA, like DEX, repressed the expression of basal IL-6. In contrast, although not statistically significant, P4 and NET-A appear to upregulate IL-6 mRNA expression. In Figure 5.6 B, although it appears that DEX may repress IL-8 expression, this is not statistically significant. Interestingly, P4 significantly upregulated IL-8 mRNA expression, while both MPA and NET-A did not affect mRNA expression (Figure 5.6 B). The ligands did not appear to regulate RANTES mRNA expression in the HeLa cells (Figure

5.6 C). However this could be due to low basal levels of RANTES in the HeLa cells (as indicated by real-time qRT-PCR Ct values, data not shown). The results on the endogenous gene models are consistent with the GRE-reporter gene results and shows that the synthetic progestins differentially regulate the expression of these genes. In addition, it confirms that MPA, unlike NET-A, acts like a GR partial agonist for transactivation on the endogenous genes; it also shows that MPA acts like a GR partial agonist for transrepression on the IL-6 endogenous gene.

The presence of Vpr did not affect basal regulation of GILZ or $\text{I}\kappa\text{B}\alpha$ mRNA expression (Figure 5.5 A and B, respectively). Also, expression of Vpr protein did not affect the DEX- and MPA-mediated regulation of GILZ or $\text{I}\kappa\text{B}\alpha$. Similar results are obtained for RANKL mRNA expression (Addendum Figure 3). Similarly to the endogenous GR-transactivation genes, Vpr did not appear to affect the ligand-mediated regulation of IL-6 (Figure 5.6 A). Although it appears that Vpr may upregulate basal IL-6 mRNA expression, statistical significance could not be established in this multi-variable experiment. Similar to the results obtained on IL-6, it appears that Vpr upregulates basal expression and has no effect on the ligand-mediated regulation of IL-8 mRNA. In addition, like the other transrepression model genes, it appears that Vpr did not affect the regulation of RANTES mRNA in the presence of the ligands. Again, although not statistically significant, it appears Vpr upregulates basal RANTES mRNA expression. Figure 5.6 D shows the expression of Vpr protein (~20 kDa) in the cells and also shows that Vpr did not affect the levels of endogenous GR protein. In addition, it shows that the ligands do not regulate Vpr protein expression. In summary, these results show that Vpr does not affect the ligand-mediated regulation of the endogenous transactivation or transrepression model genes. However, it appears that Vpr may regulate the basal levels of the endogenous transrepression model genes.

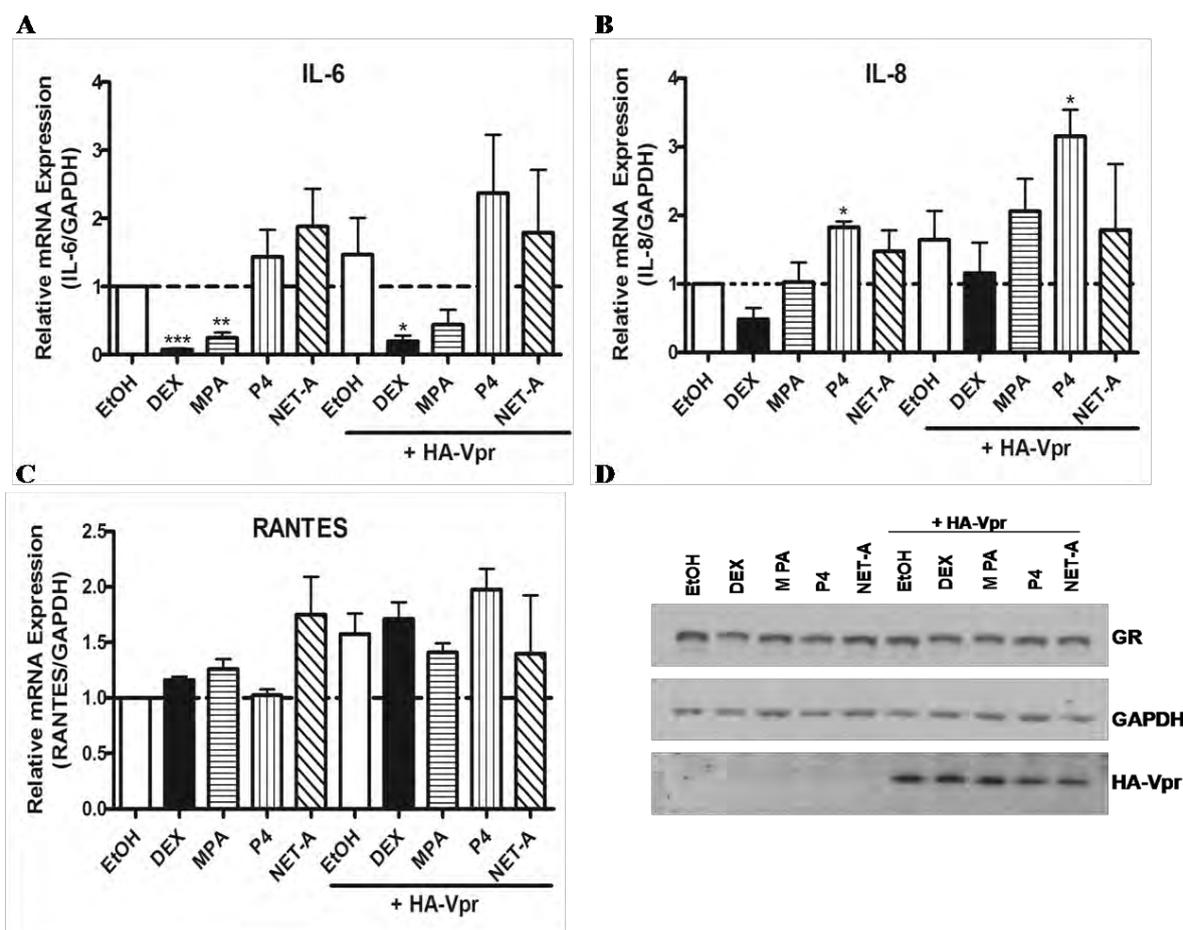


Figure 5.6: Vpr does not affect DEX- and MPA-mediated repression of IL-6 HeLa cells were seeded into a 12-well plate at a density of 15×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with $1 \mu\text{g/well}$ HA-Vpr or empty vector. After 24 hrs, the cells were treated with 100 nM DEX, $1 \mu\text{M}$ MPA, $10 \mu\text{M}$ P4, $10 \mu\text{M}$ NET-A or EtOH (0.1% v/v; vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested for either total RNA with TRIzol® and 500 ng RNA was reverse-transcribed or for whole cell lysate with 2X SDS sample buffer. Relative (A) IL-6, (B) IL-8 and (C) RANTES gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalized to –Vpr basal activity (EtOH) in order to obtain relative fold expression. **Relative protein expression.** (D) Equal amounts of whole cell lysates were loaded on SDS page gels and analyzed by Western blotting. The blots were cut in strips and probed with antibodies specific for GR, HA- and GAPDH (loading control). Graphs (A-C) represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with a Dunnett

post-test and a paired t-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

5.5 Vpr dose-dependently enhances the DEX-mediated regulation of a GRE-reporter gene

Having shown that 125 ng of the Vpr expression vector enhanced DEX- and MPA-mediated induction of a GRE-reporter gene, while 1 μg of the Vpr expression vector did not affect ligand-mediated regulation of the endogenous genes, these results led to the hypothesis that the effect of Vpr on gene regulation may be dose-dependent. Therefore it was next investigated whether Vpr affected ligand-mediated gene regulation in a dose-dependent manner. This was done by transiently transfecting HeLa cells with TAT-GRE and increasing amounts of HA-Vpr (25 ng, 125 ng and 300 ng)/well, before treating with 100 nM DEX for 24 hrs. Thereafter, luciferase activity was measured. As expected, DEX induced transactivation of the GRE-reporter gene (Figure 5.7 A). Figure 5.7 A, also shows that increasing amounts of Vpr dose-dependently enhanced the DEX-mediated induction of the reporter gene while having no effect on the basal levels of the gene. The expression of Vpr protein in the cells is shown in the Western blot (Figure 5.7 B). The smaller amounts of Vpr (25 ng and 125 ng) could not be detected using this method. This was unexpected since 125 ng Vpr was detected by Western blot in earlier experiments (Figure 5.4 B). This discrepancy could be due to differences in transfection efficiency between the experiments. This suggestion is further supported by the differences in relative fold induction shown in Figure 5.4 A and Figure 5.7 A. In retrospect, a transfection control such as the *Renilla* luciferase control reporter vector should have been used to normalize luciferase activity. The Western blot also shows that increasing amounts of Vpr do not affect GR protein levels (Figure 5.7 B). In addition, Figure 5.7 B shows that GR protein levels are reduced by DEX. The result that

Vpr dose-dependently enhances DEX-mediated regulation of the GRE reporter gene is consistent with the hypothesis that Vpr is a co-activator of the GR.

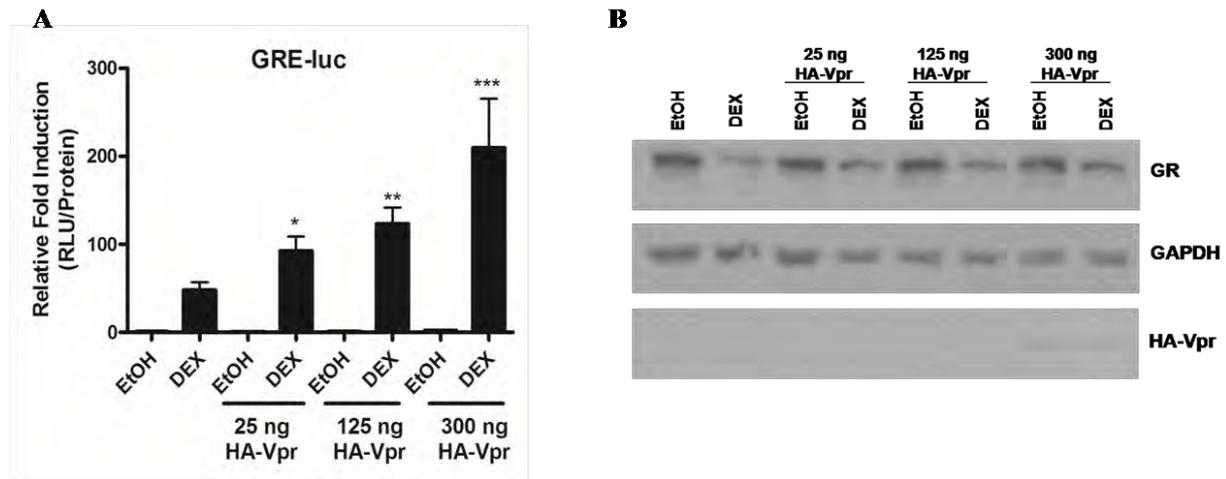


Figure 5.7: Vpr dose-dependently enhances DEX-mediated induction of a GRE-reporter gene. (A) HeLa cells seeded into a 24-well plate at a density of 7.5×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with 82 ng/well TAT-GRE; 25, 125 or 300 ng/well HA-Vpr and relative amounts of empty vector. After 24 hrs, the cells were treated with 100 nM DEX or EtOH (0.1% v/v; vehicle) for 24 hrs. Then, the cells were washed twice with PBS and lysed with 1X reporter lysis buffer and the luciferase activity was measured. Luciferase activity was normalized to protein content per well as determined by Bradford assay. In addition, in order to obtain fold induction the ligand stimulated responses were normalized with the –Vpr basal activity (EtOH). **Relative GR protein expression.** (B) After transactivation assays the remaining HeLa cell lysates were prepared for analysis by Western blotting by adding 5X SDS sample buffer. Then equal amounts of sample were loaded on SDS page gels and analyzed by Western blotting. The blots were cut in strips and probed with antibodies specific for GR and GAPDH (loading control). Graph (A) represents pooled results of at least two independent experiments, performed in triplicate, and are plotted as means \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with a Dunnett post-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

5.6 Increasing amounts of Vpr do not affect the DEX-mediated regulation of the endogenous genes

Having shown that Vpr dose-dependently co-activates the GR-mediated regulation of a reporter gene, it was next investigated whether this occurs on the endogenous genes. This was done again by transiently transfecting HeLa cells with increasing amounts of Vpr (50 ng, 250 ng, 600 ng and 1 μ g)/well and then treating the cells with 100 nM DEX for 24 hrs. Thereafter, mRNA expression was measured by qRT-PCR. DEX induced both GILZ and I κ B α gene expression (Figure 5.8 A and B). Contrary to the reporter gene results, increasing amounts of Vpr did not affect basal or DEX-mediated induction of GILZ (Figure 5.8 A). In addition, Vpr (50 ng, 250 ng and 600 ng) did not appear to affect basal or DEX-mediated induction of I κ B α (Figure 5.8 B). However, although not statistically significant, it appears that 1 μ g Vpr may upregulate basal I κ B α mRNA levels (Figure 5.8 B). The Western blot in Figure 5.8 C shows the expression of Vpr protein in the cells and further confirms that increasing amounts of Vpr do not affect GR protein levels.

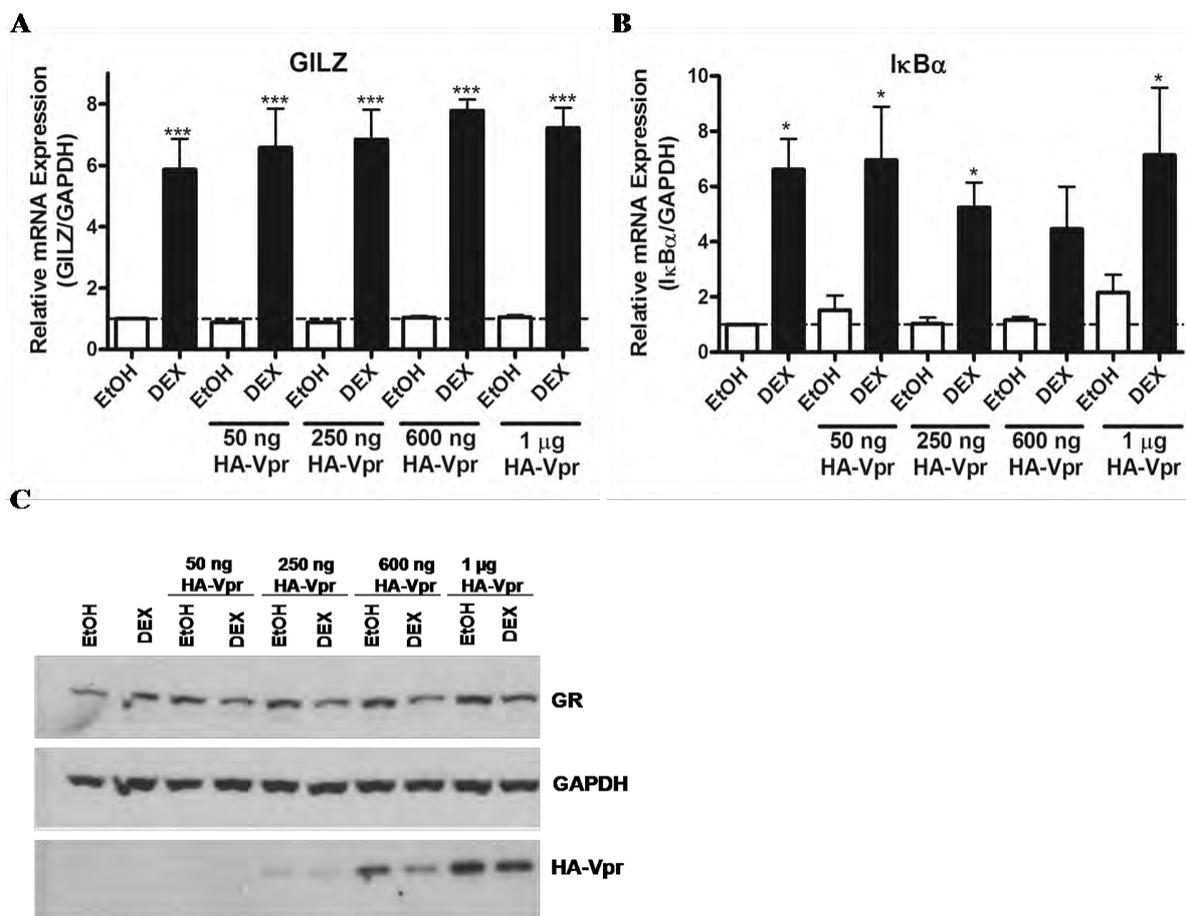


Figure 5.8: Increasing amounts of Vpr do not affect the DEX-mediated induction of GILZ and I κ B α . HeLa cells were seeded into a 12-well plate at a density of 15×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with 60 ng, 250 ng, 600 ng or 1 μ g /well HA-Vpr and relative amounts of empty vector. After 24 hrs, the cells were treated with 100 nM DEX and EtOH (0.1% v/v; vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested for either total RNA with TRIzol® and 500 ng RNA was reverse-transcribed or for whole cell lysate with 2X SDS sample buffer. Relative (A) GILZ and (B) I κ B α gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalized to –Vpr basal activity (EtOH) in order to obtain relative fold expression. **Relative protein expression.** (C) Then equal amounts of sample were loaded on SDS page gels and analyzed by Western blotting. The blots were cut in strips and probed with antibodies specific for GR, HA- and GAPDH (loading control). Graphs (A-B) represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with

Dunnett post-test and a paired t-test. Statistical significance is denoted by * or *** to indicate $P < 0.05$ or $P < 0.0001$, respectively.

DEX repressed both IL-6 and IL-8 mRNA expression (Figure 5.9 A and B). In addition, increasing amounts of Vpr do not appear to regulate the DEX-mediated repression of both IL-6 and IL-8 (Figure 5.9 A and B). Although it appears that 600 ng and 1 μ g Vpr may upregulate basal IL-6, this is not significant (Figure 5.9 A), while in Figure 5.9 B, 1 μ g Vpr significantly upregulated basal IL-8 mRNA expression. The results indicate that, unlike the reporter gene, increasing amounts of Vpr do not affect DEX-mediated regulation of the endogenous genes. However, the results indicate that Vpr may affect the basal levels of endogenous IL-6 and IL-8 mRNA in a ligand-independent manner.

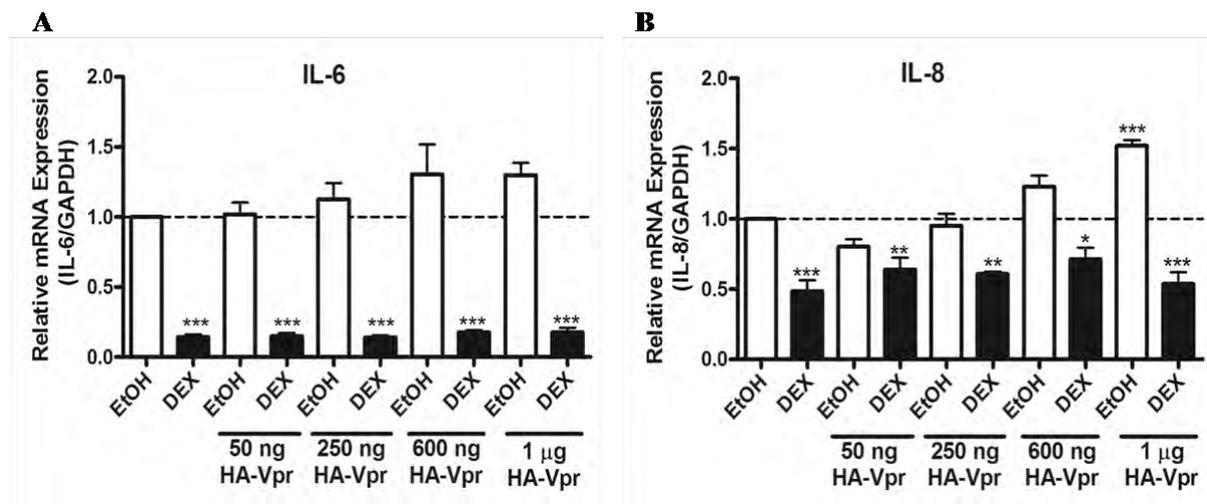


Figure 5.9: Increasing amounts of Vpr do not affect DEX-mediated repression of IL-6 and IL-8. HeLa cells were seeded into a 12-well plate at a density of 15×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with 60 ng, 250 ng, 600 ng or 1 μ g /well HA-Vpr and relative amounts of empty vector. After 24 hrs, the cells were treated with 100 nM DEX and EtOH (0.1% v/v; vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative (A) IL-6 and (B) IL-8 gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene

expression was normalized to basal activity (EtOH) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with Dunnett post-test and a paired t-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

Having shown by transient transfection experiments using a Vpr expression vector that increasing amounts of Vpr do not affect ligand-mediated regulation of the endogenous genes, the affect of Vpr on endogenous gene regulation was next investigated using HIV-1 pseudovirus to deliver Vpr. The HIV-1 pseudovirus experiments were performed in the TZM-bl cell line which is a HeLa cell line modified to express the co-receptors, CCR5 and CXCR4, which are required for HIV-1 infection. TZM-bl cells were infected with increasing amounts of HIV-1 wt (wild-type) or HIV-1 Δ Vpr (Vpr deletion mutant) and incubated for 72 hrs. The cells were then treated with 100 nM DEX for 24 hrs. Thereafter, mRNA expression was measured by qRT-PCR. As expected, DEX induced both GILZ and I κ B α mRNA levels, while the increasing amounts of HIV-1 wt did not affect basal or DEX-induced expression of GILZ and I κ B α (Figure 5.10 A and B). The absence of Vpr (HIV-1 Δ Vpr), also did not appear to affect basal or DEX-induced expression of GILZ and I κ B α (Figure 5.10 A and B).

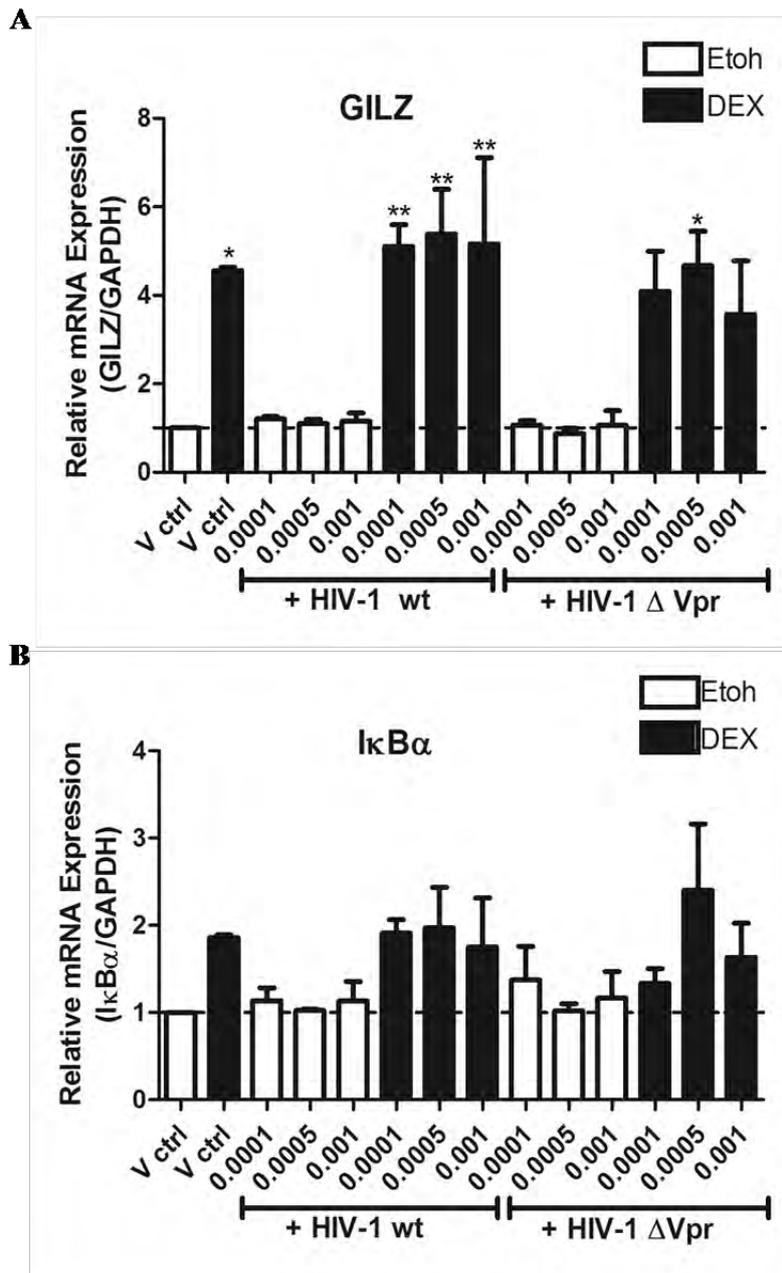


Figure 5.10: Vpr delivered by HIV-1 pseudovirus does not affect DEX-mediated induction of GILZ and I κ B α . TZM-bl cells were seeded into 12-well plates at a density of 10×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were infected for 72 hrs with either virus control (Vctrl), increasing MOI (0.0001, 0.0005 and 0.001) of HIV-1 wt (pNL4-3.E⁻ and pLET-LAI) or HIV-1 Δ Vpr (pNL4-3.E^{R-} and pLET-LAI). Then, the cells were treated with 100 nM DEX and EtOH (0.1% v/v; vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative (A) GILZ and (B) I κ B α gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene

expression was normalized to V ctrl basal activity (EtOH) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA), with Dunnett post-test and a paired t-test. Statistical significance is denoted by * or ** to indicate $P < 0.05$ or $P < 0.001$, respectively.

Again, DEX repressed both IL-6 and IL-8 mRNA levels (Figure 5.11 A and B). In addition, it appears that HIV-wt did not affect DEX-mediated repression of either IL-6 or IL-8 mRNA levels (Figure 5.11 A and B). However, HIV-1 wt appears to upregulate basal IL-6 mRNA levels and this upregulation is lost with HIV-1 Δ Vpr (Figure 5.11 A). HIV-1 wt does not appear to affect basal IL-8 mRNA levels, while the absence of Vpr (HIV-1 Δ Vpr) appears to repress basal IL-8 (Figure 5.11 B). Again, statistical significance could not be established for most of the mRNA results due to the large number of variables in the pseudovirus experiment. These results are consistent with the Vpr transient transfection experiment results and suggest that Vpr does not affect the DEX-mediated regulation of the endogenous transactivation and transrepression model genes. Results also suggest that Vpr regulates the endogenous genes ligand-dependently and this regulation is specific to the pro-inflammatory genes.

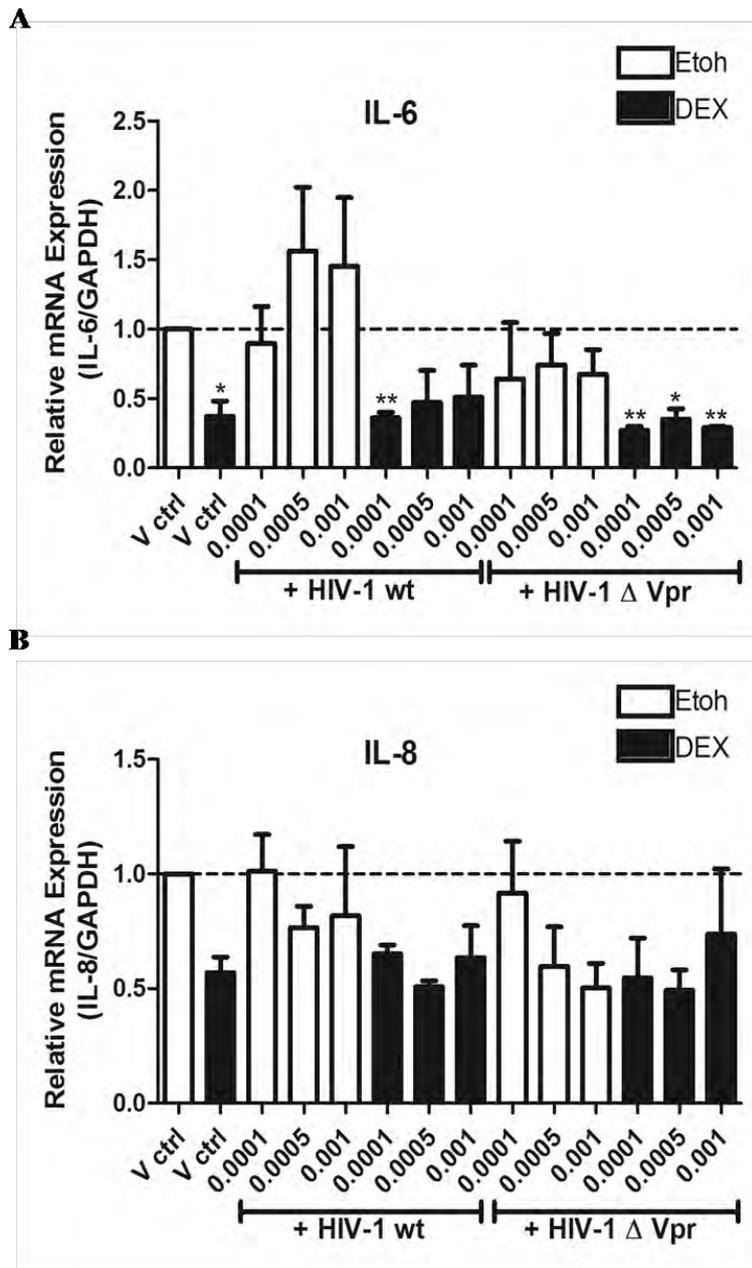


Figure 5.11: Vpr delivered by HIV-1 pseudovirus does not affect DEX-mediated repression of IL-6 and IL-8. TZM-bl cells were seeded into 12-well plates at a density of 10×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were infected for 72 hrs with either virus control (V ctrl), increasing MOI (0.0001, 0.0005 and 0.001) of HIV-1 wt (pNL4-3.E⁻ and pLET-LAI) or HIV-1 Δ Vpr (pNL4-3.E⁻R⁻ and pLET-LAI). Then, the cells were treated with 100 nM DEX and EtOH (0.1% v/v; vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative (A) IL-6 and (B) IL-8 gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was

normalized to V ctrl basal activity (EtOH) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with Dunnett post-test and a paired t-test. Statistical significance is denoted by * or ** to indicate $P < 0.05$ or $P < 0.001$, respectively.

Chapter 6: An investigation into the role of GR and Vpr in the regulation of HIV-1 LTR transcription in the presence of DEX, P4 and the synthetic progestins (Results).

Aims

The main aim of this chapter was to investigate the hypothesis that the GR and HIV-1 Vpr regulate HIV-LTR transcription in the presence of DEX, P4 and the synthetic progestins. In particular, the goals were to (i) determine whether P4 and the synthetic progestins regulate HIV-1 LTR transcription, (ii) determine whether the regulation of the HIV-1 LTR in the presence of the ligands is mediated via the GR, (iii) determine whether Vpr differentially regulates HIV-1 LTR transcription in the presence of ligands.

Results

6.1 The synthetic progestins, like DEX and P4, all repress HIV-1 LTR transcription

In order to determine whether the synthetic progestins regulate HIV-1 LTR transcription, HeLa cells were transiently transfected with HIV-1 LTR-luc and Tat expression vectors. The cells were then treated with saturating concentrations of DEX, MPA, P4 and NET-A or treated with the combination of RU-486 and DEX for 24 hrs. Figure 6.1 shows that in the absence of Tat the LTR is not activated. In addition, it is shown that all ligands; DEX, MPA, P4 and NET-A repressed Tat-activated LTR activity. The GR/PR antagonist, RU-486, also repressed LTR transcription. Interestingly, although it appears that the combination of DEX + RU-486 may additively repress LTR transcription there is no significant difference between DEX and DEX + RU-486. Since there is no significant difference between RU-486 and DEX

to deliver viral proteins by means of a more physiological method than transient transfection of a Tat expression vector, HIV-1 pseudovirus experiments were done. TZM-bl cells were infected with HIV-1 wt pseudovirus for 72 hrs, and then treated with 100 nM of DEX, MPA and NET-A for 24 hrs. Thereafter, luciferase activity was measured. For all the HIV-1 pseudovirus experiments luciferase activity was normalized to MTT activity. The MTT assay which measures viable cells with an active metabolism is a different method of normalization than the Bradford assay which measures total protein content of the cells (Sittampalam *et al.* 2004). Figure 6.2 shows that in the absence of virus (V ctrl) the LTR is not activated. Similar to results from the transient transfection of Tat and LTR-luc experiments, Figure 6.2 shows that DEX, MPA, P4 and NET-A repressed transcription of the stably integrated LTR. These findings show that there is no differential ligand regulation of the LTR i.e. the synthetic progestins, like DEX and P4; all repress the LTR to a similar extent. Since the ligands similarly repress the LTR it is suggested that the ligand-mediated repression of the LTR might not be GR-specific.

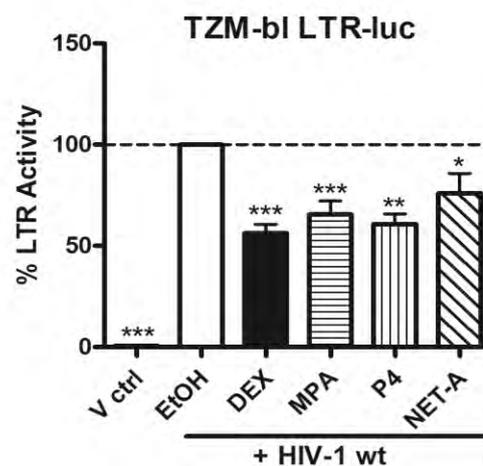


Figure 6.2: The synthetic progestins, like DEX and P4, all repress viral-activated LTR transcription. TZM-bl cells were seeded into 96-well plates at 1×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were infected with HIV-1 wt (pSg3E⁻ and pDU151A) at a MOI of 0.0005 or virus control (V ctrl) for 72 hrs. Then, the cells were

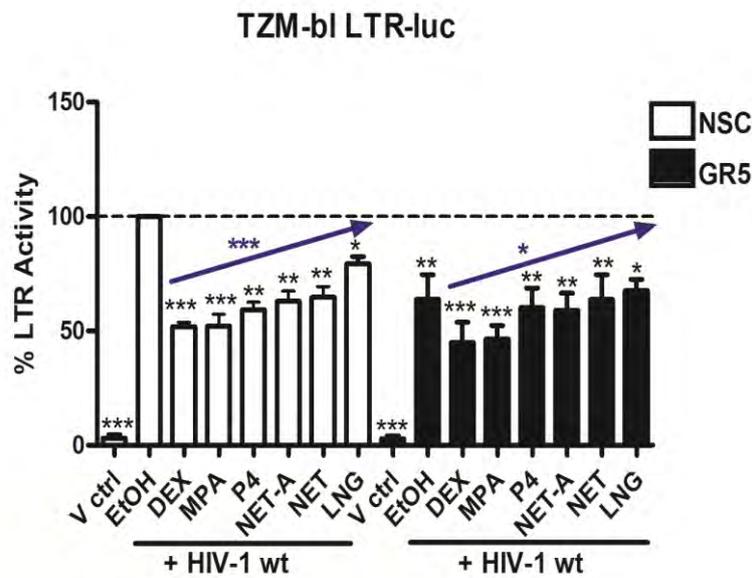
washed twice with PBS and then treated with 100 nM DEX, MPA, P4, NET-A or EtOH (0.1% v/v; vehicle) in serum free DMEM for 24 hrs. Thereafter, the cells were lysed with 1X Bright-Glo™ and the luciferase activity was measured. In addition, a parallel experiment was done and MTT activity was measured. Luciferase activity was normalized to MTT activity. Furthermore, relative LTR activity/MTT was normalized to + HIV-1 wt basal activity (EtOH) set to 100% to obtain % LTR activity. Graphs represent pooled results of at least two independent experiments, performed in duplicate, and are plotted as means +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with Dunnett post-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

6.2 Rank order of the ligand-mediated HIV-1 LTR repression is indicative of a role for the GR

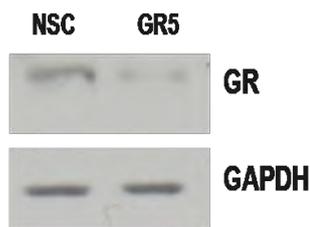
In order to determine whether the ligand-mediated repression of the LTR involves the GR, GR knockdown experiments were done. This was performed by first transfecting the TZM-bl cells with GR or NSC siRNA for 48 hrs. Then the cells were infected with HIV-1 wt pseudovirus for 72 hrs. Next, the cells were treated with 100 nM DEX, MPA, P4, NET-A, NET and LNG for 24 hrs. Thereafter, luciferase activity was measured. Here, NET was included in this experiment as a control to exclude the possibility that the acetate form (NET-A) would regulate the HIV-1 LTR transcription differently. In addition, a second generation synthetic progestin (LNG) with no reported GR activity was also included. Again, it is shown that in absence of virus (V ctrl) the LTR is not activated (Figure 6.3 A). Figure 6.3 A also shows that DEX, MPA, P4 and NET-A significantly repressed LTR transcription similar to the results in Figure 6.2. In addition, NET repressed LTR activity similar to NET-A (Figure 6.3 A). Interestingly, LNG also repressed the LTR (Figure 6.3 A). There is a significant linear trend in the order of the ligand-mediated repression and it appears that there is a rank order in which the ligands repress LTR transcription is DEX>MPA>P4>NET-A>NET>LNG. Figure

6.3 A shows that basal LTR transcription decreased considerably when the GR was knocked down. Surprisingly, the ligand-mediated repression is not lifted in the GR knockdown condition and the linear trend in the order of the ligand-mediated repression remains. The Western blot in Figure 6.3 B confirms successful knock down of GR protein in the cells and Figure 6.3 C shows that the average knockdown of GR protein is ~41%. Although the GR knock down did not lift the ligand-mediated repression, the result in which the ligands repress the LTR in a rank order similar to that of the ligands potency for GR-mediated transactivation and transrepression of transcription suggests that GR is involved in the ligand-mediated LTR repression. In addition, 59% of GR protein is still present in cells which suggest that the ligands require a small amount of GR protein in the TZM-bl cells to mediate a repression response on the LTR gene.

A



B



C

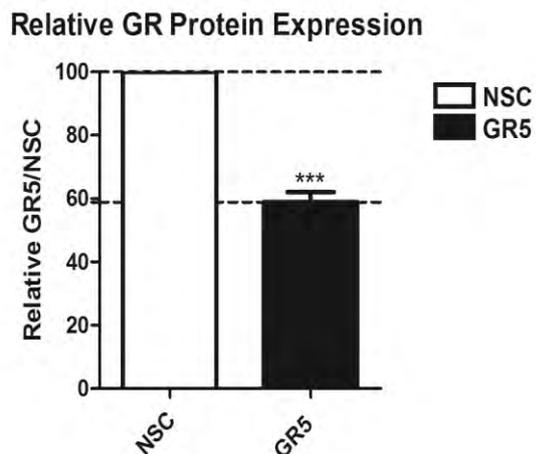


Figure 6.3: The rank order of ligand-mediated LTR repression is indicative of a role for the GR. (A) TZM-bl cells were seeded into 10 cm dishes at a density of 100×10^4 cells/dish and incubated for 24 hrs. Thereafter, the cells were transfected with 10 nM GR or NSC siRNA for 48 hrs. Then, the cells were re-seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were infected with HIV-1 wt (pSg3E⁻ and pDU151A) at a MOI of 0.0005 or virus control (V ctrl) for 72 hrs. Then, the cells were washed twice with PBS and then treated with 100 nM DEX, MPA, P4, NET-A, NET, LNG or EtOH (0.1% v/v; vehicle) in serum free DMEM for 24 hrs. Thereafter, the cells were lysed with 1X Bright-Glo™ and the luciferase activity was measured. In addition, a parallel experiment was done and MTT activity was measured. Luciferase activity was normalized to MTT activity. Furthermore, relative LTR activity/MTT was normalized to + HIV-1 wt NSC

basal activity (EtOH) set to 100% to obtain % LTR activity. Pooled results are shown of at least three independent experiments, performed in duplicate, and plotted as means \pm SEM. **(B)** For verification of GR knockdown, TZM-bl cells were seeded into 10 cm dishes and incubated for 24 hrs. Thereafter, the cells were transfected with 10 nM GR or NSC siRNA for 48 hrs. Then, the cells were harvested with 5X SDS sample buffer. Equal amounts of sample were loaded on SDS page gels and analyzed by Western blotting with antibody specific for GR and GAPDH (loading control). A representative Western blot is shown. **(C)** Western blots of at least three independent experiments were quantified to determine the relative GR protein expression and plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with a Dunnett post-test, paired t-test and linear trend post-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

6.3 Vpr does not play a role in GR-mediated regulation of HIV-1 LTR

Having shown that the synthetic progestins, like DEX and P4, all repress LTR transcription and the GR may be involved in this repression; the role of Vpr in this ligand-mediated LTR repression was next investigated. HeLa cells were transiently transfected with HIV-1 LTR-luc, Tat and HA-Vpr expression vectors. The cells were then treated with saturating concentrations of DEX, MPA, P4 and NET-A for 24 hrs. Figure 6.4 A confirms that in the absence of Vpr, the ligands repress LTR transcription. It is also shown that Vpr enhances basal LTR transcription and the ligands are still capable of repressing the Vpr enhanced LTR transcription (Figure 6.4 A). Figure 6.4 B shows that in the presence of Vpr, the % of ligand-mediated LTR repression is similar to that in the absence of Vpr. This suggests that Vpr does not play a role in GR-mediated repression of the LTR.

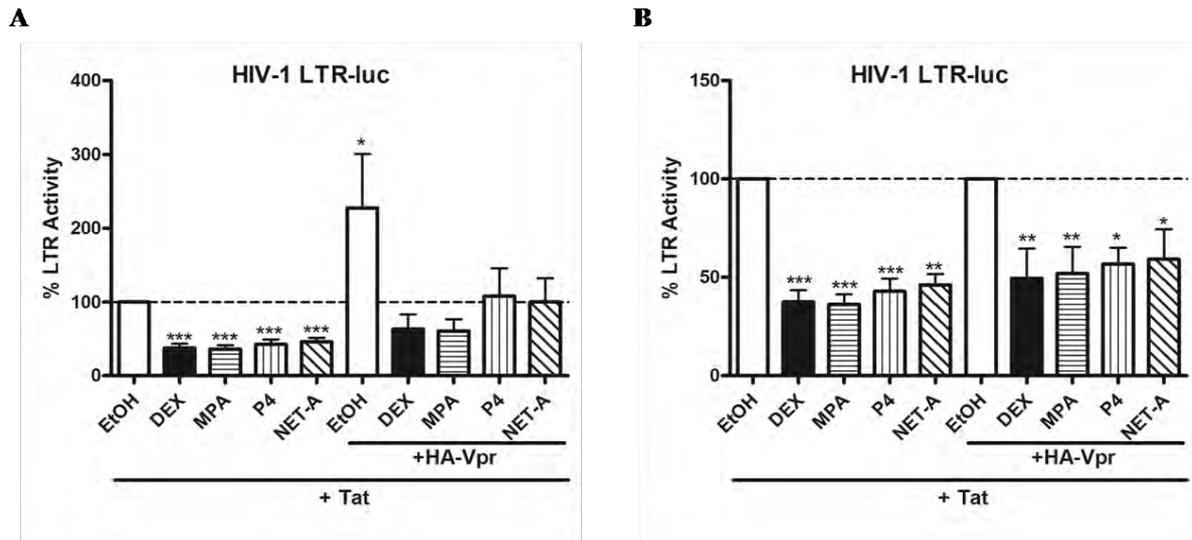


Figure 6.4: Vpr does not affect % ligand-mediated repression of the LTR. HeLa cells were seeded into 24-well plates at a density of 7.5×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with 47 ng/well HIV-1 LTR-luc and 125 ng/well Tat and HA-Vpr or empty vector and incubated for 24 hrs. Then the cells were either treated with 100 nM DEX, 1 μ M MPA, 10 μ M PROG, 10 μ M NET-A or EtOH (0.1% v/v; vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and lysed with 1X reporter lysis buffer and the luciferase activity was measured. Luciferase activity was normalized to protein content per well as determined by Bradford assay. In addition, the ligand stimulated responses were normalized with the (A) +Tat -Vpr basal activity (EtOH) set to 100 or (B) both +Tat -Vpr and +Tat +Vpr vehicle set to 100% to obtain % LTR activity. Pooled results are shown of at least five independent experiments, performed in triplicate, and plotted as means \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with Dunnett post-test or paired t-test. Statistical significance is denoted by * or *** to indicate $P < 0.05$ or $P < 0.0001$, respectively.

Having shown by transient transfection experiments that Vpr does not affect the % ligand-mediated repression of the LTR but it does enhance basal LTR transcription in the presence of Tat, it was next determined whether Vpr is involved in the DEX-mediated LTR regulation using HIV-1 pseudovirus to deliver Vpr protein. This was done by infecting the cells with an increasing MOI of HIV-1 wt and HIV-1 Δ Vpr (Vpr deletion mutant) for 72 hrs. The cells

were then treated with 100 nM DEX for 24 hrs. Thereafter, luciferase activity was measured. Figure 6.5 A shows a dose-effect in which increasing amounts of HIV-1 wt results in a corresponding increase in fold HIV-1 LTR activation. Although the trend of HIV-1 LTR activation remained consistent between each biological repeat, there were large fold differences between each repeat which produced the large error (Figure 6.5 A). Nevertheless, the results show that in the absence of Vpr (indicated by HIV-1 Δ Vpr) there appears to be a smaller increase in LTR activity compared to the presence of Vpr (indicated by HIV-1 wt). In addition, it appears that DEX may repress activated LTR transcription (Figure 6.5 A). In order to normalise the data for the differences in fold between each biological repeat, the data-set was plotted to obtain % LTR activity relative to the MOI (0.001) which produced the highest LTR activity set to 100% (Figure 6.5 B). Figure 6.5 B shows that HIV-1 wt at a MOI 0.0005 and 0.001 significantly activated LTR transcription. As expected, DEX repressed the HIV-1 wt activated LTR transcription (Figure 6.5 B). In addition, HIV-1 Δ VpR did not significantly activate the LTR. Although it appears that HIV-1 Δ Vpr activates the LTR, this small increase in LTR activity is not significant. These results are consistent with the HeLa cells results that show Vpr enhances basal LTR transcription. Taken together with the transient transfection experiments these results suggest that Vpr and Tat are required for a maximal LTR response. In addition, results suggest that in the TZM-bl cells (under these conditions) that Vpr is required for significant LTR activation. Since the LTR is lowly activated in the absence of Vpr, the role of Vpr in the DEX-mediated repression of the LTR could not be determined from this experiment.

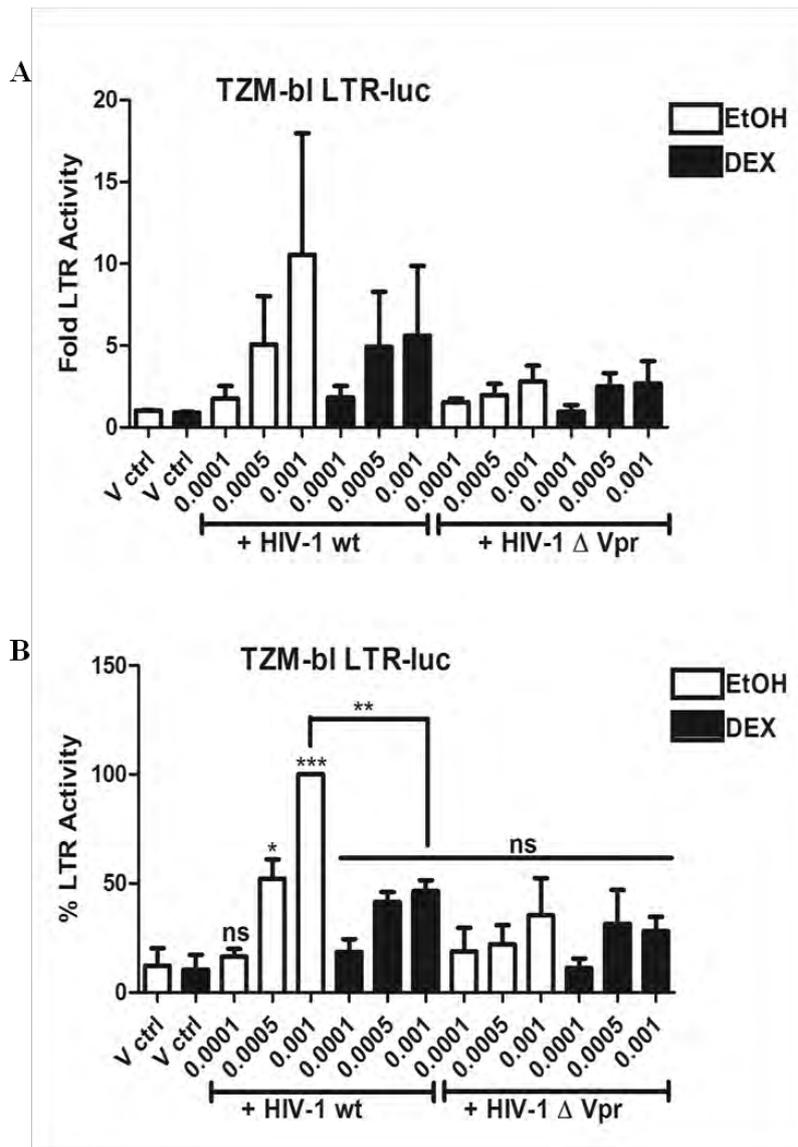


Figure 6.5: Vpr is required for optimal LTR activity. TZM-bl cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were infected with either HIV-1 wt (pNL43.E⁻ and pLET-LAI) or HIV-1 Δ Vpr (pNL43.E^{R-} and pLET-LAI) at an increasing MOI of 0.0001, 0.0005 and 0.001 or virus control (V ctrl) for 72 hrs. The cells were then washed twice with PBS and then treated with 100 nM DEX or EtOH (0.1% v/v; vehicle) in serum free DMEM for 24 hrs. Thereafter, the cells are lysed with 1X Bright-Glo™ and the luciferase activity was measured. In addition, a parallel experiment was done and MTT activity was measured. Luciferase activity was normalized to MTT activity. Furthermore, **(A)** relative LTR Activity/MTT was normalized to V ctrl basal activity (EtOH) set to 1 to obtain relative fold induction. In addition, **(B)** relative LTR Activity/MTT was normalized to HIV-1 wt MOI 0.001 treated with vehicle to obtain % LTR Activity/MTT. Pooled results are shown of at least three independent experiments, performed

in duplicate, and plotted as means \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with Dunnett post-test and a paired t-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

Chapter 7: Discussion & Conclusion

7.1 MPA, unlike NET-A and P4, acts like a full to partial GR agonist for regulation of inflammatory genes in cervical epithelial cells

The cervical mucosa of the lower FGT is the primary site of infection for HIV-1 during male to female transmission (Rametse *et al.* 2014). The epithelial cells of the endocervix express a variety of cytokines and chemokines including IL-6, IL-8 and RANTES (Fichorova & Anderson 1999). An excessive release of pro-inflammatory cytokines within the FGT may facilitate HIV transmission by either disrupting the epithelial barrier and/or providing the virus with a steady supply of target cells. However, a decrease in immune activation markers may lower the host's ability to mount a defence against pathogens. Thus, it is crucial to understand how factors such as hormonal contraception may influence the local immune response by the epithelial cells.

This novel study investigated the differential regulation of inflammatory genes by the synthetic progestins (MPA and NET-A) used in hormonal contraception and the mechanism of action of this regulation in cervical epithelial cells. In the present study, End1/E6E7 and HeLa cells were used as model cell systems for the cervical epithelium. Experiments were performed in the absence of an immune activator since the cervical epithelial cells constitutively express the selected pro-inflammatory cytokines (Fichorova & Anderson 1999). In addition, experiments in the End1/E6E7 cells in the presence of TNF- α , to mimic infection, showed a similar % repression of the pro-inflammatory genes with DEX and MPA, unlike P4 and NET-A (Govender *et al.* 2014).

Dose response analysis was done to determine whether the synthetic progestins and P4 differentially regulate the inflammatory genes at hormonal contraceptive serum

concentrations (1 nM – 65 nM) (Kirton & Cornette 1974; Hiroi *et al.* 1975). In addition, two time points (4 hrs and 24 hrs) were chosen to perform the dose response analysis in order to determine whether the differential regulation by the progestogens is time-dependent. This is the first study to perform dose-response analysis on these selected endogenous genes in endocervical cells. MPA, unlike NET-A or P4, induced the anti-inflammatory gene (GILZ) and repressed the pro-inflammatory cytokines/chemokine genes (IL-6, IL-8 and RANTES) in the End1/E6E7 cell line. These results are similar to previous studies showing that MPA and NET-A differentially regulate gene expression in cell lines and primary cells (Koubovec *et al.* 2005; Ronacher *et al.* 2009; Tomasicchio *et al.* 2013; Hapgood *et al.* 2014). The differential regulation of inflammatory genes by the progestogens could be partly explained by their different relative binding affinities (RBA) for the GR and is consistent with the reported RBA percentages for MPA (~79.1 %, reference agonist DEX), P4 (~5.6%) and NET-A (0.88 – 1.6%) (Koubovec *et al.* 2005; Hapgood *et al.* 2013). Although RBAs of ligands do not necessarily correlate quantitatively with relative agonist activity (Ronacher *et al.* 2009), for these particular ligands they do in terms of rank order. The GC agonist potency for transactivation and transrepression of MPA has previously been shown to be greater than NET-A and P4 (Koubovec *et al.* 2005).

Similar to the findings in the End1/E6E7 cells it was found in this thesis that MPA, unlike NET-A and P4, upregulates anti-inflammatory genes (GILZ and I κ B α) and downregulates pro-inflammatory genes (IL-6 and IL-8) in the HeLa cell line. The findings in both cell lines are consistent with the effects of MPA being mediated by the GR. Also, they are consistent with the GR being the predominant SR expressed in both cell lines. However, there appears to be some cell-specific differences in the ligand-mediated regulation of IL-8 and RANTES gene expression. In the End1/E6E7 cell line, 1 μ M MPA repressed IL-8 gene expression at 24 hrs but this did not occur in the HeLa cell line at 24 hrs. However, in the HeLa cell line, 100

nM MPA repressed IL-8 at 4 hrs (Addendum Figure 1). These findings are consistent with the effects of MPA being dose- and time-dependent and are further supported by results from the dose-response experiments in the End1/E6E7 cell line (Figure 4.1). Also, it appears that RANTES is regulated by hormones at 24 hrs in the End1/E6E7 cell line (Figure 4.1 H) but not in the HeLa cell line (Figure 5.6 C); however this could be due to low basal levels of RANTES in HeLa cells.

The differential regulation of the inflammatory genes by the progestogens suggests that MPA, unlike NET-A and P4, has anti-inflammatory actions in both the End1/E6E7 and HeLa cell lines. Previous reports have suggested that MPA has anti-inflammatory effects in the FGT (Elovitz & Wang 2004; Huijbregts *et al.* 2013). Consistent with this, results from this present study suggest that MPA may have immunosuppressive effects in the endocervix of the FGT. Importantly, dose response analysis from this thesis shows that MPA at 10 nM significantly represses IL-6 and RANTES mRNA levels and MPA has a potency of ~24 nM for transactivation of the anti-inflammatory GILZ gene and ~4 – 20 nM for repression of the pro-inflammatory genes (Figure 4.2). The serum concentrations for 150 mg intra-muscularly (IM) administered DMPA have been reported to be in the range of 2.5 to 65 nM MPA a few days after injection and to plateau at about 2.6 nM for about three months thereafter (Kirton & Cornette 1974; Hiroi *et al.* 1975; Hapgood *et al.* 2013; Stanczyk *et al.* 2013; Shelton & Halpern 2014). Although the levels of MPA in tissue are to the present author's knowledge unknown, it is possible that they may be higher than serum levels. Results from this current study suggest that immunosuppressive effects are likely to be relevant at physiological doses of MPA used in injectable contraception, particularly shortly after injection, and possibly during the plateau phase as well. Recently, it has been reported that Pfizer pharmaceutical company has developed a sub-cutaneously (SQ) administered and lowered dose MPA with a much better pharmacokinetic profile (Shelton & Halpern 2014). Although the lowered dose

SQ MPA has lower peak serum levels (4.1 nM – 6.2 nM) (Shelton & Halpern 2014), it is still likely that SQ MPA may have some immunosuppressive effects shortly after administration. However, due to proprietary information, this SQ MPA is considerably more expensive and therefore unlikely to be used by women in Sub-Saharan Africa who are at high risk for HIV-1 infection. Although the reported serum concentrations of 200 mg IM administered NET-EN are in a similar range (1.5 to 59 nM) (Fotherby 1983) to injectable DMPA, results from this thesis suggest that NET-EN is unlikely to exert effects on inflammation via the GR, even shortly after injection.

The concentration of endogenous P4 in serum of premenopausal women is low during the follicular phase (0.65 nM) but rises to about 80 nM during the luteal phase, and to about 600 nM during pregnancy as reviewed in (Africander, Verhoog, *et al.* 2011). Whether the effects of P4 on inflammatory gene regulation in the menstrual cycle and during pregnancy are via the GR is unknown, but is possible. Present dose response analysis shows that P4 at 10 – 100 nM may exert some anti-inflammatory effects by regulation of IL-6 most likely via the GR, the only SR expressed in the End1/E6E7 cells, while P4 at concentrations up to 100 nM shows very little effect on expression of the other genes investigated. Results from this present study also suggest that pregnancy concentrations of P4 (100 nM – 1 μ M) may exert pro-inflammatory effects on some genes, similar to previous findings in ectocervical and vaginal cell lines (Africander, Louw, *et al.* 2011). Consistent with the pro-inflammatory effects of P4 at pregnancy concentrations, a recent study has shown in Ect1/E6E7 cells that 1 μ M P4 upregulates pro-inflammatory cytokine IL-12 and downregulates anti-inflammatory cytokine IL-10 (Louw-du Toit *et al.* 2014). In addition, the Louw-du Toit *et al.* study has shown by GR siRNA that the P4-induced pro-inflammatory response is via the GR (Louw-du Toit *et al.* 2014). Although pregnancy levels of P4 generally promote an anti-inflammatory Th2 response which may aid maternal tolerance to fetus in the uterus (Miyaura & Iwata 2002;

Arck *et al.* 2007; Schumacher *et al.* 2014), present data with previous reports (Africander, Louw, *et al.* 2011; Louw-du Toit *et al.* 2014) suggest that in the lower FGT high P4 levels may be pro-inflammatory most likely via the GR. A previous study has shown that high P4 concentrations increases RANTES expression in endometrial T-cells (Ramhorst *et al.* 2006), which together with the current results, suggests that regulation of RANTES gene expression in the endometrium by P4 may be PR- and not GR-mediated. Since the effect of RANTES upregulation, necessary for implantation (Ramhorst *et al.* 2006), occurs in endometrial cells which express PR, upregulation with P4 is likely to be via the PR, for which P4 has a high affinity (RBA = 100%, Hapgood *et al.* 2013). Taken together, these findings suggest that the effects with P4 on immune function are unlikely to be mediated via the GR at conditions other than at pregnancy, given the low potency of P4 via the GR. However, some gene- and cell-specific GR-mediated effects on immune function by P4 may occur in the luteal phase of the menstrual cycle, such as promoting a Th2 response. In addition, the effects of P4, during pregnancy, on immune function in the lower FGT may be different to that of the upper FGT.

The MPA-mediated repression of the pro-inflammatory mediators; IL-6, IL-8 and RANTES in the endocervical cells via the GR may have physiological significance for cervical mucosal immunity, disease susceptibility and predisposition. However, the physiological implications are difficult to predict. These immune regulators have been reported to play a role in HIV pathogenesis (Fichorova *et al.* 2004). IL-6 has been shown to induce HIV-1 replication via nuclear factor κ B (NF- κ B) mediated HIV-1 long terminal repeat (LTR) activation, while IL-8 triggers the recruitment of HIV-1 target cells to the inflammation site and may also stimulate HIV-1 replication in T cells and macrophages (Poli *et al.* 1994; Lane *et al.* 2001; Alfano & Poli 2002; Mamik & Ghorpade 2014). RANTES is a ligand for the chemokine receptor 5 (CCR5) and has been shown to inhibit HIV-1 replication by competing with HIV for CCR5 and by causing internalization of the receptor (Colin *et al.* 2013). However, RANTES has

also been linked to enhancing HIV-1 replication by activating intracellular signalling pathways and regulating viral gene expression from the HIV-1 LTR via a mechanism that is independent of CCR5 (Gordon *et al.* 1999). Consequently, MPA-mediated anti-inflammatory effects in the lower FGT may decrease HIV-1 acquisition by decreasing the number of target cells or decrease HIV-1 replication. However, decreased pro-inflammatory mediators could also inhibit immune function, such as B-cell maturation, T-cell activation and differentiation and consequently reduce the host's ability to mount a defence against a pathogen and therefore aid initial infection (Janeway Jr *et al.* 2012). Consistent with the latter, most high quality studies show that MPA increases the risk of HIV-1 acquisition in women (Baeten *et al.* 2007; Heffron *et al.* 2012; Wand & Ramjee 2012; Crook *et al.* 2014; Noguchi *et al.* 2014; Polis *et al.* 2014). Although some studies show no significant correlation between MPA and HIV-1 risk (Polis *et al.* 2014), latest meta-analysis reports that DMPA-usage but not NET-EN increases risk of HIV-1 infection (Morrison, Chen, *et al.* 2014). However, the literature on whether MPA increases acquisition of HIV-1 or other STI's by increasing or decreasing pro-inflammatory markers in the FGT *in vivo* is inconsistent. Consistent with this present study's findings of MPA repressing pro-inflammatory mediators, a previous report found that women on DMPA displayed lower levels of the pro-inflammatory cytokine IFN- α in plasma and genital secretions compared to controls with no hormonal contraception (Huijbregts *et al.* 2013). Furthermore, more recent findings from the same group showed that MPA, unlike P4, NET or LNG, inhibits pro-inflammatory cytokine production by HIV-1 activated T-cells and pDCs (Huijbregts *et al.* 2014). Similarly in non-human primate models, it has been shown that MPA suppresses pro-inflammatory cytokines such as IFN- γ in SHIV-infected rhesus macaques (Trunova *et al.* 2006). Studies in mouse models show that MPA increases susceptibility to HSV-2 and decreases immune responses to infection (Gillgrass *et al.* 2003; Kaushic *et al.* 2003). Also consistent with this thesis, results presented at the HIV R4P

conference 2014 showed that MPA, unlike NET-A and P4, represses some pro-inflammatory cytokines like IL-6 in primary cervical tissue explants (Ray *et al.* 2014). In addition, it was shown in cervical tissue explants that MPA, unlike NET-A, increases HIV-1 replication (Ray *et al.* 2014). In contrast, a clinical study showed that DMPA-usage is associated with increased levels of pro-inflammatory cytokines and chemokines such as RANTES and increased HIV-1 acquisition (Morrison, Fichorova, *et al.* 2014). However, in this study it was not possible to discriminate between elevated RANTES levels being a cause of infection or a consequence of exposure to HIV-1 prior to seroconversion (Morrison, Fichorova, *et al.* 2014). Additionally, since ~76 % of the MPA users in this study were positive for an STI, it may be that elevated RANTES was a consequence of STI infection, despite attempts to correct for that confounding variable.

7.2 MPA-mediated anti-inflammatory gene regulation in endocervical cells is via the GR

Although evidence in the literature suggests that the MPA-mediated anti-inflammatory action is mediated via the GR (Koubovec *et al.* 2004; Koubovec *et al.* 2005; Ronacher *et al.* 2009; Huijbregts *et al.* 2013; Hapgood *et al.* 2014), this present study aimed to provide direct proof of a GR mechanism of action on endogenous genes, via a GR knock down strategy. In order to provide this evidence, the End1/E6E7 cell line was first assessed for its SR content. The presence of only GR mRNA and protein in the End1/E6E7 cell line suggested that the MPA-mediated inflammatory gene regulation is via the GR. However, for the first time it was shown with the GR knockdown strategy that the GR is required for the MPA-mediated regulation of IL-6 and IL-8 mRNA and protein (Figure 4.7), consistent with the hypothesis that the MPA-mediated anti-inflammatory action in the endocervical cells is via the GR. It is worth noting that NET was included in this experiment as a control to exclude the possibility

that the acetate form (NET-A) would regulate the genes differently. However, it is shown that NET-A acts similarly to NET. The findings from the GR knockdown experiment highlight the importance of choice of progestin used in hormonal contraception and suggest that progestins such as MPA, unlike NET-A and P4, will have anti-inflammatory actions in cells where the GR is the predominant SR expressed. Consequently, a GR-mediated immunosuppressive response in the FGT could result in a lowered immune response and thereby aid in STI susceptibility and disease progression. These results together with other *in vitro*, *ex vivo*, clinical and animal *in vivo* studies (Gillgrass *et al.* 2003; Kaushic *et al.* 2003; Koubovec *et al.* 2004; Koubovec *et al.* 2005; Trunova *et al.* 2006; Ronacher *et al.* 2009; Huijbregts *et al.* 2013; Tomasicchio *et al.* 2013; Huijbregts *et al.* 2014; Ray *et al.* 2014) suggest that high dose progestin-only contraceptives like MPA with GR properties should be avoided by women at high risk for STI infection.

In contrast to findings in this thesis, a previous study found that MPA in the presence of TNF- α upregulated IL-8 mRNA in the Ect1/E6E7 ectocervical cells (i.e. pro-inflammatory) (Africander, Louw, *et al.* 2011). However, similar to the anti-inflammatory effects of MPA in the endocervical cells, Africander *et al.* found that MPA in the presence of TNF- α repressed RANTES mRNA expression in the ectocervical cells while in the Vk2/E6E7 vaginal cells, MPA did not regulate the cytokines (Africander, Louw, *et al.* 2011). Based on results from this thesis and previous reports, MPA appears to have differential effects on cytokine/chemokine regulation in the ectocervical, vaginal and endocervical cells and this requires further investigation. Furthermore, Africander *et al.* found that the ectocervical and vaginal cell lines express a variety of SRs such as PR, GR, AR and ER (Africander, Louw, *et al.* 2011). The differences in SR expression between the endocervical, ectocervical and vaginal cell lines could account for the differential MPA-mediated effects in these cell lines. In addition, whether other GR isoforms are expressed by these cells and the effects of MPA

via the other GR isoforms is unknown but unlikely since the other isoforms are GC-resistant. The results collectively suggest that progestins will have differential effects on genital mucosal immune function and susceptibility to infections depending on which SRs are expressed. Since the AR- and PR-mediated effects of MPA are similar to P4 and NET-A as reviewed in (Hapgood *et al.* 2013), the effects of MPA are likely to be very different to those of NET-A and P4, when mediated by the GR, but not the AR or PR. It is also possible that there would be differential effects by the progestogens when mediated by the ER or MR since NET-A has estrogenic activity while P4 has anti-mineralocorticoid activity (Schindler *et al.* 2003; Hapgood *et al.* 2013).

In addition to differential inflammatory gene regulation, the ligands have previously been shown to affect GR protein levels. Specifically, the GR is degraded in a ligand-selective manner (Avenant *et al.* 2010); that is, full agonist DEX causes the fastest GR degradation followed by MPA, P4, and then NET (Avenant *et al.* 2010). In both cell lines DEX-mediated regulation is more potent than MPA, NET-A and P4 which is consistent with the observed effects of the ligands on GR protein levels at 24hr. The effects of the DEX and MPA on GR protein turnover are consistent with the hypothesis that DEX and MPA-mediated gene regulation is via the GR. In addition, in the End1/E6E7 cells the GR protein turnover with DEX is more apparent at 24 hr than at 4 hr which is consistent with the effects of DEX on gene regulation being more potent at 24 hrs. Although the effects of MPA on gene regulation in the End1/E6E7 cell line are mediated by the GR, the effect of MPA on GR protein turnover is not as apparent in the single representative Western blot. In retrospect, for the End1/E6E7 cell line multiple Western blots should have been analyzed and the effects of the ligands on GR protein levels quantitated as done for the HeLa cell line.

In order to confirm the differential regulation of the inflammatory genes by the progestogens in a primary endocervical model, dose-response and antagonist experiments were done in the

VEN-100 model which is a bio-engineered multilayer of primary endocervical epithelial cells. Unfortunately, the cells did not respond as expected since the viability of the cells decreased significantly by the time they were harvested for analysis. This was due to logistical delays in the delivery of the tissue from Ashland, MA, USA to Cape Town, RSA. Therefore, not much can be concluded from the dose-response or antagonist experiments with the primary cells. It is worth noting that it was prohibitively expensive to repeat these experiments or attempt to solve the cell viability problems of the VEN-100 cells. Fortunately, the SR content of the primary cells was assessed while the cells were still viable and it was found that the primary endocervical cells contain GR mRNA and protein, MR mRNA and ER α mRNA, but not PR or AR mRNA. This study was not able to investigate MR and ER α protein levels due to the insufficient amount of primary cell lysate. Similar to the primary endocervical cells, the End1/E6E7, HeLa and TZM-bl cervical cell lines express GR. Also similar to the primary endocervical cells, the HeLa and TZM-bl cells express MR. Like this current study ER α has previously been shown to be expressed in primary endocervical cells (Al-hendy *et al.* 2006). However, it has been previously shown by immunohistochemical analysis, Western blotting and RT-PCR that primary human endocervical cells express both PR isoforms, PR-A and PR-B, (Al-hendy *et al.* 2006) in contrast to the findings of this present study. Since the expression of sex SRs are affected by a variety of factors such as hormonal status and age of a patient (Brodowska *et al.* 2010) this could explain the differences between the current study's observations and previous reports. In particular, PR protein expression in uterine tissue is dependent on the stages of menstrual cycle. There is an increase in PR expression from early proliferative to late proliferative stage and then a decrease in PR expression from late proliferative to early secretory and late secretory stages (Ingamells *et al.* 1996). PR protein expression in cervical tissue has also been shown to be dependent on hormonal status whereby pregnant women have been found to express much

lower amounts of PR than non-pregnant women (Stjernholm *et al.* 1996). Thus it is possible that the VEN-100 endocervical cells may have been collected at a stage when the PR was not expressed. This raises interesting questions as to whether changes in SR content for e.g. PR to GR ratio during the menstrual cycle may differentially regulate inflammatory gene expression in response to progestins and future investigation is warranted.

To further investigate the mechanism of gene regulation via the GR, *de novo* protein synthesis was inhibited and mRNA expression was measured. The use of cycloheximide (CHX) is an established method to prevent *de novo* protein synthesis and thereby identify the type of gene regulation (Baliga *et al.* 1969; Ravni *et al.* 2006). This method is a useful tool to show whether the GR is directly involved in the gene regulation or indirectly (i.e. by upregulation of another protein which in turn regulates the gene of interest). The GR was found to be partially involved in the regulation of GILZ and directly involved in the regulation of $\text{I}\kappa\text{B}\alpha$, IL-6 and IL-8 genes (Figure 4.8). The GILZ and $\text{I}\kappa\text{B}\alpha$ gene promoters have multiple glucocorticoid response elements (GREs) and are examples of GR transactivation model genes (Hermoso & Cidlowski 2003; Muzikar *et al.* 2009). In addition, in A549 cells it has been shown that the GR is recruited to the GRE region of the GILZ promoter by both DEX and MPA (Hadley *et al.* 2011). In contrast, the ChIP assay results from this thesis did not show GR recruitment to the GILZ promoter with MPA. This is not unexpected since it is shown by CHX experiments that MPA-induction of GILZ may in part require new protein synthesis and may not only involve direct GR transactivation. However, as mentioned above it has been previously shown in A549 cells that the GR is recruited to the GRE region of the GILZ promoter by MPA (Hadley *et al.* 2011) therefore it is likely that a small amount of GR is recruited by MPA, but this is below the limits of detection of the ChIP assay in HeLa cells. A possible reason for this is that in comparison to the A549 cells, the HeLa cells may express lower amounts of GR protein. The IL-6 and IL-8 gene promoters

have binding sites for transcription factors that include activator protein-1 (AP-1) and NF- κ B (McKay & Cidlowski 1999; Koubovec *et al.* 2004). It is proposed that GR represses IL-6 and IL-8 gene expression by interference of these transcription factors (De Bosscher *et al.* 2003). The findings that activation with DEX recruits GR to the IL-6 and IL-8 promoter regions are consistent with previous reports (Cvoro *et al.* 2011; Verhoog *et al.* 2011). Koubovec *et al.* also showed that MPA represses both IL-6 and IL-8 promoter activity (Koubovec *et al.* 2004). However, it is shown for the first time, that stimulation with MPA recruits GR to the regions encompassing the AP-1 site of the IL-6 promoter and NF- κ B site of the IL-8 promoter, thereby repressing expression of these genes, as schematically represented (Figure 7.1). Although stimulation with MPA and DEX recruits the same amount of GR to the IL-6 promoter, this does not correlate with the ligands potency and efficacy of IL-6 mRNA regulation. This suggests that stimulation with MPA and DEX may recruit different amounts of co-factors and/or different co-factors and is consistent with a study which shows differential binding of co-factors in ligand-selective GR-mediated gene regulation (Ronacher *et al.* 2009).

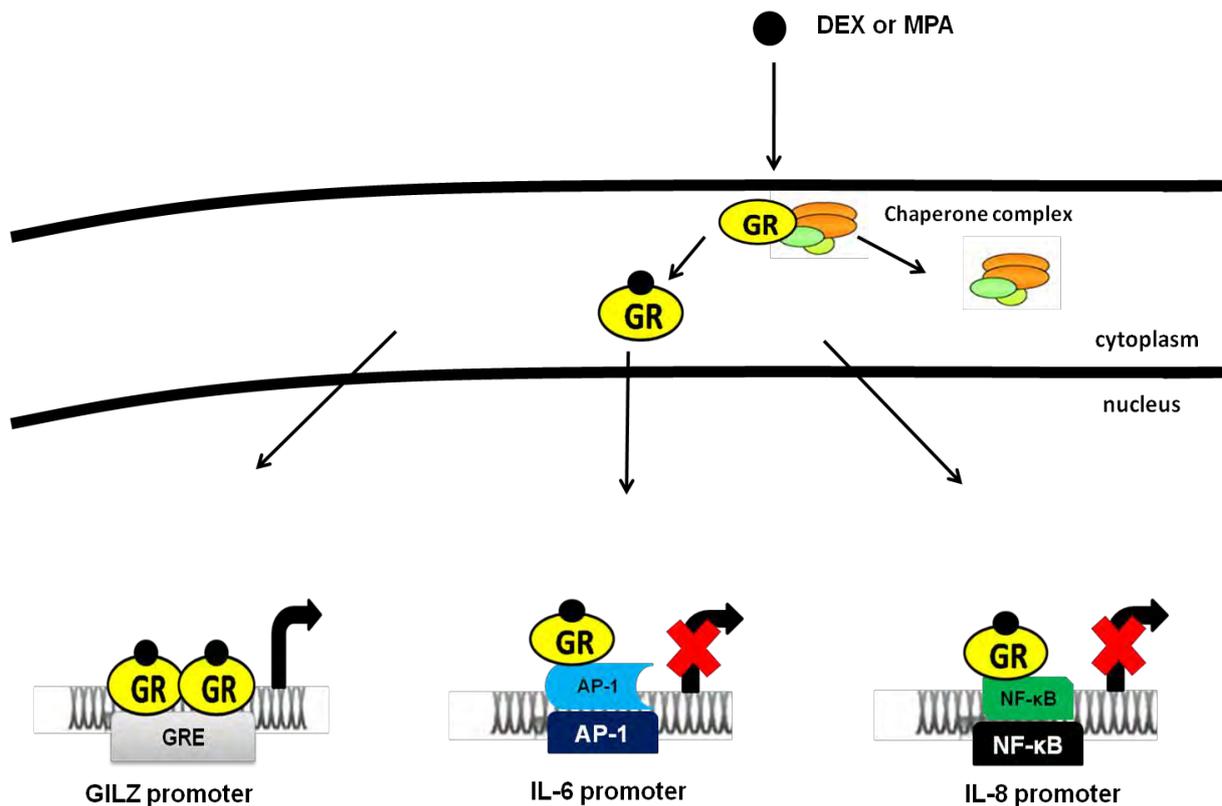


Figure 7.1: Schematic model for the role of GR in MPA- and DEX-mediated anti-inflammatory actions via regulation of GILZ, IL-6 and IL-8. Upon DEX or MPA binding to the GR, the GR undergoes a conformational change and translocates to the nucleus where it occupies GRE sites of the GILZ promoter to activate transcription of the GILZ gene or interacts with pro-inflammatory transcription factors bound to AP-1 sites of the IL-6 promoter to suppress IL-6 gene transcription or to NF-κB sites of the IL-8 promoter to suppress IL-8 gene transcription. Abbreviations: DEX = dexamethasone, MPA = medroxyprogesterone acetate GR = glucocorticoid receptor, GRE = glucocorticoid response element, AP-1 = activator protein-1, NF-κB = nuclear factor kappa B. Note: Stimulation with MPA may not necessarily recruit GR to GILZ in the HeLa cell line.

7.3 Vpr potentiates ligand-mediated reporter gene regulation but not ligand-mediated endogenous gene regulation

It has been hypothesized that the GR plays a role in HIV-1 pathogenesis whereby a decrease in pro-inflammatory cytokines results in lowered host immune response and thus aids in disease progression. The HIV-1 accessory protein Vpr has previously been shown to be immunosuppressive since it represses pro-inflammatory cytokines similar to the actions of

glucocorticoids (GCs) (Ayyavoo, Mahboubi, *et al.* 1997; Muthumani *et al.* 2000; Mirani *et al.* 2002). Crosstalk between Vpr and the GR has been proposed to be the mechanism whereby the GR modulates both host and viral gene regulation (Hapgood & Tomasicchio 2010). HIV-1 Vpr has been previously shown to potentiate GR-mediated reporter gene and endogenous gene transcription in the presence of DEX (Kino *et al.* 1999; Mirani *et al.* 2002; Fakruddin & Laurence 2005). However, there is limited data on whether Vpr modulates GR-mediated inflammatory gene regulation in the presence of DEX and no data for the synthetic progestins, MPA and NET-A. Since the actions of MPA have been shown in this current study to be mediated by the GR, it is possible that Vpr may co-activate GR-mediated inflammatory gene regulation in the presence of MPA similar to the action of DEX.

This present study investigated the effects of Vpr on the regulation of inflammatory genes in the presence of DEX, P4 and the synthetic progestins. In this study the HeLa and TZM-bl cell lines were used as the model systems. The TZM-bl cell line is a HeLa cell line modified to express CCR5 and CXCR4. This allows the cells to be infected by HIV-1 and thereby to deliver physiological levels of viral proteins such as Vpr.

The HeLa and TZM-bl cells were assessed for their SR content to determine whether they express GR. It was found that they both express GR mRNA and protein similar to the End1/E6E7 cell line and primary endocervical cells (as discussed earlier). In addition, both the cell lines expressed AR and MR, mRNA whereas only the TZM-bl cells expressed detectable AR protein. The HeLa cell line required further investigation to determine whether the endogenous GR, AR and MR were functional. It was found by reporter assays, using a transactivation model, that endogenous GR was transcriptionally active. The AR and MR specific agonists (MIB and ALD, respectively) only produced a transcriptional response in the presence of overexpressed AR and MR. Furthermore, it was confirmed on the endogenous inflammatory genes that GR is active whereby the GR agonist, DEX, induced

anti-inflammatory gene expression (GILZ) and repressed pro-inflammatory gene expression (IL-6). This is a typical GR-mediated response (De Bosscher *et al.* 2000) and suggests that the GR plays a role in ligand-mediated anti-inflammatory responses in the cervical cells. Interestingly, ALD induced expression of the pro-inflammatory gene, IL-8. This would indicate that MR is active in the HeLa cells and suggests that the MR could be responsible for the mediation of a pro-inflammatory response. This is consistent with previous studies which showed that ALD induced pro-inflammatory cytokines in renal cells and caused vascular inflammation by upregulation of pro-inflammatory genes in heart tissue (Rocha *et al.* 2002; Blasi *et al.* 2003). Results suggest that the MR-mediated response may be promoter-specific since ALD only promoted a MR-mediated response on endogenous IL-8 but not on the simple reporter gene. The assays to determine whether the SRs are functional were not done in the TZM-bl cell line. It was assumed that they would have similar SR functionality since they are derived from the HeLa cell line and have the same SR content as them.

In order to determine whether Vpr co-activates the GR-mediated response in the presence of MPA, it was first established that MPA acts as a partial agonist for the GR in the HeLa cell line. It was found by promoter-reporter assays that MPA acts as a partial agonist for the GR, while P4 and NET-A have no agonist activity, consistent with a previous study done in COS-1 cells (Ronacher *et al.* 2009). The ligands did not produce the expected responses on the transrepression model reporter genes (Addendum Figure 2). It could be that the IL-6 reporter gene promoter may require an additional co-factor for the GR to mediate repression and this co-factor is lacking in the HeLa cells. In hind sight, this should have been confirmed by performing reporter gene assays using the AP-1 reporter gene in the presence of the ligands in the HeLa cell line. It was decided that the IL-6-luc reporter genes were not a suitable transrepression model for the HeLa cell line and consequently the endogenous transrepression genes were investigated.

The hypothesis that HIV-1 Vpr acts as a co-activator of the GR is supported by the result that shows Vpr enhances the DEX-mediated transactivation of a reporter gene. In addition, these results are consistent with previous studies which show that Vpr enhances the GC-mediated response of TAT-GRE and mouse mammary tumour virus (MMTV) reporter genes, in a variety of cell lines such as human embryonic kidney 293T cells and the T lymphoblastoma-derived CEM and Jurkat cells (Kino *et al.* 1999; Sherman *et al.* 2000). It has also been previously shown by *in vitro* binding and co-immunoprecipitation assays that this co-activation response may be due to Vpr interacting with the GR via the LXXLL motif (Kino *et al.* 1999). However, for the first time it is shown here that Vpr also enhances MPA-mediated transactivation of a TAT-GRE reporter gene. Western blots show that Vpr does not affect the expression of GR protein and therefore rules out the possibility that Vpr increases GR protein expression which could enhance transactivation. Consistent with this it has been shown that Vpr does not affect ligand-dependent serine 203, 211 or 226 GR phosphorylation (Grantham 2012). It has been previously hypothesized that tissue hypersensitivity to GCs in HIV-1 patients occurs via Vpr enhancing transactivation of the GR (Kino & Chrousos 2001). The results from this present study support this hypothesis and suggest a mechanism whereby Vpr enhances the efficacies of both DEX and MPA for transactivation. Again, results from this present study highlight the importance of choice of progestin used in contraception. Results also suggest that injectable MPA, unlike NET-A and P4, could mimic the effects of GC hypersensitivity and/or potentially enhance GC hypersensitivity in HIV-1 patients. Consistent with this, a previous study has shown that MPA, unlike NET-A and P4 increases apoptosis of T-cells via the GR and this is potentiated by Vpr (Tomasicchio *et al.* 2013). This may potentially decrease the ability of T-cells to mount an anti-viral defence and thereby contribute to immunodeficiency and therefore MPA may not be the best contraceptive option for women infected with HIV-1.

Investigation into the endogenous genes revealed that Vpr does not modulate the DEX- or MPA-mediated regulation of the selected inflammatory genes. This is in contrast to a previous report that shows Vpr potentiates DEX-mediated repression of the pro-inflammatory cytokine IL-12 in human monocytes (Mirani *et al.* 2002). This present study only investigated the cytokine genes (IL-6, IL-8 and RANTES) which are known to be constitutively expressed in cervical epithelial cells. Thus it is possible that Vpr acts as a GR co-activator on other cytokine genes such as IL-12. Previous reports also show that Vpr enhances DEX-mediated induction of Receptor of Activated NF- κ B Ligand (RANKL) protein levels (Fakruddin & Laurence 2005). However, this present study showed that although DEX upregulates RANKL mRNA, Vpr does not enhance this DEX-mediated upregulation (Addendum Figure 3). This suggests that the Vpr-mediated co-activation of RANKL may be post-transcriptional. Alternatively, since the Fakruddin & Laurence study transfected higher amounts of Vpr expression vector (2 – 6 μ g) than this present study and since this present study shows that effects with Vpr are dose-dependent, it is possible that at higher concentrations Vpr may act as a co-activator on RANKL (Fakruddin & Laurence 2005). Another possibility is that the Vpr co-activation response seen by Fakruddin *et al.* is a non-specific response and an artefact due to the very high amounts of DNA transfected (Fakruddin & Laurence 2005). On the other hand, the lack of Vpr co-activation in this present study may be due to cell type differences. The experiments done in this present study were in HeLa cells while the experiments done in the Fakruddin & Laurence study were done in the Jurkat cell line and PBMCs (Fakruddin & Laurence 2005). It is possible that the Jurkat cells and PBMCs may have the transcription factor/s required for the Vpr co-activation response on RANKL and this transcription factor/s may be lacking in the HeLa cell line.

There are several possible explanations for the different findings with Vpr between the reporter gene assays and endogenous genes. Although the reporter gene and endogenous

genes investigated have GRE sites, again, it is possible that Vpr regulation could be promoter-specific. Since the aim of this study was to investigate the role of Vpr in GR-mediated inflammatory gene regulation, the GILZ and I κ B α inflammatory endogenous GRE genes were investigated. However, the promoter-reporter assays were done using a model GRE-reporter gene, TAT, which encodes for a metabolic liver enzyme. Although the promoters of TAT, GILZ and I κ B α all contain 2 or more GRE sites, it is possible that the presence of not only GRE sites but other factors such as GRE distance from basal promoter may dictate Vpr co-activation. In addition, it has been previously shown that minor changes in the TATA-box region of a gene promoter can modulate GR-mediated responses (Meyer *et al.* 1997). It is therefore likely that discrepancy in the responses could be attributed to the differences in TATA-box element of the genes. Thus, it is still possible that Vpr may act as a GR co-activator on other GRE inflammatory genes. The differences in the promoter architectures between a reporter gene and an endogenous gene could also account for the contrasting results. Endogenous genes have more complicated promoters than the simple reporter gene promoters. In addition, endogenous genes are surrounded by chromatin which could affect the accessibility and spatial organization at transcription sites unlike the reporter genes which are not packaged into native chromatin (Müller *et al.* 2007). Thus it is possible that Vpr may only regulate gene transcription in the absence of chromatin. Another explanation for the differing results is that there could be differences in the amount of Vpr protein expressed in the cells for the reporter vs. the endogenous gene experiments. The reporter gene assays had a lower ratio of Vpr plasmid DNA to cells and thus it is possible that lower Vpr protein was expressed in the cells than in the endogenous gene experiments. If this were the reason, this would suggest that Vpr modulation of DEX-mediated gene regulation is dose-specific and thus this hypothesis was tested.

This hypothesis was investigated by performing Vpr dose response analysis in the HeLa cells. The reporter gene assay revealed that Vpr dose-dependently enhances DEX-mediated induction of the TAT-GRE reporter gene. This is consistent with previous studies that showed Vpr dose-dependently enhances the DEX-mediated induction of TAT-GRE and MMTV reporter genes in the 293T, CEM, Jurkat and A204 cell lines (Kino *et al.* 1999; Sherman *et al.* 2000). However, investigation into the endogenous GRE genes showed that the increasing amounts of Vpr did not affect the DEX-mediated induction of GILZ or I κ B α mRNA. Similarly, the increasing amounts of Vpr did not affect the DEX-mediated repression of the pro-inflammatory IL-6 or IL-8 genes. To determine whether Vpr delivered in a more physiologically relevant context i.e. in the context of whole virus could act as a co-activator on the endogenous inflammatory gene, HIV-1 pseudovirus experiments were performed in the TZM-bl cell line. Consistent with the transient transfection experiments, HIV-1 pseudovirus experiments showed that Vpr does not affect the DEX-mediated regulation of the endogenous inflammatory genes in TZM-bl cells. Taken together, results from this present study suggest that Vpr can dose-dependently co-activate GR-mediated transactivation (as illustrated in Figure 7.2) in the presence of GCs and MPA. However, while this co-activation occurs on synthetic reporter genes, the response on endogenous genes may be highly promoter- and/or cell-specific and/or dependent on the absence of chromatin. The current results suggest that Vpr does not co-activate the GR-mediated regulation of endogenous GILZ, I κ B α , IL-6 and IL-8 genes in the presence of ligands in HeLa and TZM-bl cells, even when exogenously expressed, or delivered in HIV-1 pseudovirus particles. Nevertheless, it is possible that Vpr may co-activate other GR-regulated endogenous inflammatory genes in the presence of ligands, or these genes in other cell types such as monocytes (Mirani *et al.* 2002).

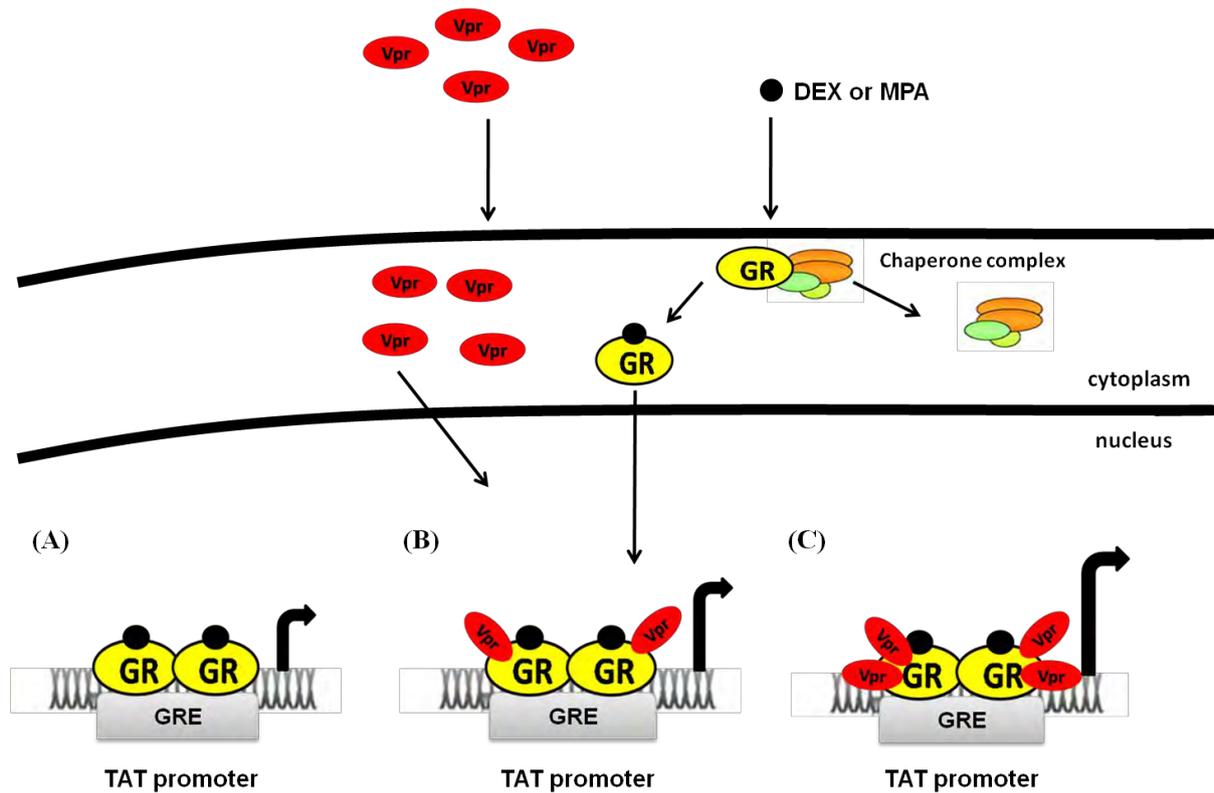


Figure 7.2: Schematic model of Vpr dose-dependent co-activation of GR-mediated transactivation in the presence of DEX and MPA. Upon ligand binding, GR undergoes a conformational change and translocates to the nucleus. Activated GR-ligand complex binds to GRE sites of the promoter region of the TAT gene and (A) activates transcription in the absence of Vpr, (B) in the presence of small amounts of Vpr, GR-mediated transactivation is enhanced and (C) in the presence of high amounts of Vpr, GR mediated transactivation is further enhanced. Abbreviations: DEX = dexamethasone, MPA = medroxyprogesterone acetate, GR = glucocorticoid receptor, Vpr = viral protein R, GRE = glucocorticoid response element, TAT = tyrosine aminotransferase. Note: evidence for Vpr directly associating with GR taken from Kino *et al.* 1999.

In the absence of DEX, although Vpr does not affect the DEX-mediated regulation of the inflammatory genes, it does appear to regulate the pro-inflammatory cytokine genes ligand-independently. In particular, it was shown in this thesis by transient transfection experiments in the absence of GCs that exogenously expressed Vpr upregulates IL-6 and IL-8 mRNA levels in the HeLa cells, suggesting that Vpr is pro-inflammatory. This is supported by a previous study that showed exogenously expressed Vpr upregulates IL-8 protein levels in Jurkat, A549 and U937 cell lines (Roux *et al.* 2000). However in the present study, delivery

of Vpr by pseudovirus did not affect IL-8 mRNA levels, unlike the result with exogenously expressed Vpr. Work by others has shown that delivery of Vpr by pseudovirus upregulates IL-8 protein levels in A549 and U937 cell lines and primary human T-cells and macrophages (Roux *et al.* 2000). Consistent with the effects of Vpr being dose-dependent, the difference between the findings for pseudovirus delivery between the current study and that of Roux *et al.* could be explained by the difference in amount of virus used, or by cell-specific effects. In support of the former possibility, it is notable that the current study infected cells with an apparently lower dose of pseudovirus compared to the Roux *et al.* study (Roux *et al.* 2000). In addition, the pseudovirus constructs used in the Roux *et al.* study is different to the ones used in the present study. Consequently, differences in stoichiometry of the Vpr protein complexes between the different pseudovirus and the exogenously expressed Vpr could also be attributed for the discrepancies. Contrary to previous studies that suggest Vpr is immunosuppressive (Ayyavoo, Mahboubi, *et al.* 1997; Muthumani *et al.* 2000; Mirani *et al.* 2002) in the absence of GCs, findings from this thesis suggest that Vpr may promote a pro-inflammatory response under certain conditions (Figure 5.9 and 5.11 A). It is proposed based on the current findings and literature reports that Vpr may have both anti-inflammatory and pro-inflammatory properties, depending on the life cycle of HIV and concentration of Vpr in the cells, Vpr may switch between these two effects to aid in viral pathogenesis.

Although Vpr regulates the selected pro-inflammatory genes in the absence of GR ligand, it is still possible that the GR plays a role in this regulation. Previous reports have shown that the GR antagonist, RU-486, reverses the GC ligand-independent Vpr-mediated regulation of NF- κ B reporter gene and endogenous inflammatory genes (Mirani *et al.* 2002; Muthumani *et al.* 2006). Furthermore, a previous study from the present author's laboratory has shown that the GR can act ligand-independently to regulate inflammatory genes (Verhoog *et al.* 2011).

Therefore, it is also proposed that the unliganded GR may play a role in the Vpr-mediated anti-inflammatory response (as illustrated in Figure 7.3 A).

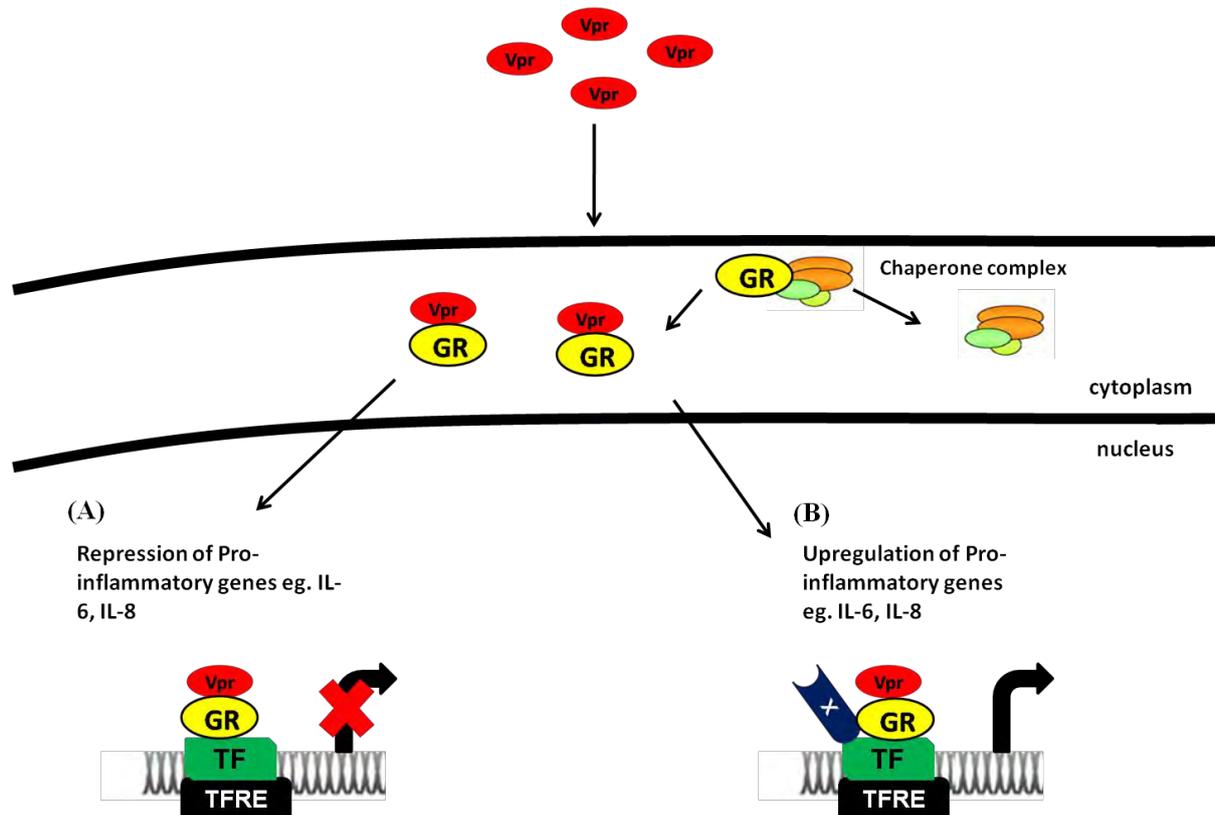


Figure 7.3: A model for the mechanism of unliganded GR involvement in Vpr-mediated regulation of pro-inflammatory genes. It is proposed that depending on the concentration of Vpr and stage of infection, Vpr associates with unliganded GR to either (A) repress or (B) upregulate pro-inflammatory gene expression. Abbreviations: GR = glucocorticoid receptor, Vpr = Viral protein R GRE = glucocorticoid response element, TF = transcription factor, TFRE = transcription factor response element, X = unknown transcription factor.

Attempts were made to investigate the role of unliganded GR in Vpr-mediated upregulation of IL-6 and IL-8 by GR knockdown in the HeLa cells. However, this was unattainable due to the difficulty to obtain efficient GR knockdown whilst preserving cell viability of the HeLa cell line. Nevertheless, experiments in End1/E6E7 cells showed that Vpr upregulation of IL-8 is attenuated when GR is knocked down (unpublished, C. Avenant), consistent with a role for

the unliganded GR in modulating Vpr-mediated regulation of pro-inflammatory genes. Although the mechanism of this regulation is currently under investigation by others in the present author's laboratory, results from this present study rule out the possibility that Vpr promotes a pro-inflammatory response by regulation of GR protein levels. Results from this thesis have important implications for HIV-1 pathogenesis and suggest that depending on the stage of HIV-1 infection that Vpr may be pro-inflammatory in the absence of GCs as illustrated in Figure 7.3 B.

7.4 The GR plays a role in ligand-mediated repression of HIV-1 LTR transcription

In addition to a role for the GR in regulating host inflammatory genes in response to HIV-1 infection, the GR has been shown to play a role in HIV-1 LTR transcription. However, there have been conflicting reports on whether the GR mediates an increase or decrease in HIV-1 LTR transcription (Laurence *et al.* 1989; Furth *et al.* 1990; Kolesnitchenko & Snart 1992; Mitra *et al.* 1995; Kurata & Yamamoto 1999; Russo *et al.* 1999; Kino *et al.* 2000). An increase in HIV-1 transcription would result in an increase in viral replication and whereas a decrease in HIV-1 LTR transcription would most likely decrease viral replication. On the other hand, a decrease in HIV-1 replication could increase viral latency. Thus, it is important to understand the exact role of the GR in regulating HIV-1 transcription.

Previous studies have observed DEX-mediated regulation of HIV-1 LTR transcription in multiple cell lines (Laurence *et al.* 1989; Mitra *et al.* 1995; Kino *et al.* 2000). However, there are no data on whether the synthetic progestins, MPA and NET-A, regulate HIV-1 LTR transcription. Given that MPA acts like a partial to full agonist for the GR, it is proposed that MPA, like DEX, regulates HIV-1 LTR transcription via the GR. This present study investigated the effects of DEX, P4 and the synthetic progestins on HIV-1 LTR transcription in

the HeLa and TZM-bl cell lines. Again, the TZM-bl cell line was used for its ability to be infected by HIV-1. In addition, it was used since it contains a stably integrated HIV-1 LTR-luc reporter gene.

It was found that DEX repressed Tat-activated LTR transcription in the HeLa cell line similar to previous reports (Laurence *et al.* 1989; Mitra *et al.* 1995; Kino *et al.* 2000). It was also found that 100 nM P4 represses Tat-activated LTR transcription which is similar to results from a previous study in U937 cells (Lee *et al.* 1997). In addition, in attempts to determine whether P4 could re-activate LTR activity in the absence of virus, a previous study has also shown in TZM-bl cells that 100 nM P4 represses basal LTR activity (Asin *et al.* 2008). Results from this thesis suggest that mid-secretory phase concentrations of P4 may reduce HIV-1 replication and are consistent with previous findings that show by p24 levels in PBMCs that 100 nM P4 alone and the combination of 100 nM P4 + 1 nM E2 reduces HIV-1 replication (Asin *et al.* 2008). Taken together, results presented here and by others suggest that P4 may reduce HIV-1 replication by a mechanism of transcriptional repression of the LTR. Interestingly, Asin *et al.* also found that mid-proliferative phase concentrations of E2 and P4 may enhance HIV-1 replication in PBMCs (Asin *et al.* 2008) suggesting that HIV-1 viral loads may increase or decrease depending on the stage of the menstrual cycle, consistent with clinical reports that show cyclic changes in HIV-1 levels (Greenblatt *et al.* 2000; Reichelderfer *et al.* 2000).

For the first time it was shown here that both the synthetic progestins, MPA and NET-A, repress LTR transcription. Consistent with these findings the HIV-1 pseudovirus experiment results showed that all the ligands repressed the stably integrated LTR transcription in the TZM-bl cell line. These findings show that the delivery of Tat by transient transfection is comparable to the HIV-1 pseudovirus delivery of Tat protein. In addition, it shows that the transiently transfected LTR-luc expression vector which is not surrounded by chromatin

yielded similar results to the stably integrated LTR-luc in the presence of chromatin. These results have implications for HIV-1 pathogenesis *in vivo* and suggest that progestins, like DEX, may decrease transcription of the HIV-1 LTR in HIV-1 infected cells.

The GR antagonist, RU-486, has been suggested to have anti-viral effects on HIV-1 (Schafer *et al.* 2006; Benton *et al.* 2013). In particular, it has been previously shown that RU-486 decreases HIV-1 replication in monocyte-derived macrophages and PBMCs (Schafer *et al.* 2006; Benton *et al.* 2013). This is consistent with this present study's findings which show RU-486 represses Tat-activated LTR transcription in the HeLa cells. This present study also shows that the combination of DEX and RU-486 represses LTR transcription. The mechanism of RU-486-mediated repression of the LTR has been suggested to be via the antagonism of GR (Kino *et al.* 2000; Schafer *et al.* 2006; Benton *et al.* 2013). The current findings are not consistent with a mechanism of GR antagonism. The results suggest that the unliganded GR is required for Tat-activated LTR transcription, and that this effect of the unliganded GR can be inhibited by RU486, acting in agonist mode. In the presence of DEX, RU486 also does not act as a DEX antagonist, but rather appears to act in agonist mode, resulting in no lifting of the DEX effect, or possibly even more repression. RU-486 has been shown to manifest both GR antagonist and agonist properties depending on the GR density in the cells (Zhao *et al.* 2003). At high GR density, RU-486 can behave as a full agonist for GR-mediated transrepression while at low GR density, RU-486 can act a full GR antagonist (Zhao *et al.* 2003). The current results suggest that the HeLa cell line has a GR density that promotes agonist properties of RU-486 in GR-mediated transrepression of the LTR, in the absence and presence of DEX. RU-486 has also been shown to bind GR β and have GR β -mediated transcriptional effects (Lewis-Tuffin *et al.* 2007). Alternatively, results suggest that the HeLa cells may express GR β and transrepression of the LTR by RU-486 may be via GR β .

The role of the GR in the ligand-mediated repression of HIV-1 LTR transcription was investigated by GR knockdown experiments. The GR knockdown revealed that all the ligands still repressed LTR transcription. This suggests that the GR may not be involved in the ligand-mediated repression or it could suggest that the ligands require very little GR to mediate transcriptional repression of the LTR gene in the TZM-bl cells. The latter explanation seems likely since ~60% of the GR was still present. Also consistent with the latter explanation, depending on the percentage GR knockdown, some transrepression model genes have shown to be hypersensitive to GR whereby ligand-mediated repression still occurs in the presence of GR siRNA transfection e.g. IL-6 in A549 cells (unpublished, C. Kemp). Attempts to increase the % GR knockdown were unsuccessful due to cell death. The addition of NET and LNG to the panel of ligands revealed a linear trend in the rank order in which the ligands repress LTR transcription i.e. DEX>MPA>P4>NET-A>NET>LNG. The rank order of DEX>MPA>P4>NET-A>LNG is similar to that of the ligands relative binding affinity for the GR (Koubovec *et al.* 2005; Hapgood *et al.* 2013). In addition, it is similar to the ligands potency for GR-mediated transactivation of GRE-regulated genes and transrepression of NF- κ B-regulated genes (Koubovec *et al.* 2005; Ronacher *et al.* 2009). Furthermore, a previous study showed by GR overexpression experiments that the GR is involved in the DEX-mediated repression of the LTR (Mitra *et al.* 1995). Taken together, it is most likely that the GR mediates the responses the ligands in LTR repression. In addition, insensitivity to ~40% GR knockdown suggests that very little GR is required for the regulation of the LTR. In comparison to the effects of the ligands on inflammatory gene regulation, it appears as if the presence of virus has blunted the differences between ligand activities via the GR on LTR regulation. That is, in the presence of virus the LTR gene is hypersensitive to GR bound to any of the ligands. For example, GR-mediated repression of the pro-inflammatory cytokines IL-6 and IL-8 in the HeLa and End1/E6E7 cells occurs with DEX and MPA and not with P4

and NET-A. In addition, GR-mediated repression of HIV-1 LTR transcription in the HeLa and TZM-bl cells occurs with both DEX and the progestogens. Furthermore, although the low dose intra-vaginal progestin contraceptive LNG has a reported RBA for the GR (> 1%); it has no reported GR activity (Hapgood *et al.* 2013). However, for the first time, results from this thesis suggest that LNG may regulate LTR transcription via the GR. Results from this present study suggest that both high dose progestins like MPA and NET-A and low dose progestins like LNG used in contraception and GCs used in therapeutics may regulate HIV-1 replication via the GR. This has important implications for both women on progestin-only contraception and patients with chronic inflammation on GCs. As mentioned earlier, a decrease in HIV-1 replication could have positive effects for the host. On the other hand, a decrease in HIV-1 replication by the progestins could possibly promote viral latency in women on hormonal contraception and this would allow the virus to persist at low levels for years prior to the onset of AIDS (Levy *et al.* 1994). A leading theory for how HIV-1 latency is initially established involves infection of activated CD4⁺ T cells as they are returning to a resting state to form long-lived memory T cells (Siliciano & Greene 2011; Hakre *et al.* 2012). During this transition, HIV-1 gene expression is largely suppressed (Abbas & Herbein 2012). Consequently, a stably integrated but transcriptionally silent provirus in a memory T cell is formed. Results presented in this thesis provide a possible molecular mechanism for the role of progestins in viral latency. As suggested earlier, the progestins repress LTR transcription via the GR. The classical GR mechanism of action for transrepression is via recruitment of co-repressors and chromatin remodelling complexes such as HDACs which facilitate chromatin condensation and consequently gene repression (Chinenov *et al.* 2013). HDACs have previously been shown to be responsible for suppression of HIV-1 gene expression (Kumar *et al.* 2014). In addition, HDAC-1 recruitment has been shown to promote latency of HIV-1 LTR (Williams *et al.* 2006). Furthermore, HDAC inhibitors such as vorinostat have

been used therapeutically to reactivate HIV-1 from latency in patients undergoing HAART (Archin *et al.* 2012). Therefore, it is suggested that the progestins acting via the GR may promote recruitment of HDACs to the LTR and consequently promote viral latency. To the present author's knowledge there have been no reports on the effects of progestins on viral latency.

Although results from this thesis are contrary to previous reports from non-human primate models and clinical studies involving humans that suggest MPA-usage is associated with higher mean peak and acute viral loads as reviewed in (Stringer & Antonsen 2008), it is possible that the effects of hormones may differ during different stages of infection. Clinical reports also show that use of hormonal contraception is associated with increased HIV-1 disease progression in women not yet on anti-retroviral therapy (Stringer *et al.* 2009) while others show that MPA-usage is not associated with deleterious consequences for HIV-1 disease progression (Heffron *et al.* 2013; WHO 2014). Future research should assess how these hormones affect acute vs. chronic infection in the context of disease progression. Results from this thesis also suggest that further investigation into the biological activity of low dose progestins such as LNG and etonogestrel (ETG) in the context of HIV-1 infection is warranted. In particular, emphasis should be placed on LNG since this low dose progestin used in implants (Norplant) has recently been chosen to be investigated in a new randomised clinical trial (announced at the HIV R4P meeting, October 2014) (Rees 2014). In addition, LNG (vaginal ring) in combination with microbicides for multi-purpose prevention technologies is under current investigation by CONRAD (Thurman *et al.* 2013). Furthermore, ETG used in the implant Implanon has been recently rolled out at public health facilities across South Africa (SA to offer women free implant contraceptives, 2014). It would be crucial to determine whether progestins, like LNG and ETG, have differential off-target

biological effects in the context of HIV-1 before being considered for clinical trials and public use.

Interestingly, in this current study when GR was knocked down, basal LTR transcriptional activity also decreased. This suggests that the unliganded GR protein is required for efficient HIV-1 LTR transcription. Alternatively, the decrease in basal LTR transcription could be due to fewer virus particles entering the cells in the knockdown condition. This is consistent with a previous study which showed by GR knockdown that GR protein is required for optimal provirus integration (Wiegers *et al.* 2008). Taken together, this may have implications for HIV-1 pathogenesis whereby unliganded GR protein levels may be regulated by the virus to ensure optimal infectivity and is consistent with a clinical study which suggests there is a link between changes in GR levels and HIV-1 infection (Guo *et al.* 1996). Although, this present study showed that Vpr does not regulate GR protein levels, this does not rule out the possibility that other non-Vpr related mechanisms by HIV-1 may regulate GR protein levels. Consequently, it would be important to determine whether HIV-1 infected individuals have significantly different GR levels than uninfected individuals. These results also imply that factors such as GCs and some progestin contraceptives that are shown here and by others to regulate GR protein levels may influence HIV-1 replication.

7.5 Vpr does not play a role in GR-mediated regulation of HIV-1 LTR transcription

Evidence in the literature suggests that Vpr plays an important role in HIV-1 LTR transcription. In particular, Vpr has been shown to both activate viral transcription in the absence of Tat and enhance Tat-activated viral transcription (Forget *et al.* 1998; Zhu *et al.* 2001; Kino *et al.* 2002; Varin *et al.* 2005; Cui *et al.* 2006). However, there is limited data on role of Vpr in the regulation of HIV-1 LTR transcription in the presence of DEX and no data

for the synthetic progestins, which was thus explored in this thesis. In this study, the HeLa and TZM-bl cell lines were again used as the model systems.

Similar to previous results obtained in this thesis; DEX, P4, MPA and NET-A all repressed LTR transcription in the absence of Vpr but in the presence of Tat. It was found that Vpr enhances Tat-activated LTR transcription in the HeLa cell line, consistent with previous reports in human primary microglial cells, Jurkat cells and A204 cells (Kino *et al.* 2000; Sawaya *et al.* 2000; Kino *et al.* 2002). This is also consistent with the idea that Tat and Vpr are required for optimal HIV-1 replication (Sawaya *et al.* 2000). Similar to a previous report, this current study shows that Vpr does not modulate the suppressive effects of DEX on Tat-activated HIV-1 LTR promoter (Kino *et al.* 2000). For the first time it is shown that Vpr also does not affect the progestin-mediated suppression of Tat-activated HIV-1 LTR transcription. This suggests that unlike host gene regulation Vpr does not modulate GR-mediated LTR repression in the presence of the ligands.

HIV-1 pseudovirus experiments were performed in the TZM-bl cell line to confirm the result that Vpr does not affect the GR-mediated HIV-1 LTR repression. Consistent with previous findings in this thesis, DEX repressed HIV-1 wt activated LTR transcription. It was found that wt virus (Tat + Vpr protein) dose-dependently activated the LTR while the LTR activation with Δ Vpr virus (Tat protein alone) was not statistically significant. This is consistent with the suggestion that Vpr and Tat are required for optimal HIV-1 replication. Since the LTR activation with Δ Vpr virus was too low to detect using luciferase assays in the TZM-bl cells, the DEX-mediated suppression of the LTR in the absence of Vpr could also not be detected. Therefore, transient transfection experiment results could not be confirmed using this method.

7.6 Conclusions

In summary the present study demonstrates directly for the first time that the anti-inflammatory effects of MPA, unlike NET-A and P4, in endocervical epithelial cells are via the GR. In addition, this study shows that the GR mechanism of action is via transcriptional regulation of the inflammatory genes. Furthermore, it is shown that at physiological doses particularly shortly after injection, MPA is anti-inflammatory for the genes investigated. Although the endocervical cells do not get productively infected by pathogens such as HIV-1, these cells play an important role in mucosal barrier protection. Therefore results from this thesis have implications for genital mucosal immune function and disease susceptibility and progression. In particular, effects of MPA are likely to be very different to those of NET-A and P4, when mediated via the GR, whereby a decrease in immune activation markers by MPA may inhibit T-cell activation and result in a lowered T-cell immune response. This is consistent with literature that shows MPA like the GC, DEX, has other consequences for T-cell function such as increased apoptosis of T-cells. Accordingly, this may lower the host's ability to mount a defence against pathogens such as HIV-1. Consequently, results from this thesis provide a mechanism for most observational reports that suggest injectable MPA increases the risk of HIV-1 acquisition in women whereby the GR is likely to play a major role in HIV-1 susceptibility in the FGT in the presence of MPA.

In addition, it was established that the GR may play a role with Vpr in HIV-1 pathogenesis. In HIV-1 infection, extracellular Vpr may enter host cells and thereby modulate host gene regulation via the GR in the absence or presence of GR ligands. In the presence of GR ligands, Vpr may promote an anti-inflammatory response via the GR which could lead to an immunocompromised individual and this could allow opportunistic pathogens to infect the host. Vpr may enhance GR-mediated gene regulation in the presence of DEX and MPA, but

not NET-A or P4 in some contexts. The physiological implications could be that MPA, unlike NET-A and P4, may mimic the effects of GC hypersensitivity and/or potentially enhance the GC hypersensitivity observed in AIDS patients. In addition, it is possible that Vpr could discriminate between MPA vs. NET-A in HIV-1 disease progression. While in the absence of hormones, Vpr may hijack the unliganded GR to promote a pro-inflammatory response which would then most likely recruit more target cells to be infected. Results from this thesis suggest that depending on its concentration in host plasma and/or cells, Vpr may switch between anti-inflammatory or pro-inflammatory properties and this will also most likely be dependent on stage of HIV-1 infection. In addition, Vpr modulation of host genes may be highly promoter-specific and/or dependent on the absence of chromatin.

The GR may also play a role in HIV-1 pathogenesis by viral LTR regulation in the absence and presence of GR ligands. In the absence of GR ligands the unliganded GR may be required for efficient viral replication. While in the presence of GR ligands; the activated GR may be involved in the ligand-mediated regulation of HIV-1 LTR transcription. It was shown for the first time that MPA, NET-A and LNG like DEX and P4 suppress LTR transcription, possibly via the GR, and Vpr does not modulate this regulation. These results have implications for HIV-1 replication and disease progression. Results suggest that, depending on the stage of HIV-1 disease, a decrease in HIV-1 replication by the progestogens and GCs may either have positive effects for the host or aid viral latency and consequently support HIV-1 pathogenesis.

Of most significance, results from this thesis highlight the importance of choice and concentration of progestin used in contraception. Collectively present *in vitro* results with previous *ex vivo*, animal *in vivo* and clinical studies suggest that relatively high dose progestins such as injectable MPA, that have off-target biological effects via the GR may not be the best contraceptive option for women at high risk for HIV-1 infection. Since injectable

contraception is the method of choice in high risk areas, an alternative contraceptive for women in these areas must not only have good contraception efficacy but also be practical, acceptable and discrete. The other available injectable progestin-only contraceptive, NET-EN, may represent a safer alternative to MPA, based on the results reported here. Since the observational clinical data on whether injectable contraceptives, in particular DMPA, increases risk of HIV-1 acquisition and disease progression is controversial it was not considered to be persuasive enough by the WHO to recommend discontinuation of any particular form of contraception (WHO 2014). Therefore a randomized clinical trial designed to compare the effects of MPA to other contraceptives may be the only way to get a clearer answer on whether there is an increased HIV-1 risk associated with DMPA-usage.

7.7 Future perspectives

Based on literature reports and results from this thesis, there appears to be major differences in SR content between the three compartments of the FGT and this may explain the differences in the MPA-mediated regulation of inflammation in these compartments. It would be interesting to directly compare the role of MPA in inflammation in the three different compartments of the FGT (vaginal vs. ectocervix vs. endocervix) and possibly determine the mechanism of action of the MPA-mediated regulation in each compartment. This could be done by performing experiments in the vaginal (Vk2/E6E7), ectocervical (Ect1/E6E7) and endocervical (End1/E6E7) cell lines. This should also be confirmed in primary cells by isolating cells from each compartment. Dose-response analysis could be done to determine whether the synthetic progestins regulate inflammatory genes at hormonal contraception concentrations. To gain insight into the mechanism of action, the SR content of primary cells should be assessed, if not previously known.

The question as to whether changes in SR content for e.g. PR to GR ratio during the menstrual cycle differentially regulate inflammatory gene expression in response to progestins was raised in thesis. The relative PR/GR ratio in different compartments of the FGT should be investigated and then could be mimicked in the End1/E6E7 cell lines to determine whether SR ratios affect gene regulation. Varying amounts of PR could be overexpressed to mimic different stages of the menstrual cycle and inflammatory gene regulation could be assessed in the presence of progestins. It would also be interesting to investigate whether changes in PR to GR ratio during the menstrual cycle influence HIV-1 infection and/or replication.

Results from this thesis suggest that MPA-mediated anti-inflammatory effects in endocervical cells may have important implications for HIV-1 susceptibility and progression in the lower FGT. In order to investigate this hypothesis, the effects of MPA-regulated cytokine secretions on T-cell susceptibility to HIV-1 infection need to be determined. This could be done by incubating the endocervical cell line and/or primary endocervical cells with different doses of MPA, NET-A, P4 and DEX for 24 hrs and/or 48 hrs. After incubation, the medium which would contain the ligand-regulated cytokine protein secretions could be collected. Thereafter, T-cells could be pre-incubated with the cytokine secretions and then infected with HIV-1 pseudovirus or infectious molecular clones (IMCs). Then p24 levels could be measured to determine whether the ligand-regulated cytokine secretions affect HIV-1 infection.

Another strategy to determine whether progestins such as MPA increase HIV-1 susceptibility would be to perform experiments on cervical tissue explants. Cervical tissue could be collected from pre-menopausal women undergoing hysterectomies for benign reasons. Primary cervical explants could be used to measure the productive infection of HIV-1 target cells such as CD4⁺ T-cells. Cervical explants could be treated with different doses of MPA,

NET-A, P4 and DEX and then infected with HIV-1 IMCs. Then, as above, p24 levels could be measured.

In this present study it is suggested that the effects of the progestins when mediated via the GR may have negative consequences for genital mucosal immune function and possibly HIV-1 pathogenesis. In addition, although LNG has no reported GR activity results from this thesis shows that it may act via the GR in regulation of HIV-1 LTR transcription. All these findings highlight the importance of choice of progestin used in contraception. LNG is a 2nd generation contraceptive and is used in vaginal rings and implants. Therefore it would be crucial to determine whether this progestin and other newer generation progestins such as ETG used in implants and vaginal rings would affect genital mucosal immune function. This could be done by treating the endocervical cell line and/or primary endocervical cells and/or cervical explants with a panel of newer generation progestins and then measure inflammatory gene and cytokine protein regulation. If the newer generation progestins affect inflammatory gene regulation, it would be important to determine whether this affects HIV-1 susceptibility and could be done as explained above.

Given the controversy around MPA-usage, it would also be important to determine the effects of new and existing contraceptives on a broad panel of genes such as genes involved in innate and adaptive immunity, cell proliferation, cell integrity and structure. For existing contraceptives this could be done by performing RNA sequencing analysis on blood, cytobrush and/or cervical tissue samples from women.

It would also be important to determine whether the synthetic progestins, MPA and NET-A, differentially regulate cytokine expression in *in vivo* models. This could be done using 'humanized' mouse models. This is a mouse model in which various types of human cells and tissues are engrafted and function as they would in humans (Ito *et al.* 2012). Experiments

could be done by having 4 groups of mice which represent the panel of ligands (control, DEX, MPA and NET-A). These mice could be treated with injectable hormonal contraceptive concentrations of MPA (every 3 months) and NET-A (every 2 months). Thereafter blood and cervical tissue sample could be collected to determine the effect of the hormones of inflammatory gene expression. The serum levels of natural hormone P4, MPA and NET-A in the blood and cervical tissue could also be tested. Furthermore, the SR content and the effects of the ligands on SR levels could be determined. It would also be important to determine whether MPA increases HIV-1 susceptibility and investigate the effect of MPA on cytokine regulation in the presence of HIV-1 infection *in vivo*. This could also be done using the ‘humanized’ mouse model for HIV-1 infection (Denton & Garcia 2011).

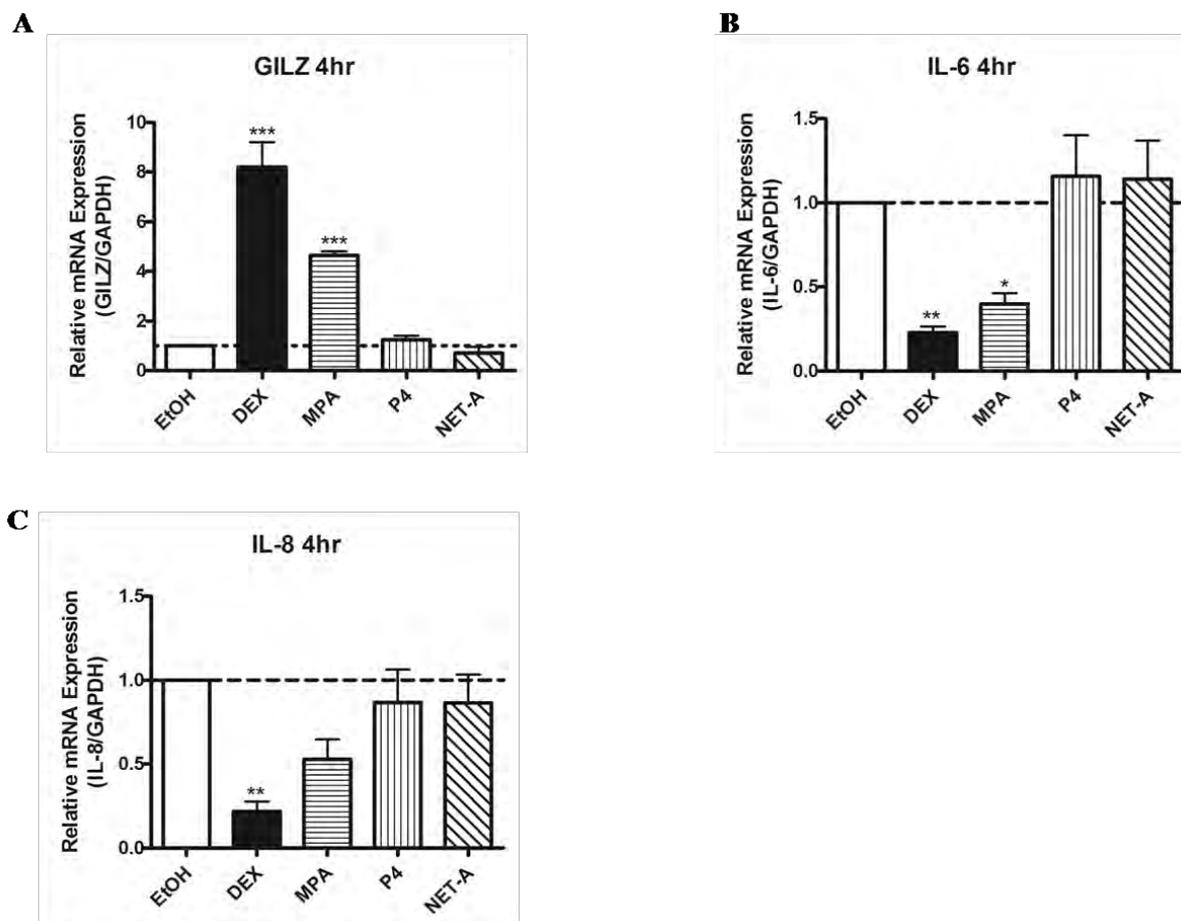
Results from this thesis show that Vpr dose-dependently co-activates GR-mediated transactivation but this co-activation may be highly promoter-specific. In order to investigate this hypothesis, first the expression of a large number of genes in the absence of presence of DEX and/or Vpr needs to be analyzed. This could be done by performing RNA sequencing which has the capacity to analyze the expression of thousands of genes at one time. Once the genes that are co-activated by Vpr are identified, GR siRNA knockdown can be done to determine whether the GR is required for the gene regulation. Thereafter, the promoters of the GR-regulated genes that are co-activated by Vpr can be analyzed further to identify if the promoter of these genes have a common site/region that allows this co-activation response.

Evidence in the literature suggests that Vpr is anti-inflammatory similar to GCs by the regulation of inflammatory mediators (Ayyavoo, Mahboubi, *et al.* 1997; Muthumani *et al.* 2000; Mirani *et al.* 2002). This current study showed that Vpr-mediate an induction of pro-inflammatory cytokines in the absence of ligands. This result taken together with previous reports in the literature suggest that Vpr may be anti-inflammatory and pro-inflammatory depending on the HIV-1 life cycle and concentration of Vpr. Results also suggest that the

unliganded GR may be involved in the Vpr-mediated pro-inflammatory response. This could be further investigated in the peripheral blood mononuclear cells (PBMCs). Varying amounts of HIV-1 pseudovirus/IMC could be used to deliver different doses of Vpr protein and GR knockdown could be done to assess whether the GR is involved in the Vpr-mediated regulation of the inflammatory genes. To gain further insight into mechanism of action, CHIP assays could be done to determine whether the GR and/or Vpr are recruited to the promoters of the inflammatory genes.

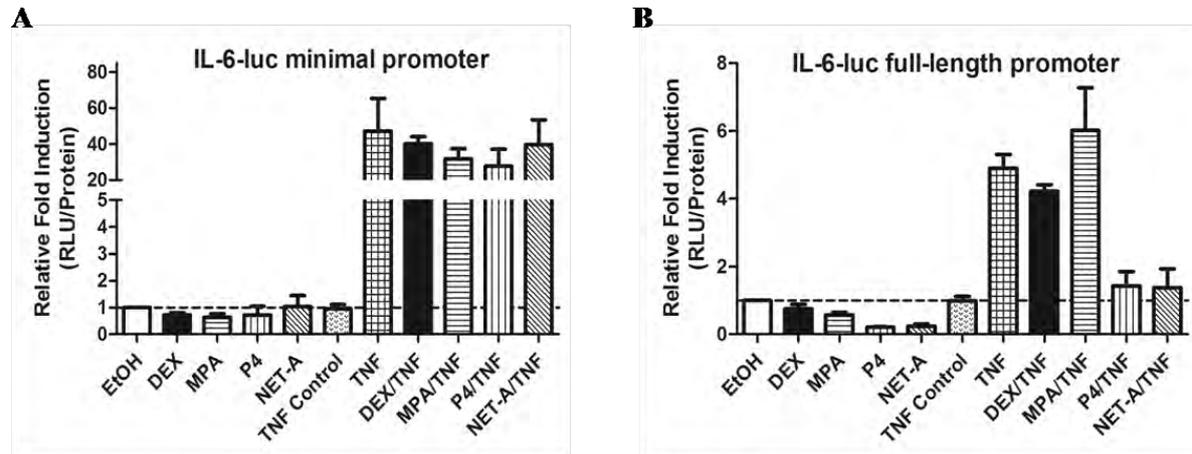
In addition to the role of the GR in inflammatory gene regulation, this present study suggests that the GR is involved in the regulation of HIV-1 LTR transcription in the presence of DEX and the progestins. Although antagonist experiments using RU-486 and GR knock down using siRNA were performed to establish the role of the GR in LTR regulation, these experiments were inconclusive. Since 41% knockdown of GR protein did not reverse the ligand-mediated repression of the LTR, it was suggested that the LTR gene is hypersensitive to GR. To test this hypothesis dose response analysis with the ligands could be done. It is predicted that at lower concentrations of ligands, there would be less repression of the LTR. Since a higher protein knockdown using GR siRNA transfection could not be achieved while preserving cell viability, lentiviral knockdown using GR shRNA could be performed to confirm GR involvement in LTR regulation.

Addendum: Supplementary Data

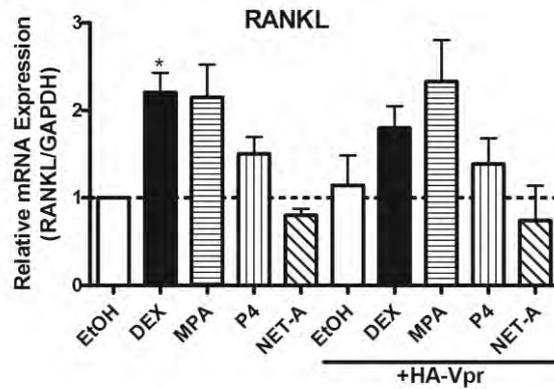


Addendum Figure 1: MPA acts like a GR agonist in the regulation of inflammatory genes. HeLa cells were seeded at a density of 15×10^4 cells/well and incubated for 24 hrs. The cells were then treated with 100 nM DEX, MPA, P4, NET-A or EtOH (0.1% v/v, vehicle) for 4 hrs. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. **(A)** Relative GILZ, **(B)** IL-6 and **(C)** IL-8 gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (EtOH) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA, with Dunnett

post-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.



Addendum Figure 2: DEX does not appear to repress IL-6 reporter gene. HeLa cells were seeded into a 24-well plate at a density of 7.5×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with either 250 ng/well **(A)** IL-6 minimal promoter (p(IL-6 κ B)3-50hu-IL-6P-luc) or **(B)** IL-6 full length promoter (p1168hu-IL-6P-luc). After 24 hrs, the cells were treated with/without TNF- α in combination with 100 nM DEX, 1 μ M MPA, 10 μ M P4, 10 μ M NET-A or EtOH (0.1% v/v; vehicle) for 24 hrs. Then, the cells were washed twice with PBS and lysed with 1X reporter lysis buffer and the luciferase activity was measured. Luciferase activity was normalized to protein content per well as determined by Bradford assay. In addition, the ligand stimulated responses were normalized with the basal activity (EtOH) in order to obtain a fold induction. Graph represents pooled results of at least two independent experiments, performed in triplicate, and are plotted as means \pm SEM.



Addendum Figure 3: Vpr does not affect DEX- and MPA-mediated transactivation of RANKL. HeLa cells were seeded into a 12-well plate at a density of 15×10^4 cells/well and incubated for 24 hrs. Thereafter, the cells were transiently transfected with $1 \mu\text{g}/\text{well}$ HA-Vpr or empty vector. After 24 hrs, the cells were treated with 100 nM DEX, 1 μM MPA, 10 μM P4, 10 μM NET-A or EtOH (0.1 % v/v; vehicle) for 24 hrs. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. Relative RANKL gene expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalized to basal activity (EtOH) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism™ software (version 5) using a one-way ANOVA followed by a paired t-test. Statistical significance is denoted by * to indicate $P < 0.05$.

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

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Abstract

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

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Introduction

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

Pathogens such as herpes simplex virus (HSV), human papillomavirus (HPV), and HIV have been shown to infect epithelial cells of the FRT and the process is affected by treatment with hormones such as progesterone (P4) [9,10].

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

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

Clinical research on the effects of contraceptives on HIV-1 acquisition, transmission and disease progression has been hampered by a lack of understanding of the molecular mechanisms of action of the progestin components of contraceptives and a lack of appreciation of the differences between progestins, which cannot be considered to act as a single class of compounds regarding their side-effect profiles [36–38]. Although MPA and NET elicit similar progestational effects to P4 [39,40], differences in biological effects mediated via steroid receptors other than the progesterone receptor (PR) could be expected [36–38], and have been demonstrated for the GR [41]. Synthetic progestins were designed to mimic the actions of the natural ligand P4 but with better bioavailability [38]. Both NET-EN and NET-A are metabolised to the active molecule NET, as well as other metabolites, unlike MPA, which is itself the active compound [42]. Progestins were also designed to be potent, high affinity PR agonists. However, many progestins bind to other members of the steroid receptor family, including the GR, the androgen receptor (AR) and mineralocorticoid receptor (MR) [43], thereby exhibiting off-target effects via these receptors [37,38]. It has been shown that MPA has a higher relative binding affinity compared to NET-A and P4 for the human GR (relative binding affinity % of 79.1, 0.88 and 5.57 for MPA, NET-A and P4, respectively) [41] and unlike NET-A and P4, acts as a potent partial to full GR agonist for both transactivation and transrepression [41,44]. The GR, a ligand activated steroid receptor, has potent anti-inflammatory and immunosuppressive activity [45]. Consistent with this idea, we have previously shown that MPA, at doses in the range found in serum of contraceptive users, represses expression of mRNA and protein levels of the pro-inflammatory cytokine IL-6 and the chemokine IL-8, in mouse fibroblast cells, most likely via the GR

[46]. Similarly, Bamberger *et al.* showed that MPA represses IL-2, IL-1, and IL-6 protein expression in normal human lymphocytes, most likely via the GR [47]. Thus it is possible that MPA used as contraceptive modulates immune function and inflammation, and hence responses to pathogens, by changes in cytokine gene expression, particularly in the genital mucosa. A key question that remains to be investigated is what the effect is of different synthetic progestins as compared to P4 on cytokine gene expression and immune function in the FRT. These are likely to vary since we have previously shown that MPA, compared to NET-A and P4, elicit very different effects on IL-8 promoter expression in HEK293 cells, mediated via the GR [41], as well as exhibit differential effects in several steps of the GR pathway [44]. In support of an immunosuppressive role of MPA in increasing HIV-1 pathogenesis, MPA was recently shown to have immunosuppressive effects on key regulators of cellular and humoral immunity and increased HIV-1 replication in activated peripheral blood mononuclear cells (PBMCs) *ex vivo* [48,49]. The Hel laboratory also showed that women using DMPA displayed lower levels of IFN α in plasma and genital secretions compared to controls with no hormonal contraception, consistent with an immunosuppressive effect of DMPA *in vivo* [48,49]. A possible mechanism for differential effects of progestins and P4 on HIV-1 acquisition may include differential effects on inflammation in the FRT. However the direct effects of MPA, as compared to NET and P4, on expression of inflammatory markers in endocervical cells, the prime site for HIV-1 acquisition, have not been previously investigated. Using a human immortalised endocervical (End1/E6E7) epithelial cell line [50] as a model for the mucosal surface of the endocervix, as well as the HeLa cervical cell line, the present study aimed to determine the relative effects, molecular mechanisms and steroid receptor involvement of MPA, P4 and NET-A in expression of key inflammatory response genes.

Materials and Methods

Antibodies and Compounds

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

Cell Culture

Human epithelial cervical cancer cells (HeLa) purchased from America Type Culture Collection (ATCC, USA) were cultured in 75 cm² flasks (Greiner Bio-one International, Austria) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 10% (v/v) foetal bovine serum (Highveld Biological, South Africa) 100 IU/mL penicillin and 100 μ g/mL streptomycin (Gibco, Invitrogen, UK). End1/E6E7 (human endocervical cells immortalized with human papillomavirus E6/E7 [54] were obtained from Dr Fichorova, OB/GYN Depart-

ment, Brigham & Women's Hospital, Boston, USA. The End1/E6E7 cells were cultured in 75 cm² flasks (Greiner Bio-one International, Austria) in keratinocyte serum-free medium (ker-sfm; Sigma-Aldrich, South Africa) supplemented with keratinocyte growth supplement, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen, UK). All cells were maintained at 37°C in a 5% CO₂ incubator. Cells were passaged with 0.25% trypsin/0.1% EDTA in PBS (Highveld Biological, South Africa). Trypsinization was terminated with neutralization medium [DMEM (Sigma-Aldrich, South Africa), 10% (v/v) calf serum (Highveld Biological, South Africa), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen, UK)]. The cell lines were regularly tested for mycoplasma infection by means of Hoechst staining [55], and only mycoplasma-negative cells were used in experiments.

Plasmids

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

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

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

Western Blotting

For the steroid receptor controls, COS-1 cells were seeded into 12-well plates (Greiner bio-one, Cellstar, Austria) at a density of 25×10⁴ cells/well. The next day the cells were transfected with 1 µg/well of empty vector, GR, AR or PR and 2 µg/well of MR or ER using FuGENE 6 (Roche Diagnostics, South Africa). After 24 hrs, the cells were washed once with PBS and lysed with 50 µl 2X SDS sample buffer (5 X SDS sample buffer: 100 mM TRIS-HCL pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% β-mercaptoethanol and 0.1% (w/v) bromophenol-blue) and boiled for 10 min at 100°C. In addition, lysates were prepared from

End1/E6E7 and HeLa cells seeded into 12-well plates at a density of 35×10⁴ cells/well and 15×10⁴ cells/well, respectively. Equivalent amounts of protein were loaded on either a 6% or 8% SDS-PAGE before being transferred onto a Hybond-ECL nitrocellulose membrane (Amersham, South Africa) using the Mini Protean III blotting system (Bio-Rad, South Africa). Blots were probed for anti-GR (1:4000), anti-PR (1:1000), anti-AR (1:1000), anti-ER (1:500), anti-MR (1:1000), anti-GAPDH (1:20 000) at 4°C overnight. Blots were washed 3 times with TBS containing 0.1% Tween for 5 mins each and subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:10000) or goat anti-mouse (1:5000) secondary at room temperature for 1 hr. Protein detection was performed using ECL substrate (Thermo Scientific, South Africa) with visualization on X-ray hyperfilm (Amersham, South Africa). Bands on the X-ray film were quantified using AlphaEaseFC software version 3.1.2 (Alpha Innotech Corporation).

GR Knockdown by Small Interference RNA (siRNA)

GR knockdown was performed as previously described [56], but briefly End1/E6E7 cells were seeded in 12-well plates at a density of 35×10⁴ cells/well and incubated for 24 hrs. Thereafter, the cells were transfected with 10 nM validated GR HS_NR3C1_5 (cat #SI02654757) (Qiagen, South Africa) siRNA directed against the human GR or validated non-silencing scrambled sequence control (NSC) siRNA (cat #1027310) (Qiagen, South Africa) using HiPerfect transfection reagent (Qiagen, South Africa) as per the manufacturer's instructions. Cells were incubated for 48 hrs before being treated for 24 hrs with 100 nM test compounds. RNA was then harvested and mRNA levels were analysed by qRT-PCR, as described above. To verify the protein knockdown, cells were transfected in parallel and analysed by Western blotting as described above.

Luminex

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

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed as described in Verhoog *et al* 2011 with modifications [56]. HeLa cells were plated at 3×10⁶ cells per dish in 15 cm dishes and grown for 24 hrs in full DMEM, before changing to phenol red-free DMEM (Sigma-Aldrich, South Africa) for an additional 24 hrs. Thereafter, the cells were incubated with serum-free, phenol-free DMEM for 2 hrs, before treatment with 100 nM DEX, MPA, P4 and NET-A for 1 hr. Cells were crosslinked for 10 min at 37°C with 1% formaldehyde and the reaction was stopped with 0.1 mM glycine for 5 min, shaking at room temperature. Cells were scraped in PBS, pelleted by centrifugation and resuspended in 500 µl nuclear lysis buffer (1% SDS, 50 mM TRIS-HCL, pH 8.0, 10 mM EDTA, 1x protease inhibitor cocktail), before sonication. For immunoprecipitation, 100 µg DNA was pre-cleared with protein A/G agarose beads (sc-2003, Santa Cruz Biotechnology, USA) for 1 hr, rotating at 4°C, before incubating with 2 µg anti-GR (H300) (Santa Cruz Biotechnology, USA) or 2 µg anti-goat (Santa Cruz Biotechnology, USA), as IgG control, overnight on a rotator at 4°C. The following day, the complex was precipitated with protein A/G agarose beads for 6 hrs at 4°C, before being washed sequentially with 1 ml each of wash buffer I, II and III [57], followed by three washes with 1 ml TE buffer [10 mM Tris pH 8, 0.1 mM EDTA]. Proteins were eluted from the beads by addition of 300 µl elution

buffer (1% SDS, 100 nM NaHCO₃), before the addition of 300 nM NaCl and incubation at 65°C overnight to reverse crosslinks. The following day 15 nM EDTA, 125 nM TRIS-HCL pH 6.5 and 20 µg proteinase K (Roche, South Africa) were added and samples were then incubated at 45°C for 1 hr. DNA was purified using PCR cleanup columns (Qiagen, South Africa). Real time qRT-PCR was performed on a Corbett Rotorgene, using the Sensimix (Quantace, South Africa), which measures SYBR Green fluorescence. ChIP primers used: for IL-6 5'-TCTACAA-CAGCCGCTCACAG-3' (forward primer) and 5'-AGCGTTC-CAGTTAATTTGTATTTGT-3' (reverse primer), for IL-8 5'-GGGCCATCAGTTGCAAAT-3' (forward primer) and 5'-TTCCTTCCGGTG GTTCTTC-3' (reverse primer).

Primary Cervical Epithelial Cells

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

Results

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

We investigated the effects of the synthetic progestins on the expression of GR regulated inflammatory genes in the End1/E6E7 endocervical epithelial cell line as well as the HeLa cervical epithelial cell line. These cell lines were chosen as the model systems for this study due to the ability to perform mechanistic studies using current methodology. Furthermore, the End1/E6E7 cell line displays similar morphological and immunocytochemical properties to those of primary endocervical epithelial cells [50]. The genes investigated were chosen based on their established mechanism of regulation via the GR, and their constitutive expression in endocervical epithelial cells [7]. GILZ and IκBα are anti-inflammatory genes that are upregulated by glucocorticoids (GCs) such as DEX, while IL-6, IL-8 and RANTES are pro-inflammatory genes that are downregulated by DEX [59,60]. The GILZ and IκBα genes contain multiple glucocorticoid response elements (GREs) and are commonly referred to as GR transactivation genes [61,62]. The IL-6 and IL-8 gene promoters have binding sites for transcription factors that include activator protein-1 (AP-1) and nuclear factor κB (NFκB) [63], and these genes are transrepressed by the liganded GR via tethering mechanisms [64]. Cells were treated with P4, MPA and NET-A, as well as the GR agonist DEX for 24 hrs. Thereafter, cytokine gene mRNA was measured by real time qRT-PCR. As expected the GR synthetic agonist DEX upregulated both GILZ and IκBα mRNA in both the cell lines (Figure 1). In addition, MPA upregulated GILZ and IκBα mRNA in both End1/E6E7 and

HeLa cell lines (Figure 1). P4 and NET-A have no effect on the expression of GILZ or IκBα mRNA in either of the cell lines (Figure 1). Figure 2 A and B show that DEX and MPA, unlike NET-A and P4, repress both IL-6 and IL-8 mRNA levels, respectively, in the End1/E6E7 cell line. Interestingly, RANTES mRNA levels are repressed by DEX, MPA and P4 (Figure 2 C). The regulation of IL-6 mRNA levels by the ligands in the HeLa cells (Figure 2 D) is similar to the End1/E6E7 cells (Figure 2 A), where both DEX and MPA repress IL-6 mRNA levels. Furthermore, it appears that NET-A upregulates IL-6 mRNA levels in the HeLa cells (Figure 2 D). Similar to the End1/E6E7 cell line, DEX appears to repress IL-8 mRNA levels in the HeLa cells (Figure 2 E). However, unlike the End1/E6E7 cell line, at the 24 hr time point MPA does not appear to effect IL-8 mRNA expression in the HeLa cells (Figure 2 E). Interestingly though, at a 4 hr time point both DEX and MPA repress IL-8 gene expression in the HeLa cell line (Figure 2 H). It appears that NET-A and P4 upregulate IL8 mRNA levels in the HeLa cells (Figure 2 E). In contrast to the End1/E6E7 cells, the DEX, MPA and P4 repression of RANTES mRNA levels does not occur in the HeLa cells (Figure 2 F), although this could be due to low basal levels of RANTES in HeLa cells (as indicated by real time qRT-PCR Ct values, data not shown). Interestingly, it appears that NET-A upregulates RANTES mRNA levels (Figure 2 F). Taken together, these results show that MPA acts like the GR agonist DEX in upregulating GILZ and IκBα anti-inflammatory gene and generally downregulating IL-6, IL-8 and RANTES pro-inflammatory gene mRNA levels, unlike P4 and NET-A, with some exceptions. The results also suggest cell-specific, gene-specific and temporal differences in the regulation of some of the genes in response to the ligands, such as undetectable repression of IL-8 and RANTES mRNA by MPA in HeLa cells at 24 hrs, but similar repression of IL-8 in HeLa cells at 4 hrs compared to IL-8 in End1/E6E7 cells at 24 hrs. In addition, some experiments show repression by P4 of RANTES in the End1/E6E7 cells at 24 hrs, unlike in HeLa cells. Furthermore, it appears that regulation of mRNA levels by the ligands may be time dependent (Figure 2). The experiments in Figure 1 and 2 were performed in the absence of induction of the cytokine/chemokine genes with a pro-inflammatory ligand, since these genes are constitutively expressed in cervical epithelial cells. Experiments performed in the presence of TNFα, to mimic infection, showed a similar % repression of the pro-inflammatory genes with DEX and MPA, unlike P4 and NET (Figure S1). All further experiments were performed in the absence of TNFα.

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

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

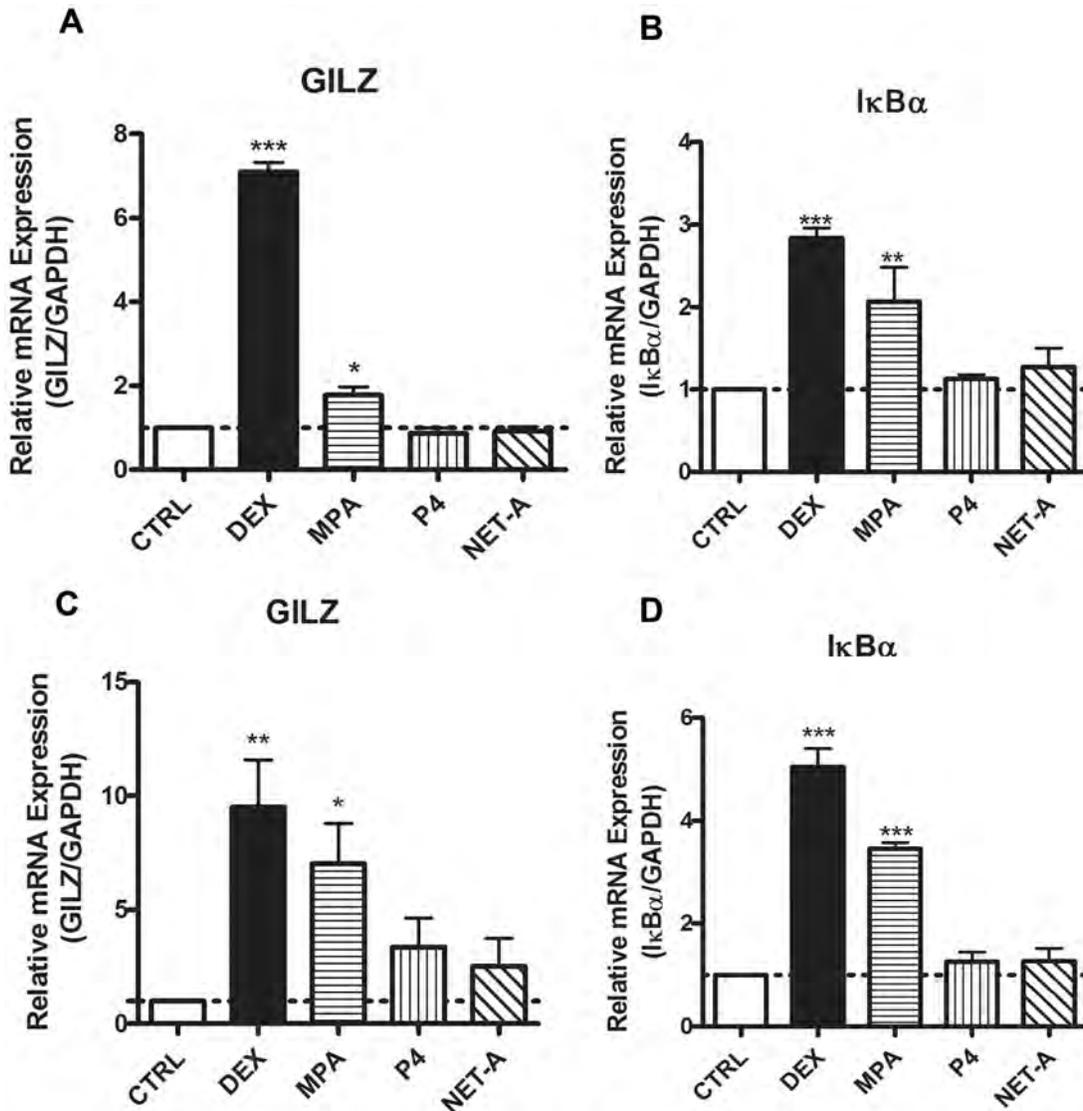


Figure 1. MPA, but not NET-A or P4, acts like a partial GR agonist for upregulation of anti-inflammatory mRNAs. (A and B) End1/E6E7 cells were treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). **(C and D)** HeLa cells were treated for 24 hrs with 100 nM DEX, 1 μ M MPA, 10 μ M P4, 10 μ M NET-A or vehicle (ethanol) (CTRL). Thereafter the cells were harvested; total RNA was isolated and reverse-transcribed. Relative GILZ and I κ B α gene expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalized to basal activity (CTRL) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with Dunnett post-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively. doi:10.1371/journal.pone.0096497.g001

1 μ M NET-A may upregulate IL-6 mRNA at 24 hrs. Figure 3 E and F show that IL-8 mRNA levels are also dose-dependently repressed by DEX and MPA at both 4 hrs and 24 hrs, with the MPA dose-dependent repression of IL-8 being more robust at 24 hrs. In addition, P4 and NET-A appear to be upregulating IL-8 at 4 hrs only. No repression of RANTES mRNA is apparent at 4 hrs (Figure 3 G). However, at 24 hrs RANTES mRNA levels are repressed by DEX and MPA in a dose-dependent manner, while NET-A and P4 appear to show some partial agonist activity (10–20%) for repression at high concentrations (Figure 3 H). MPA appears to have a potency (EC_{50}) of \sim 24 nM for transactivation of GILZ and a potency of \sim 21, 4 and 5 nM for repression of IL-6, IL-8 and RANTES mRNA, respectively at 24 hrs.

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

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

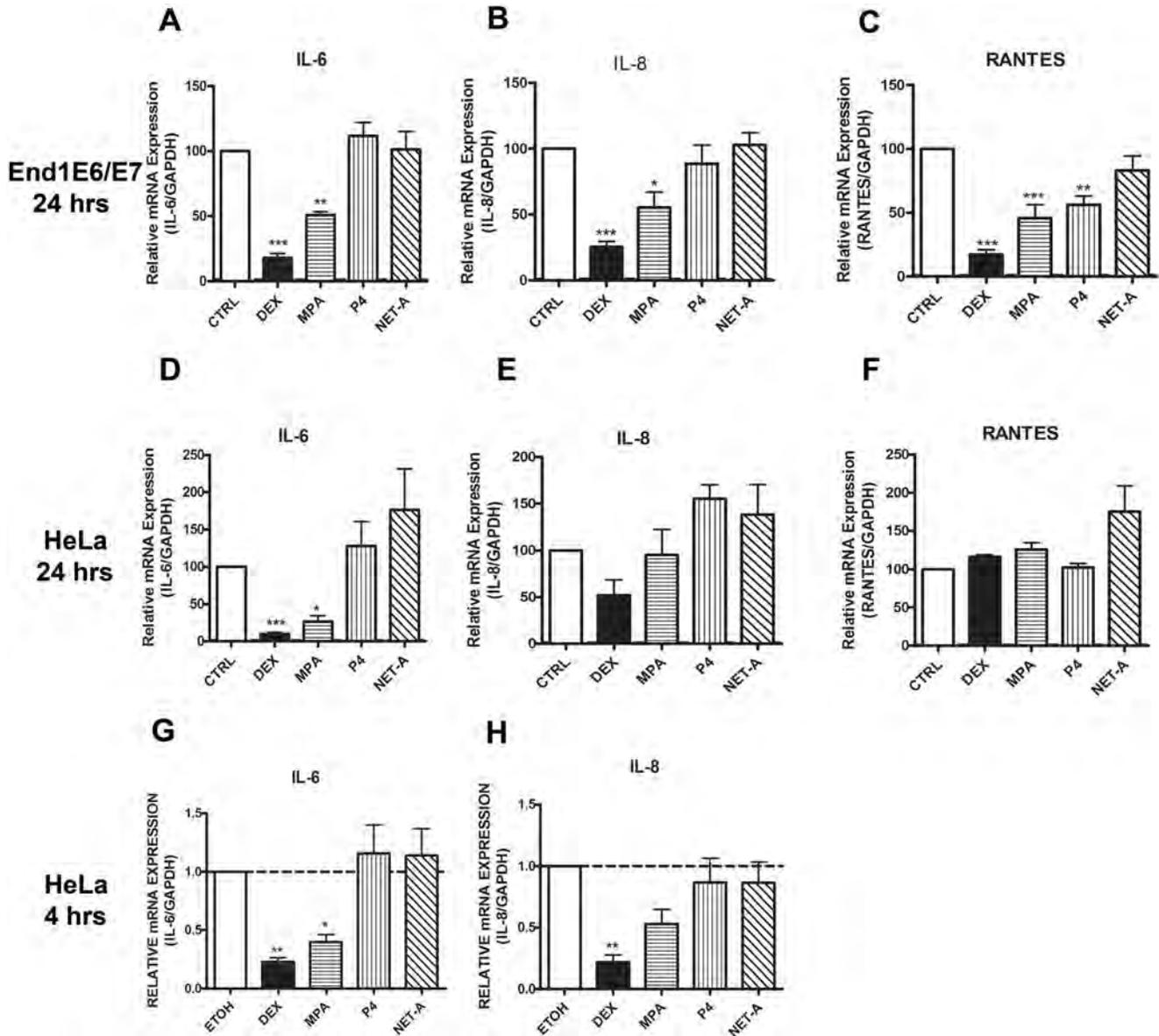


Figure 2. MPA, but not NET-A, acts like a full/partial GR agonist for repression of pro-inflammatory mRNAs. (A–C) End1/E6E7 cells were treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). (D–H) HeLa cells were treated for 24 hrs (D–F) or 4 hrs (G–H) with 100 nM DEX, 1 μ M MPA, 10 μ M P4, 10 μ M NET-A or vehicle (ethanol) (CTRL). Thereafter the cells were harvested, total RNA was isolated and reverse-transcribed. Relative IL-6, IL-8 and RANTES gene expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expression was normalised to basal activity (CTRL) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with Dunnett post-test. Statistical significance is denoted by *, ** or *** to indicate $P < 0.05$, $P < 0.001$ or $P < 0.0001$, respectively.

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reveals that in HeLa cells only endogenous GR protein is detectable. Although it appears that the HeLa and End1/E6E7 cells express MR protein (Figure 4 B), this is a non-specific band that also appears in the negative control. Since the End1/E6E7 cells do not express detectable MR mRNA it is highly unlikely that the cells express MR protein. However, it is possible that the HeLa cells do express low levels of the AR and MR that are beyond the detection level of the Western blots. It was therefore determined if the repression of cytokine genes in this cell line could be mediated via the AR and MR. HeLa cells were treated with the GR, AR and MR specific agonists (DEX, mibolone and aldosterone,

respectively) and cytokine gene expression was measured by qRT-PCR. IL-6 and IL-8 gene expression was measured since it was established above that RANTES is not regulated by the ligands of interest in the HeLa cells. Figure 4 C shows that only DEX represses IL-6 gene expression, while it appears that aldosterone, and possibly mibolone, upregulate IL-6 mRNA expression. In addition, it appears that DEX represses IL-8, while aldosterone, and possibly mibolone, upregulate IL-8 gene expression (Figure 4 D). These results indicate that DEX- and MPA-mediated repression of the cytokine genes in HeLa cells is likely to occur via the GR. Additionally, since only GR mRNA and protein were

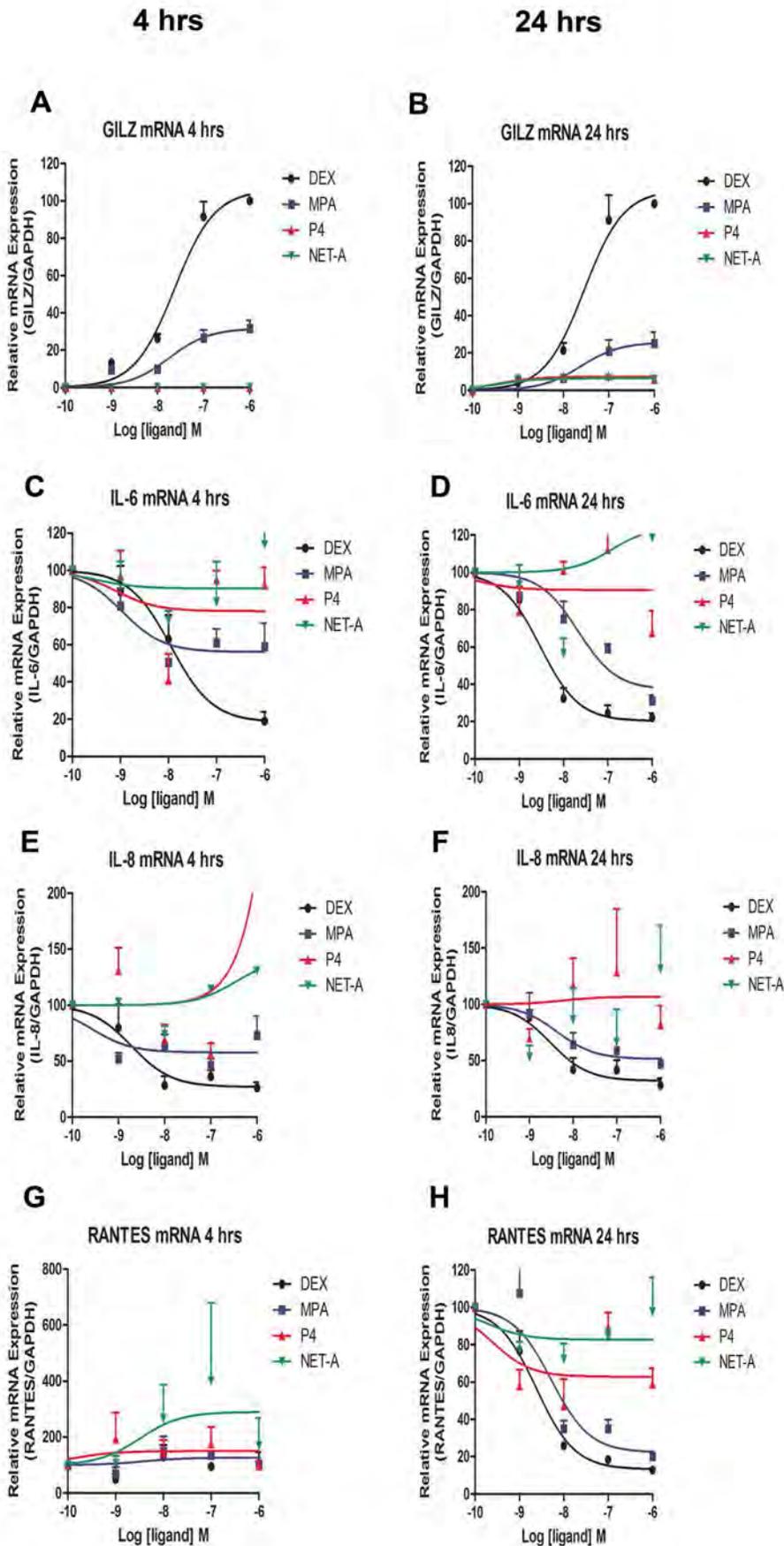


Figure 3. MPA-mediated regulation of inflammatory gene mRNA levels is dose- and time-dependent. End1/E6E7 cells were treated with increasing amounts (1 nM, 10 nM, 100 nM and 1 μ M) of DEX, MPA, P4 or NET-A, or vehicle (ethanol) (CTRL) for 4 and 24 hrs, respectively. Thereafter, the cells were harvested,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

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

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

In order to provide direct proof that the GR is involved in the regulation of the inflammatory genes in response to the synthetic progestin MPA, GR knockdown experiments were performed in the End1/E6E7 cell line. Reduction of GR protein in these cells was verified by Western blotting (Figure 6 A and 6B). As expected DEX and MPA upregulated GILZ mRNA, while P4, NET-A and NET did not (Figure 6 C). Notably, NET was included in this experiment as a control to exclude the possibility that the acetate form (NET-A) would regulate the genes differently. However, it is shown that NET-A acts similarly to NET. Both the DEX- and MPA-induced upregulation of GILZ mRNA is diminished when GR is knocked down. Figure 6 D shows that DEX upregulates I κ B α mRNA levels and this induction is repressed when GR is knocked down. Here the MPA induction of I κ B α is not significant, possibly due to the blunting of the response in the NSC knockdown conditions, and therefore a loss of induction is not apparent with the knockdown. Western blotting revealed that, unlike for the mRNA levels, DEX, MPA and NET-A all significantly increased total I κ B α protein levels (Figure S2). Protein levels could not be determined for GILZ due to the unavailability of a suitable antibody. As expected DEX and MPA repress IL-6 mRNA levels (Figure 7 A), which is lifted when the GR is knocked down. In addition, DEX-mediated repression is also evident on IL-6 protein levels (Figure 7 B). Consistent with the mRNA data, MPA appears to repress IL-6 protein levels and the repression is lifted in the knockdown. Interestingly P4 also appears to repress IL-6 protein levels, although significance could not be established. Similarly, a significant difference is observed for both DEX and MPA responses upon GR knockdown for IL-8 mRNA levels (Figure 7 C). Figure 7 D shows that DEX and MPA also appear to repress IL-8 protein levels, while GR knockdown

appears to lift this repression. RANTES mRNA levels are shown in Figure 7 E to be repressed by both DEX and MPA, but not by P4 or NET-A, in a GR-dependent manner. We were unable to detect secreted RANTES protein, possibly due to its instability in the medium (data not shown). Gene expression studies could not be performed with the primary cells since they did not maintain cell viability for the long periods of time required for the assessment (Figure S3). Taken together, these results show that DEX- and MPA-mediated regulation of the inflammatory gene mRNA levels is mediated via the GR in the endocervical cell line. This GR dependence is mimicked at the protein level for DEX and appears to also be mimicked at the protein level for MPA, for IL-6 and IL-8.

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

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

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

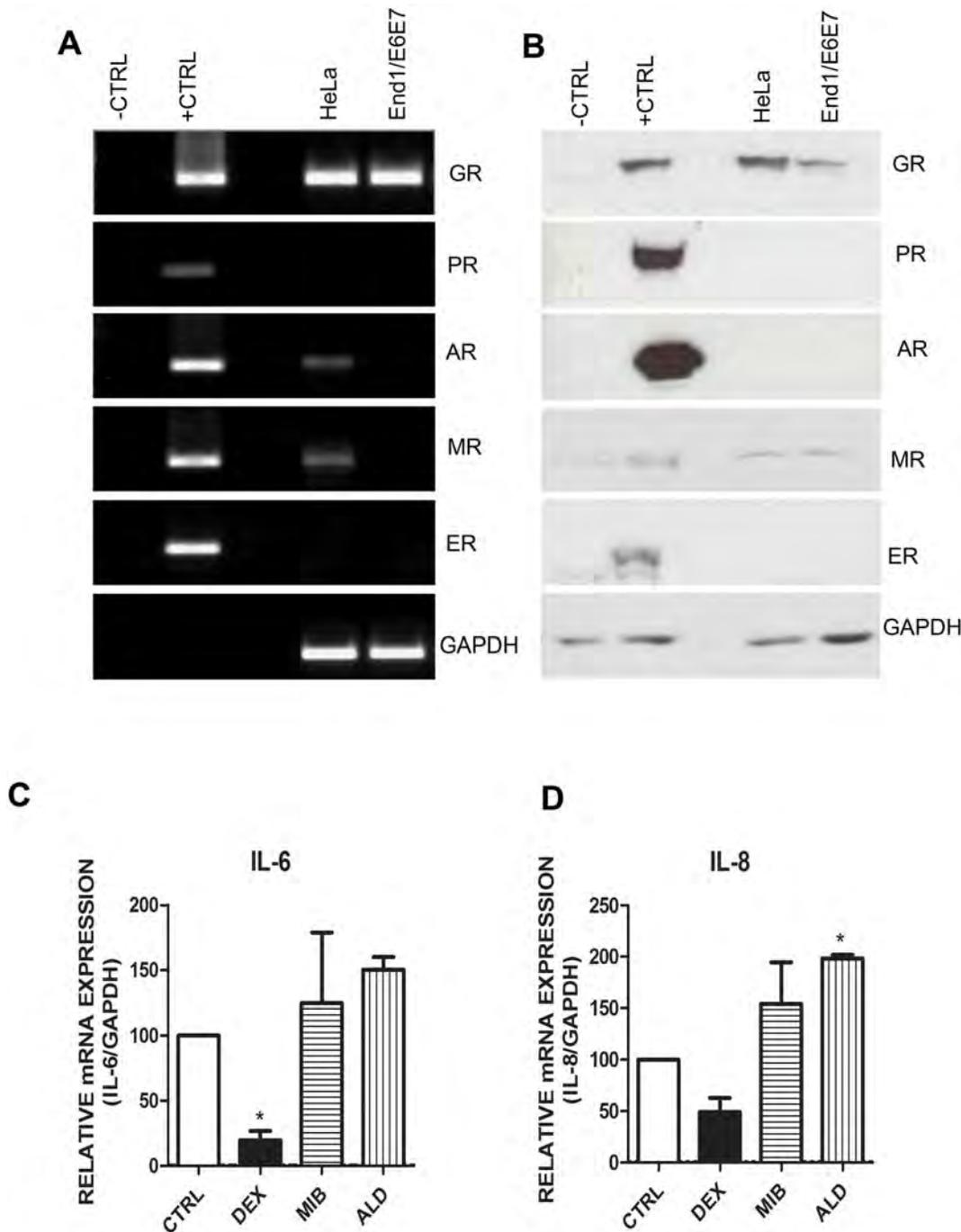


Figure 4. End1/E6E7 and HeLa cells only express detectable GR protein. (A and B) (A) HeLa and End1/E6E7 cells were harvested, total RNA was isolated and reverse-transcribed. Steroid receptor (SR) gene expression was measured by real time qRT-PCR. SR expression vectors (pcDNA3-hGR, pMT-PR-B, pSV-hAR, pRS-hMR and pSG5-hER) served as positive controls (+CTRL) for the GR, PR-B, MR and ER, respectively. COS-1 cells transiently transfected with pcDNA3 (empty vector) served as negative control (-CTRL). (B) Whole cell lysates were prepared from the HeLa and End1/E6E7 cell lines. Equal volumes of lysate were analysed by Western blotting with antibodies against specific SRs and GAPDH as loading control. (C and D) **SR agonist screen indicates that in the cervical cells the GR, but not the MR or AR repress IL-6 and IL-8 in the presence of receptor-specific agonist.** HeLa cells were treated with 100 nM DEX, 100 nM mibolerone (MIB), 10 nM aldosterone (ALD) or vehicle (ethanol) (CTRL) for 4 hrs. Total RNA was isolated and reverse-transcribed. Relative (C) IL-6 and (D) IL-8 gene expression was measured by real-time qRT-PCR and normalised to GAPDH mRNA expression. In addition, relative gene expressions were normalized to basal activity (CTRL) in order to obtain fold expression. The primers and antibody used to investigate PR levels are capable of detecting both PR-A and PR-B isoforms, however the positive protein control shown is specific for PR-B isoform only. Graphs represent pooled results of at least three independent experiments and are plotted as mean \pm SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with Dunnett post-test. Statistical significance is denoted by * to indicate $P < 0.001$. doi:10.1371/journal.pone.0096497.g004

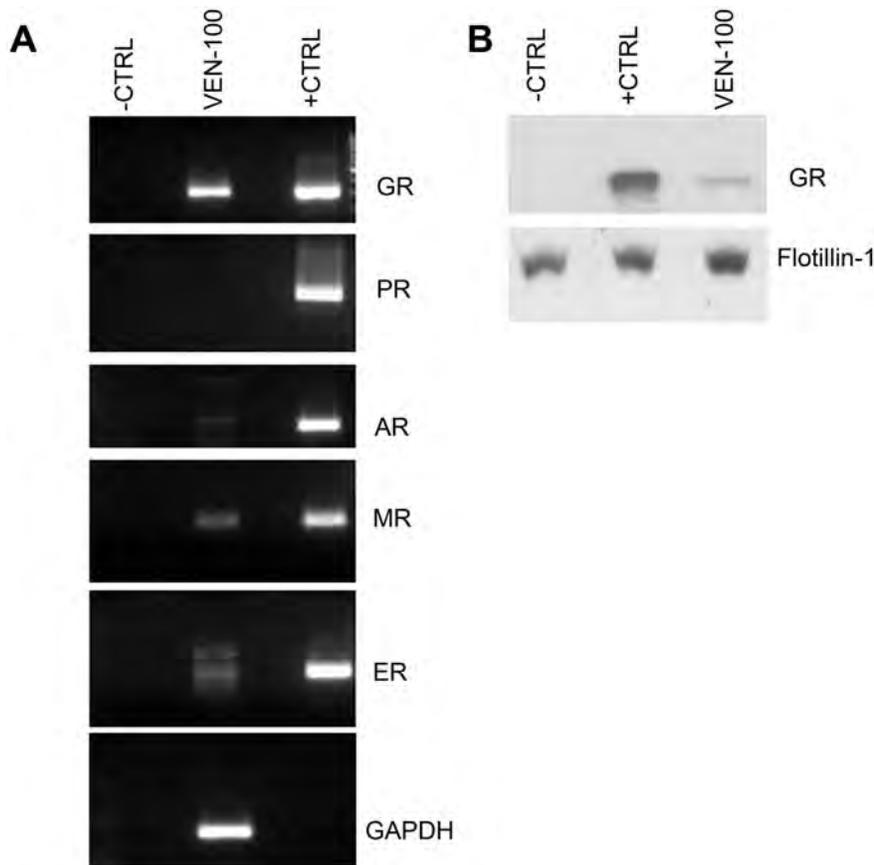


Figure 5. Only GR protein is detected in primary cervical epithelial cells (VEN-100). (A) Upon arrival the VEN-100 cells were rested overnight before being washed once with PBS and harvested with TRIzol[®]. Total RNA was isolated and 500 ng RNA was reverse-transcribed. Steroid receptor gene expression was measured by qRT-PCR with receptor-specific primers, followed by gel electrophoresis to confirm the PCR products. (B) VEN-100 cells were rested overnight before harvesting in 2X SDS sample buffer. COS-1 cells were transiently transfected with 1 μ g/well pcDNA3 (empty vector) which served as negative control (-CTRL) or with 1 μ g/well steroid receptor expression vectors (GR, PR-B, AR, MR and ER α) which served as positive controls (+CTRL). Twenty fourhrs later, the COS-1 cells were washed once and lysed with 2X SDS sample buffer. Equal volumes of cell lysate (VEN-100 and COS-1 ctrls) were analysed by Western blotting with antibodies specific for the GR and Flotillin-1 (loading control), respectively.

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matory genes investigated are at least in part regulated by direct effects of DEX without a requirement for new protein synthesis, and where this could be established, similar trends are observed for MPA.

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

In order to further investigate the mechanism of transcriptional regulation of these cytokine genes via the GR, ChIP assays were performed in HeLa cells. Attempts to perform ChIP assays in the End1/E6E7 cells were unsuccessful. This may be due to high background and low sensitivity for ChIP signals in these cells. Figure 9 A shows that stimulation with DEX, but not MPA results in the recruitment of the GR to the GILZ promoter. Furthermore, both DEX and MPA stimulation resulted in significant recruitment of the GR to the IL-6 and IL-8 promoters (Figure 9 B and C). The inability to observe GR recruitment to the GILZ promoter with MPA may be because some of the effects of MPA on GILZ are not direct, as suggested by the CHX experiments. However, since we have previously shown in A549 cells that the GR is recruited to the GRE region of the GILZ promoter by both DEX and MPA [67], it is more likely that a

small amount of GR is recruited by MPA, but this is below the limits of detection of the ChIP assay in these cells. In summary these results strongly support a model whereby both DEX and MPA suppress inflammation in the cervical epithelial cells by activating and thereby recruiting the GR to promoters of these genes and consequently inducing transcription of the anti-inflammatory gene GILZ, while repressing transcription of the pro-inflammatory genes IL-6 and IL-8.

Discussion

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

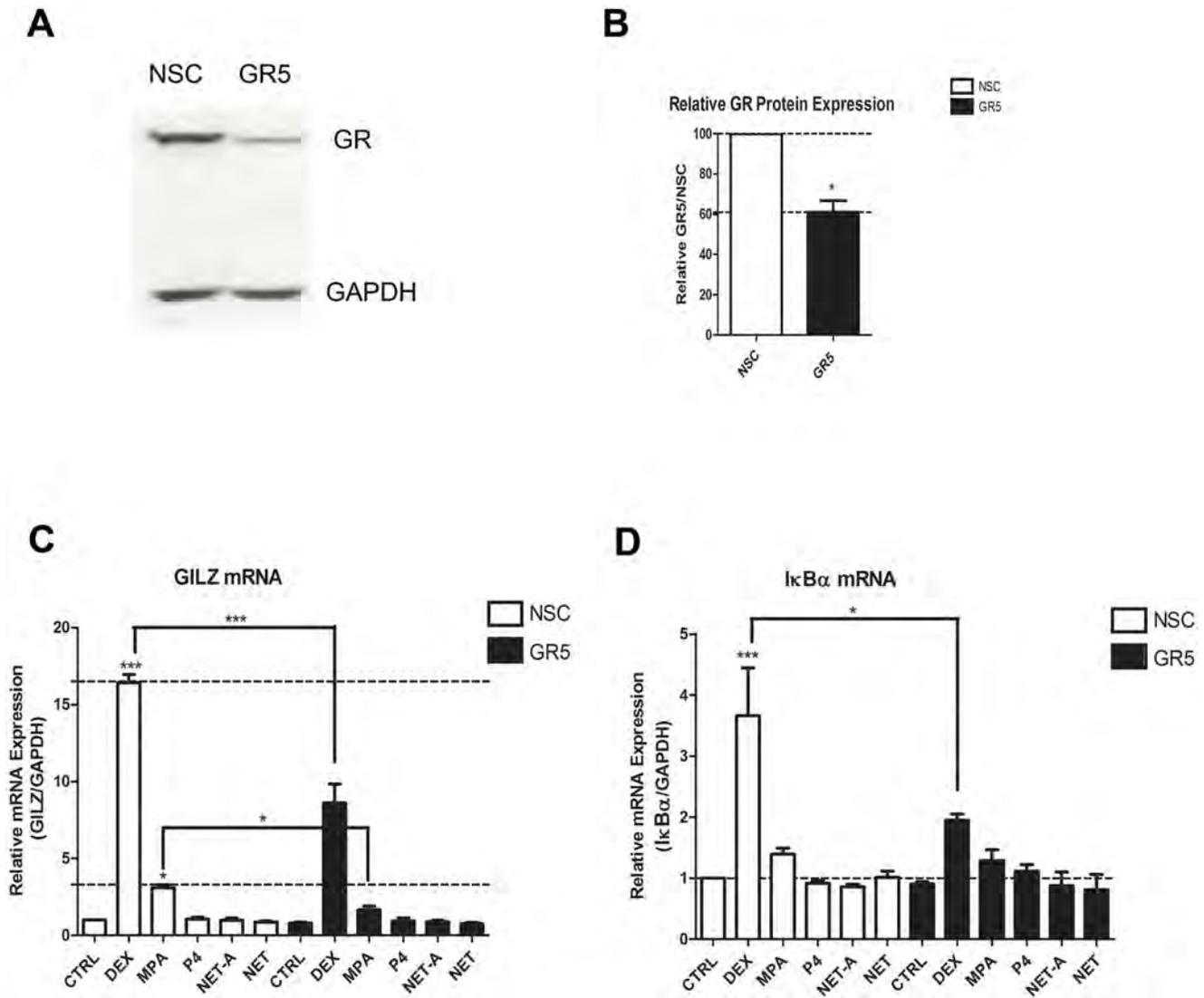


Figure 6. MPA- and DEX-mediated upregulation of anti-inflammatory mRNAs is mediated via the GR. End1/E6E7 cells were transfected with 10 nM GR or NSC siRNA (A–D) and then treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A, NET or vehicle (ethanol) (CTRL). For verification of GR knockdown a representative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

MPA unlike NET-A, decreases expression of the pro-inflammatory IL-6, IL-8 and RANTES genes in the endocervical epithelial cell line, as well as IL-6 and IL-8 in the HeLa cell line. These effects are mimicked at the protein levels for IL-6 and IL-8 in the epithelial cell line. Thus MPA, unlike NET-A and P4, shows an anti-inflammatory profile in both cell lines, for most genes investigated. Furthermore, we show for the first time that the predominant steroid receptor protein detected in the endocervical epithelial cell line and in primary endocervical epithelial cells is the GR, with no detectable PR mRNA or protein. Consistent with this finding, we also demonstrate by a combination of GR knockdown and ChIPs, that MPA, unlike NET-A, represses pro-inflammatory cytokine gene expression in cervical epithelial cells via a mechanism involving recruitment of the GR to cytokine gene

promoters. These results are consistent with a direct effect of the GR without a requirement for new protein synthesis, as shown by cycloheximide experiments. Our findings that DEX recruits GR to the IL-6 and IL-8 promoter regions are consistent with previous reports [56,73], while we show here for the first time, that stimulation with MPA recruits GR to the IL-6 and IL-8 promoter regions, thereby repressing expression of these genes. These results are consistent with our hypothesis and our previously published data that MPA, unlike NET-A or P4, acts like a partial to full GR agonist with a relatively high affinity for the GR on endogenous genes in other cells and via synthetic reporter genes [36,41,44,74]. The findings of the present study suggest that in the context of the genital mucosa, these GR-mediated effects of MPA in cervical epithelial cells are likely to play a critical role in discriminating

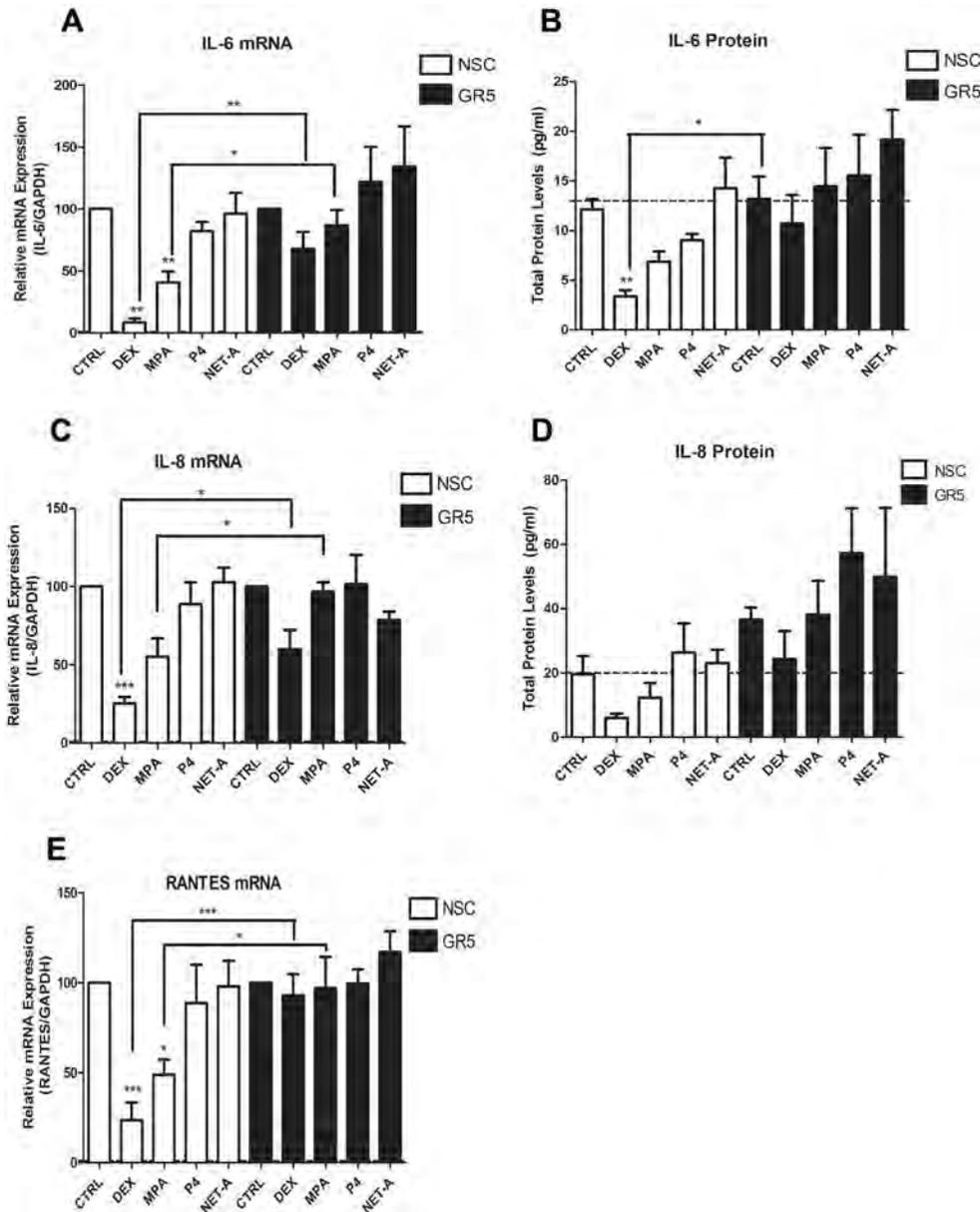


Figure 7. MPA- and DEX-mediated repression of pro-inflammatory cytokine gene mRNA is mediated via the GR. End1/E6E7 cells were transfected with 10 nM GR or NSC siRNA (A–F) and then treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). Total RNA was isolated and reverse-transcribed.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

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

Our findings that MPA has anti-inflammatory gene expression effects in the endocervical cells are consistent with previous reports

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

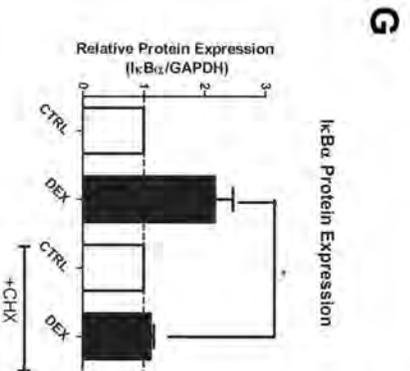
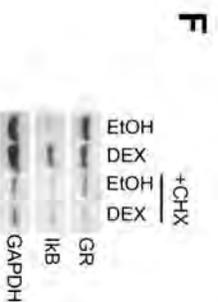
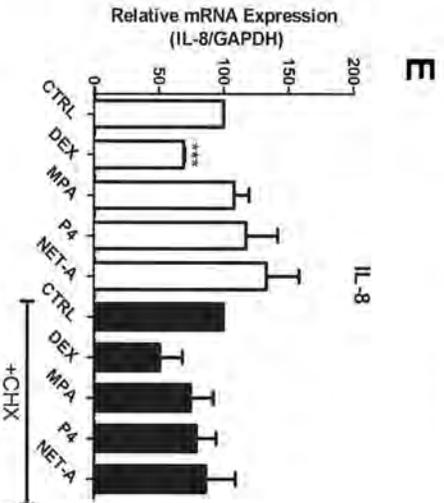
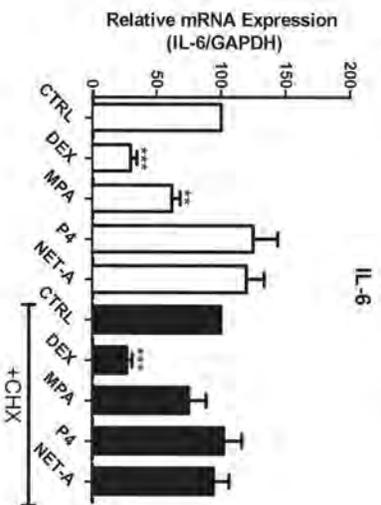
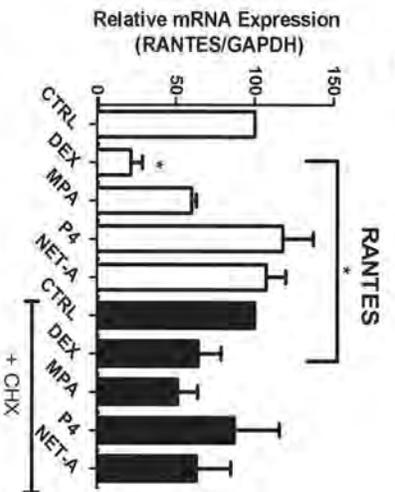
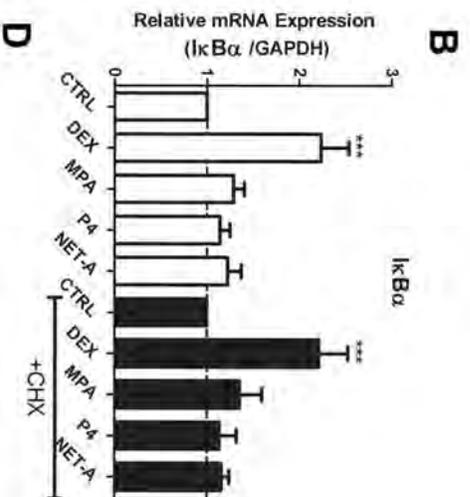
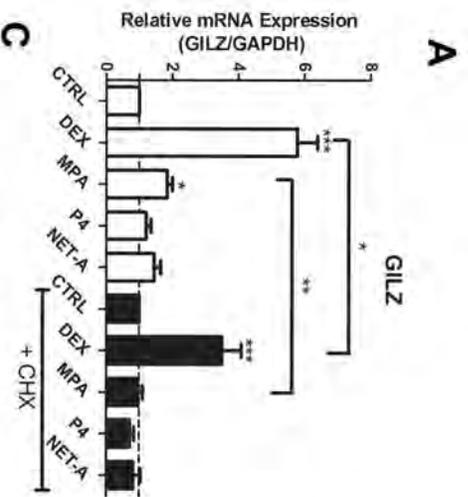


Figure 8. The GR at least in part directly regulates mRNA levels of the inflammatory genes. End1/E6E7 cells were pretreated with 1 μ g/ml cycloheximide (CHX) then treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL), in the absence or presence of CHX. Total RNA was isolated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

suggest that different compartments of the genital tract are likely to exhibit different inflammatory responses to MPA vs NET vs P4, with their associated different effects on susceptibility to genital infections. Our lack of detection of PR expression in the endocervical primary cells or cell lines raises the question as to what is the role of the PR in mediating responses to progestins and progesterone in the endocervix. It is possible that other cells besides epithelial cells in the cervix express functional PR, as is suggested from one report [79], while others report the expression of both the GR and ER α [80].

Whether the observed effects of MPA, NET and P4 are relevant to the physiological doses of these ligands *in vivo* is a critical question, which we investigated here by dose response analysis [81] to determine potencies (EC₅₀s) and efficacies (maximal response). The MPA serum concentrations of DMPA-users are reported to be in the range 2.5 to 65 nM a few days after injection and to plateau at about 2.6 nM for about three months thereafter [38,48,82], while serum concentrations for injectable NET-EN, in the range of 1.5–59 nM have been reported [83]. The concentration of endogenous P4 in serum of premenopausal women is low during the follicular phase (0.65 nM) but rises to about 80 nM during the luteal phase, and to about 600 nM during pregnancy [37]. We show that MPA at 10 nM significantly represses both IL-6 and RANTES at 24 hrs (Figure 3 C and H). Furthermore our dose response analysis show that MPA has a potency of \sim 24 nM for transactivation of the anti-inflammatory GILZ gene and a potency of \sim 4–20 nM for repression of the pro-inflammatory IL-6, IL-8 and RANTES genes. This suggests that these immunosuppressive effects are likely to be relevant at physiological doses of MPA used in injectable contraception, particularly shortly after injection, while any possible effects of NET-EN injectable

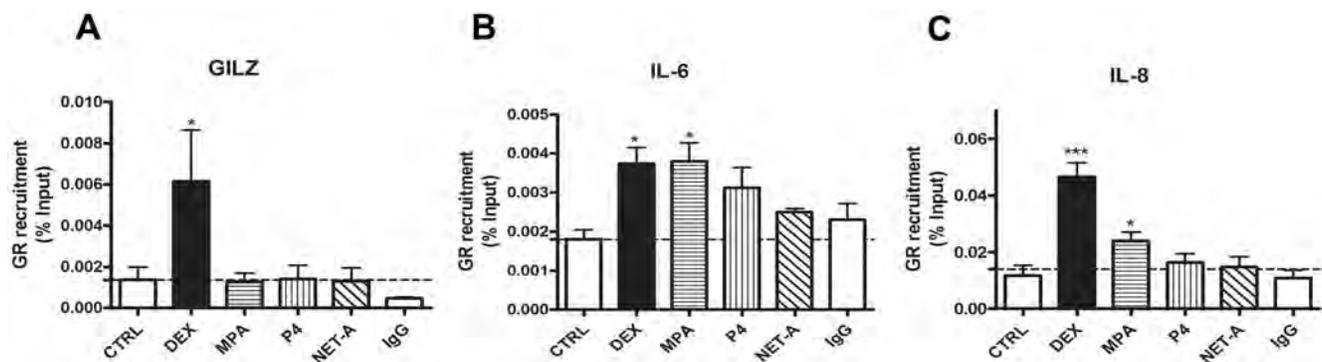


Figure 9. DEX and MPA recruit GR to the IL-6 and IL-8 cytokine gene promoters. HeLa cells were serum starved for 2 hrs and then treated for 1 hr with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). ChIP was carried out using an anti-GR antibody to immunoprecipitate endogenous GR and an anti-IgG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

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

and that choice and concentration of progestin in contraception are likely to be critical factors.

Supporting Information

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

Figure S2 DEX, MPA and NET-A induce total I κ B α protein. End1/E6E7 cells were treated for 24 hrs with 100 nM DEX, MPA, P4, NET-A or vehicle (ethanol) (CTRL). Thereafter, cells were harvested and equal volumes of lysate were analysed by (A) Western blotting with an antibody specific for total I κ B α and a GAPDH specific antibody as loading control. (B) Western blots of five independent experiments were quantified to determine the relative GR protein expression. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with a Dunnett post-test followed by a student's t-test to compare specific conditions to each other. Statistical significance is denoted by *, ** or *** to indicate P<0.05, P<0.001 or P<0.0001, respectively. (TIF)

Figure S3 Cell Viability of VEN-100. VEN-100 cells were either incubated for 24 hrs (day 1, treatment day) or 72 hrs (day 3, end of treatment day), followed by, analysis for cell viability (MTT assay). Absorbance readings were measured at 570 nm. Cell culture media served as the control (CTRL). CTRL for each day was set to 1 to obtain relative fold cell viability. The graph represents results of at least three independent experiments, plotted mean \pm SEM. Statistical analysis was carried out using GraphPad Prism software (version 5) using a one-way ANOVA with a Dunnett post-test followed by a student's t-test to compare specific conditions to each other. Statistical significance is denoted by ** or *** to indicate P<0.001 or P<0.0001, respectively. (TIF)

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

Author Contributions

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

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

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Introduction

Whether hormonal contraceptives increase HIV-1 acquisition is a critical and unresolved issue, in particular for young women in high risk areas, and is the subject of intense worldwide research and debate.^{1,2} Furthermore, whether hormonal contraceptives increase HIV-1 transmission and disease progression, as well as increase susceptibility to other infectious diseases and STIs, are also critical health issues. These issues are also mostly unresolved and are topics of ongoing research. Clinical research in these areas has been hampered by a lack of understanding of the molecular mechanisms of action of the progestin components of contraceptives and by a

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

lack of appreciation of the differences between progestins, which cannot be considered to act as a single class of compounds regarding their side effect profiles.^{3–5} Although progestins are designed to act like the natural ligand progesterone (P4) via the progesterone receptor (PR), they are likely to exert very different side-effects due to their differential affinities and activities via other members of the steroid receptor family.^{3–5} Currently a wide range of progestins is used in contraceptives, with several different delivery methods and varying progestin doses. Until recently, clinical studies investigating effects of contraceptives on HIV-1 have grouped together data based on methods of delivery (injectable versus oral), while ignoring both the type of progestin and

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

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

MPA, administered for contraception as Depo-MPA (DMPA) or Depo-Provera, is a 150 mg 3-monthly intramuscular injection that is used by millions of women worldwide, particularly in sub-Saharan Africa with high HIV-1 prevalence.^{1,6} Norethisterone enanthate (NET-EN) is a 200 mg 2-monthly injectable that is used less than MPA globally, although its usage is high in some regions of South Africa.⁷ Injectable contraceptive usage and HIV-1 prevalence are particularly high in young women in some areas of South Africa, such as at the KwaZulu-Natal site for the CAPRISA microbicide trial. In this trial, about 82% of the women investigated were on injectable progestin-only contraceptives, as compared to 15% on oral contraceptives.⁸ The majority of high-quality studies, in which the most common progestin used was levonorgestrel (LNG), showed no significant increase in HIV-1 acquisition for oral contraceptive pills.⁹ Although only a few studies have investigated the risks associated with the use of injectable NET-EN on HIV-1 acquisition, none have shown a significant association with HIV-1 acquisition.⁹ However, the majority of high-quality studies do show an increased risk associated with only or predominantly injectable DMPA usage.^{9–11} Many clinical observational studies do not investigate risks associated with subgroups of women, such as those grouped by age or HSV-2 status, usually due to a lack of sufficient study size for a particular subgroup. However, of particular concern is the finding of an adjusted hazard ratio (HR) for DMPA of 9.29 in women aged 18–20.¹² Although others have not reported such age-related findings, they may not have had sufficient young women with high HIV-1 exposure in their studies.^{13–16} Also of concern is the

finding of an HR of 4.5 for DMPA for HSV-2 negative women.¹² However, establishing indisputable evidence from such studies is extremely difficult due to methodological challenges and multiple confounding factors, such as the degree of exposure to HIV-1, condom usage, HSV-2 exposure as well as varying ages of women that have been enrolled in these studies.⁹ Furthermore increases in both HIV-1 and HSV shedding have been reported in women using contraception,^{17–19} as well as the presence of more viral variants and higher viral loads in HIV-1-infected DMPA users than non-users.²⁰ These findings are consistent with an increase in HIV-1 transmission found for injectable DMPA users.¹⁶ DMPA usage has also been associated with increased acquisition of cervical chlamydial and gonococcal infections.²¹ These STIs have also been linked to increased HIV-1 acquisition. Consistent with the clinical data, MPA increases susceptibility to vaginal simian–human immunodeficiency virus (SHIV) transmission and suppresses the antiviral cellular immune response in SHIV-infected rhesus macaques.²² No information is available regarding other progestins and HIV-1 acquisition. Contraceptives may also affect diseases progression. Most of the clinical studies to date indicate no effect of hormonal contraception on disease progression. However, this may be due to several study limitations such as insufficient power to discriminate between progestin groups or methods of contraception delivery, analysis of only chronically infected patients, and confounding variables such as antiretroviral (ARV) usage and pregnancy. This suggests that effects of specific progestins like MPA on disease progression from the time of HIV-1 acquisition, cannot as yet be excluded.^{23,24} Collectively, these findings raise questions as to the choice of progestin, dose and method of administration in determination of susceptibility to and transmission of pathogens, such as HIV-1, particularly in young women. Since the current data from observational studies are not considered persuasive enough to recommend using methods of contraception other than DMPA,^{1,2} more research is urgently required. The key question is what information is needed and how feasible it is to obtain. Some have argued that more large-scale randomized trials are needed,²⁵ but these may not be ethical or conclusive. Another approach is to investigate direct biological effects and mechanisms *ex vivo* of different progestins at specific doses for different target cells relevant to HIV-1 pathogenesis.

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

Effects of progestins on HIV-1 acquisition and transmission are proposed to occur via multiple mechanisms, including changes in vaginal structure, endometrial thinning, effects on genital flora, effects of other sexually transmitted infections, as well as changes in immune function.²⁶ At the cellular level, progestins and sex steroid hormones mediate their effects via alterations in transcription of specific genes via binding to and regulating the activity of steroid receptors.⁴ Progestins differ widely in their steroid receptor selectivity profiles.^{3–5} MPA, NET and LNG all exhibit some binding to the androgen receptor with undetectable anti-androgenic activity and some androgenic activity. MPA and NET exhibit very little antimineralocorticoid activity at contraceptive doses,²⁷ unlike some reports for LNG.^{4,27} However, MPA binds to the GR with an affinity (K_d of 4–11 nM) similar to that of the endogenous glucocorticoid cortisol and acts as a full to partial agonist for the GR, whereas P4 and NET bind to the GR with about a 50- to 100-fold lower affinity and are very weak partial GR agonists with much lower potency and efficacy.^{4,5} In contrast, the fourth-generation progestins are much more selective for the PR. The GR is a well-established regulator of immune function via regulation of transcription of a wide variety of immune function genes. Glucocorticoids are potent immunosuppressive agents which exert multiple complex actions on dendritic cells, myeloid cells and B- and T-lymphocytes, including effects on apoptosis and differentiation fate and inhibition of cytokine release and cell migration.^{28–30} Consequently, glucocorticoids are widely used as anti-inflammatory and immunosuppressive drugs. The immunosuppressive actions of glucocorticoids are largely due to their repression of transcription of target genes such as cytokine and chemokine genes, via inhibition of the transcription factors nuclear factor kappa B (NFκB) and activator protein 1 (AP-1).³⁰ Glucocorticoids, like cortisol, bind to inactive GR in the cytoplasm. Ligand-bound GR is subsequently hyperphosphorylated and translocates into the nucleus where it is able to modulate transcription. Once in the nucleus, ligand-bound GR binds as a dimer to glucocorticoid response elements (GRES)

resulting in positive transcriptional regulation, called transactivation.^{28–30} Additionally, ligand-bound monomeric GR is also known to negatively regulate transcription by interfering either directly or indirectly with other DNA-bound transcription factors, like NFκB or AP-1.³⁰ This mechanism, known as transrepression, prevents NFκB- and AP-1-mediated transcription.^{28–30} Given that MPA, unlike other progestins, acts as a relatively potent full to partial agonist for the GR, MPA is likely to exert much more potent and efficacious effects on gene promoters involved in immune function than P4 or NET via the GR, as we have demonstrated in cell lines.^{31–33} We proposed in 2004 that due to the differential activity of MPA versus NET via the GR, MPA is likely to exert different effects on immune function and hence modulate susceptibility to infections like HIV-1.³ MPA has been reported to modulate transcription of a number of genes via the GR, including IL2 in normal human lymphocytes,³⁴ and IL6 and IL8 in a mouse fibroblast cell line.³¹ We have also recently shown that MPA, unlike NET and P4, exhibits differential regulation of apoptotic genes and dose-dependently enhances HIV-1-mediated apoptosis in primary human CD4⁺ T cells via a GR-dependent mechanism,³⁵ consistent with the steroid receptor selectivity of these ligands. Furthermore, MPA, to a greater extent than P4, suppresses *ex vivo* the production of key regulators of cellular and humoral immunity involved in orchestrating the immune response to invading pathogens such as HIV-1, most likely via the GR.³⁶ It remains to be determined how different progestins affect expression of key genes involved in immune function in primary cells relevant to HIV-1 pathogenesis.

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

and adaptive immune function cells.³⁸ RANTES (CCL5), also upregulated by NF κ B, is a chemokine that is involved in the recruitment of mononuclear cells to sites of chronic inflammation³⁹ and interferes with HIV-1 entry into target cells by competitive binding to the CCR5 co-receptor.⁴⁰ A decline in RANTES protein expression is also associated with an increase in HIV-1 disease progression.³⁹ Freshly isolated PBMCs were stimulated with varying concentrations of the different ligands for 48 hrs, followed by RNA isolation, reverse transcription and real-time quantitative PCR. The results show that DEX and MPA, unlike NET and P4, repress IL6 and IL8 pro-inflammatory gene mRNA levels while increasing GILZ anti-inflammatory mRNA levels (Fig. 1), while RANTES mRNA repression was only detectable with DEX under these conditions (Fig. 1). Concurrent with mRNA expression analysis, protein expression analysis by flow cytometry indicated that the progestins mediated differential regulation on IL6 and GILZ protein levels (Fig. 1 E and F). MPA and DEX significantly repressed IL6 protein expression after 48 hrs, while P4, NET-A, NET and LNG did not affect basal expression in CD14⁺ monocytes (Fig. 1e). DEX significantly increased GILZ protein expression levels in CD4⁺ T cells, while MPA indicated a moderate, but not significant, increase in basal expression of GILZ after 48 hrs. P4, NET, NET-A and LNG did not affect GILZ protein expression levels (Fig. 1 e and f). Both NET and NET-A were included in these experiments to show that the lack of an effect with NET-A is not due to an inability to be metabolized to NET in PBMCs. We did not detect substantial GILZ expression in monocytes or IL6 expression in CD4⁺ T cells. These results showing immunosuppressive effects of MPA on gene expression are consistent with our previous data,^{31–33} as well as with results reported by others in Bacillus Calmette–Guerin-stimulated PBMCs and murine *M. tuberculosis* models,^{41,42} and those from the Hel laboratory in activated T cells and in primary vaginal mucosal mononuclear cells (VMMC)s³⁶ and cyto-brushes from patients on DMPA.³⁶

To investigate the role of the GR in mediating the responses, PBMCs were treated with DEX and MPA in the absence and presence of RU486, a known GR antagonist. In Fig. 2, IL6, IL8 and RANTES mRNA repression by DEX and MPA was lifted in the presence of RU486, while DEX and MPA induction of GILZ mRNA was attenuated by RU486 (Fig. 2 a–d). Similarly Huijbregts et al. found that RU486 treatment

together with MPA lifted the MPA-mediated repression of IFN- γ protein expression in VMMCs and activated PBMCs, CD3⁺ and CD14⁺ cells.³⁶ Although RU486 can also antagonize the PR, we have recently shown that PBMCs under our conditions express no detectable PR mRNA or protein.³⁵ Thus, our RU486 results support a role for the GR in mediating these anti-inflammatory effects in PBMCs. Importantly we show for the first time that the GR is likely to play a key role in discriminating between immunosuppressive responses by MPA versus NET and P4 in primary human cells representing key targets for HIV-1 infection and replication. We have recently shown that this discriminatory role of the GR is also observed in the End1/E6E7 endocervical epithelial cell line, a model for the endocervical mucosa and site of heterosexual HIV-1 transmission (data not shown). Interestingly in these cells, as well as in primary endocervical epithelial cells, the GR is the predominant steroid receptor expressed (data not shown).

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

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

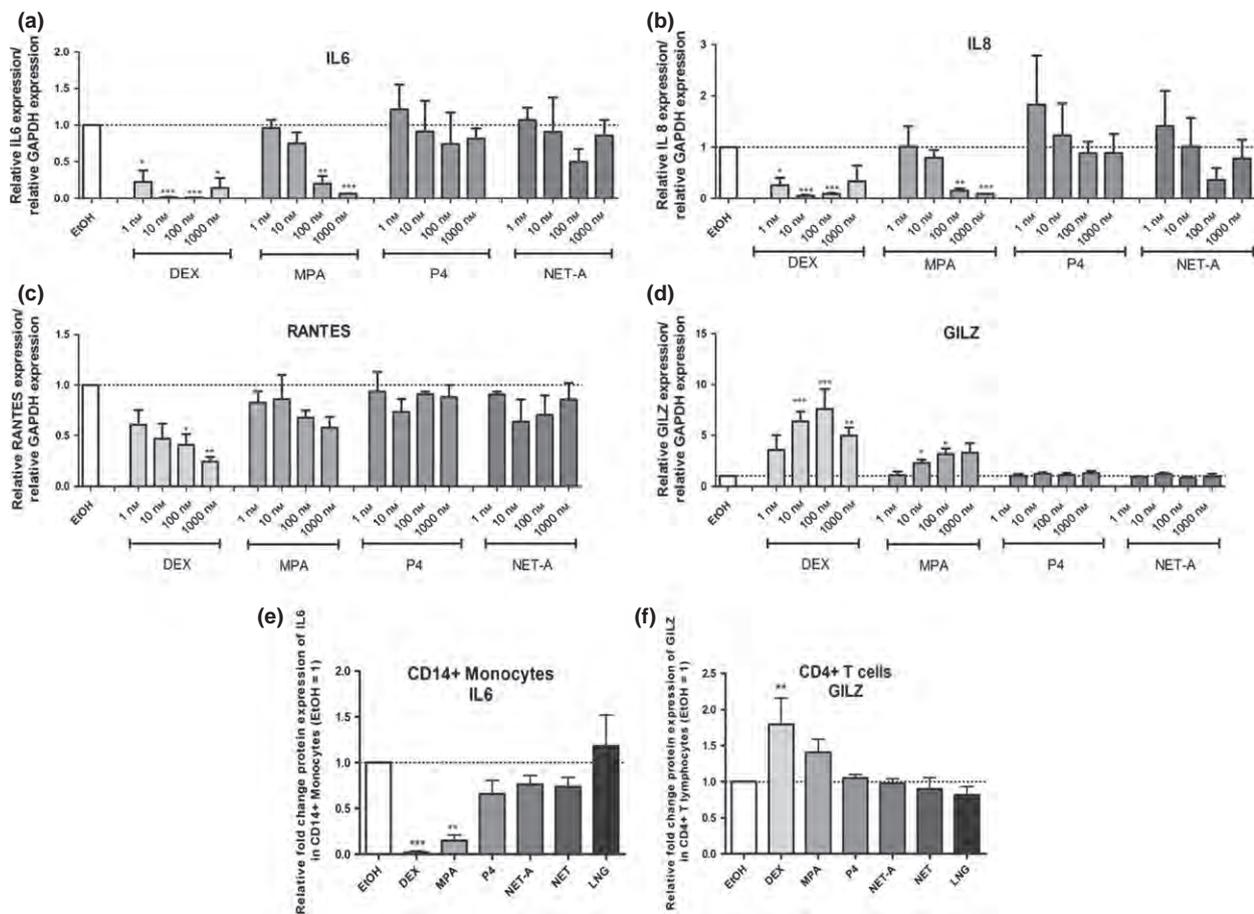


Fig. 1 DEX and MPA, dose-dependently regulate IL6, IL8, RANTES and GILZ mRNA levels in primary human PBMCs and decrease IL6 protein expression in CD14⁺ monocytes and increase GILZ expression in CD4⁺ T cells. (a-d) PBMCs were stimulated with increasing amounts of DEX, MPA, P4, NET-A or vehicle (EtOH)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

injectable contraceptive users (1–100 nM), as well as possibly on IL6 and IL8, particularly shortly after injection. However, the immunosuppressive effects of NET-EN on PBMCs via the GR are likely to be negligible, even shortly after injection, while P4 is unlikely to exert immunosuppressive effects in PBMCs even during pregnancy via these genes. Interestingly, we have recently shown that the GR may also play a key role in PBMCs in discriminating between apoptotic effects by MPA, unlike NET and P4, at doses within the contraceptive range, in the absence and presence of HIV-1 infection.³⁵ The physiological consequences of immunosuppressive and apoptotic effects of MPA

on PBMCs could include a wide range of effects beneficial to the virus. These could include compromising the ability of the host to mount effective innate and adaptive immune responses, as well as increasing binding of HIV-1 to CCR5 co-receptors. Effects seen with MPA in PBMCs may also occur in T cells and monocytes in the genital mucosa.

Conclusions

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

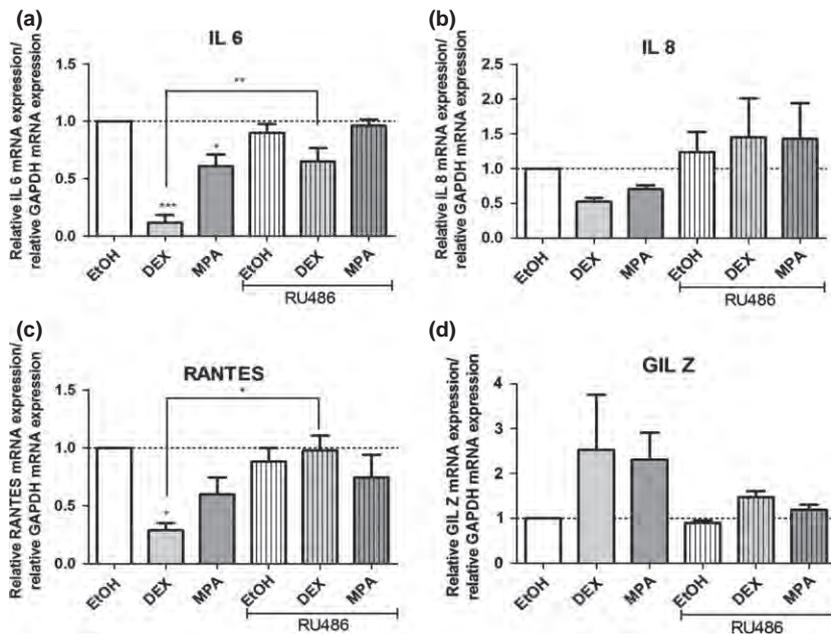


Fig. 2 DEX- and MPA-mediated regulation of GILZ, IL6, IL8 and RANTES mRNA requires the presence of the GR. (a-d) PBMCs were stimulated with 100 nM DEX and MPA in the presence and absence of 1 μ M RU486 for 48 hrs. Relative levels of IL6 (a), IL8 (b), RANTES (c) and (d) GILZ transcripts were determined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

acquisition and transmission, in particular in young women with high exposure to HIV-1, unlike other progestin contraceptives, such as the injectable contraceptive NET-EN. Several modelling studies have attempted to weigh up the competing risks between contraception and HIV-1 acquisition versus withdrawal of contraception and associated maternal mortality and other factors associated with unwanted pregnancies.^{44–46} Their results suggest that with HRs <2, the risks of withdrawal from DMPA outweigh potential benefits. However, it should be noted that risk assessments have not, to our knowledge, been published for HRs associated with only younger women in high risk areas for HIV-1 infection, or for changing to another method of contraception such as NET-EN, which has similar pregnancy risks and compliance factors to DMPA. This would seem highly relevant, given that risks for young women in sub-Saharan Africa carry a disproportionate percentage of the worldwide HIV-1 burden and are the highest users of DMPA. Although the findings showing increased HIV-1 acquisition and transmission with DMPA could be due to confounding behavioural factors, the finding that NET-EN does not increase HIV-1 acquisition argues against this, since DMPA and NET-EN users would be expected to display similar behavioural factors such as condom usage. Furthermore, both animal and *ex vivo* biochemical studies, using contraceptive concentrations of MPA, support the possibility that MPA increases HIV-1 infection

and transmission.⁴⁷ We show directly for the first time that the GR is likely to play a key role in discriminating between immunosuppressive responses by MPA versus NET and P4 in primary human cells representing key targets for HIV-1 infection and replication. Biochemical studies such as we report here provide strong support for a mechanism whereby MPA, unlike other progestins like NET-EN, is likely to modulate gene expression and immune function, at concentrations within the range of peak serum MPA concentration in injectable users, acting via the GR. The GR is ubiquitously expressed, unlike the PR. More research is needed to investigate the concentrations of different progestins in target tissues and to assess their likely dose–response effects. Given its central role in transcription regulation in all mammalian cells, effects of MPA on gene expression via the GR are likely to be the molecular basis for several differential effects of MPA versus other progestins that do not act via the GR. These could include effects on HIV-1 replication, host cell apoptosis, expression levels of several host cell receptors, cell cycle and proliferation effects and epithelial cell barrier function. Future research on the effects of different progestins on modulation of HIV-1 pathogenesis, at doses within the contraceptive range, in primary cell and tissue models will undoubtedly provide necessary insight into the question of choice of progestin and dose effects. However, it remains to be seen whether confirmatory information from such studies will be

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

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