A study of the molecular mechanism of progestin-induced regulation of IL-12 and IL-10 and implications for HIV pathogenesis

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Dissertation presented in fulfilment of the requirements for the degree of PhD in Biochemistry in the Faculty of Science at the University of Stellenbosch

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March 2013

DECLARATION

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ABSTRACT

Medroxyprogesterone acetate (MPA) and norethisterone (NET) and its derivatives (norethisterone enanthate (NET-EN); norethisterone acetate (NET-A)), designed to mimic the actions of the endogenous hormone progesterone (Prog), are extensively used by women as contraceptives and in hormone replacement therapy (HRT). A number of reports have indicated that these synthetic progestins affect immune function in the female genital tract thereby increasing the risk of acquiring sexual transmitted infections. Despite these findings, very little is known about their mechanism of action at the cellular level, in particular their steroid receptor-mediated effects on cytokine gene expression. In the first part of this thesis, the effect of Prog, MPA and NET-A on the expression of the endogenous pro-inflammatory cytokine gene, interleukin (IL)-12p40, and anti-inflammatory cytokine gene, IL-10, was investigated in a human ectocervical epithelial cell line, Ect1/E6E7. Quantitative realtime PCR (qPCR) showed that all three ligands significantly upregulated the tumor necrosis factor alpha (TNFα)-induced IL-12p40 gene expression, while IL-10 gene expression was downregulated. Moreover, by reducing the glucocorticoid receptor (GR) levels with siRNA, these effects were shown to be mediated by the GR. A more detailed investigation into the molecular mechanism of the progestogen-induced upregulation of IL-12p40 gene expression, using chromatin immunoprecipitation (ChIP), siRNA, co-immunoprecipitation and re-ChIP analyses, showed that the progestogen-bound GR is recruited to the CCAAT enhancer binding protein (C/EBP)-β regulatory element of the IL-12p40 promoter, most likely via an interaction with the transcription factor C/EBPβ. Similar experiments for the progestogen-induced downregulation of IL-10 gene expression showed that the progestogen-bound GR is recruited to the signal transducer and activator of transcription (STAT)-3 regulatory element of the IL-10 promoter, most likely via an interaction with the transcription factor STAT-3. The second part of this study elucidated the influence of the HIV-1 accessory viral protein R (Vpr) on progestogen-induced regulation of IL-12p40, IL-12p35 and IL-10 in the Ect1/E6E7 cell line. Results showed that in these cells, the overexpression of Vpr significantly modulated the effects of Prog, MPA and NET-A on the mRNA expression of IL-12p40 and IL-10, while only the NET-A effect was modulated on IL-12p35. Moreover, reducing the GR protein levels by siRNA suggested that the GR is required by Vpr to mediate its effects. Taken together, these results show that Prog, MPA and NET-A promote the pro-inflammatory milieu in the ectocervical environment, and that during HIV-1 infections, this milieu is modulated. Furthermore, the results suggest that the use of MPA or NET *in vivo* may cause chronic inflammation of the ectocervical environment, which may have important implications for ectocervical immune function, and hence susceptibility to infections such as HIV-1.

OPSOMMING

Medroksieprogesteroon asetaat (MPA), noretisteroon (NET) en derivate daarvan noretisteroon enantaat (NET-EN); noretisteroon asetaat (NET-A), ontwerp om die funksies van die natuurlike hormone progesteroon (Prog) na te boots, word wêreldwyd deur vroue as voorbehoedmiddels sowel as vir hormoon vervangingsterapie (HVT) gebruik. Daar is verskeie aanduidings dat hierdie sintetiese progestiene die immuunfunksie in die vroulike geslagskanaal kan beïnvloed en ook die moontlike vatbaarheid van seksueel oordraagbare infeksies kan verhoog. Ten spyte hiervan, is baie min bekend oor hulle meganisme van werking op ‘n molekulêre vlak, veral in die besonder hul effek op sitokinien geenuitdrukking. Die effek van Prog, MPA en NET-A op die geenuitdrukking van ’n endogene pro-inflammatoriese sitokinien, interleukin (IL)-12, en ’n anti-inflammatoriese sitokinien, IL-10, asook die onderliggend meganisme van werking, in ’n menslike ektoservikale sellyn, Ect1/E6E7, is in die eerste deel van hierdie studie ondersoek. Kwantitatiewe “realtime” polimerisasie ketting reaksie (PKR) het getoon dat al drie die ligande die tumor nekrosis faktor alfa (TNF-α)-geïnduseerde IL-12p40 geenuitdrukking opreguleer en IL-10 geenuitdrukking onderdruk. Verder is gevind dat induksie van IL-12p40 en inhibisie van IL-10 deur Prog, MPA en NET-A deur die glukokortikoïed reseptor (GR) gedryf word, aangesien volledige opheffing van die effekte op hierdie sitokinien gene waargeneem is wanneer die GR proteïen vlakke deur middel van kort inmengende ribonukleïensuur (siRNS) verminder is. 'n Meer beskrywende ondersoek in die molekulêre meganisme is uitgevoer deur gebruik te maak van chromatien immunopresipitasie (ChIP), siRNS, mede-immunopresipitasie en her-ChIP analises. Hierdie resultate het voorgestel dat die progestogeen (Prog en die sintetiese progestiene)-gebonde GR tot die CCAAT verbeterende bindings protein (C/EBP)-β regulatoriese element van die IL-12p40 promotor betrek word en dat die transkripsie faktor C/EBPβ benodig word om transkripsie van die IL-12p40 geen te aktiveer. Met betrekking tot IL-10, het die resultate voorgestel dat die progestogeen-gebonde GR tot die sein transduksie en aktiveerder van transkripsie (STAT)-3 regulatoriese element van die IL-10 promotor betrek word en dat die transkripsie faktor STAT-3 benodig word om transkripsie van die IL-10 geen te onderdruk. Die tweede deel van die studie het die invloed van die MIV-1 aksesorale virale proteïen R (Vpr) op sitokinien geenuitdrukking, spesifiek die progestogeen-geïnduseerde regulering van IL-12p40, IL-10 en IL-12p35, in die Ect1/E6E7 sellyn ondersoek. Resultate het getoon dat ooruitdrukking van Vpr in hierdie sellyn die effekte van Prog, MPA en NET-A op die mRNS uitdrukking van IL-12p40 en IL-10, en slegs die NET-A effek op IL-12p35, aansienlik moduleer. Vermindering van die GR proteïen vlakke deur middel van siRNS het getoon dat Vpr die GR benodig om hierdie veranderinge mee te bring. In samevatting, die resultate van hierdie proefskrif stel voor dat Prog, MPA en NET-A die pro-inflammatoriese milieu in die ektoservikale omgewing bevorder, en dat hierdie milieu gedurende MIV-1 infeksies verander. Verder, die resultate van hierdie studie impliseer dat die gebruik van MPA en NET *in vivo* nadelige lokale immuunonderdrukkende effekte mag hê wat kan lei tot kroniese inflammasie van die ektoservikale omgewing en ‘n moontlike verhoging in die vatbaarheid van infeksies soos MIV-1.

I would like to dedicate this thesis to my mom, Annette, and my soon to be husband, Francois…

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to everyone who has made this thesis possible. This process was not always very easy and without the support of the people mentioned below, I would not have made it this far. I would like to especially thank the following people:

My supervisor, mentor, friend and role model, Dr Donita Africander: Thank you very, very much for everything you have done for me over the past few years. I really appreciate your guidance, support and patience. You are an extraordinary and excellent mentor, and any student will be blessed to have a supervisor like you. Thank you for always carrying my best interest at heart and encouraging me when things were really tough. Thank you for all the time and effort you have put into this thesis. You have inspired me in various aspects of my life, and I hope to one day be an excellent researcher as well as a wonderful wife and mom, like you are.

My mom, Annette: There are no words to express how thankful I am for everything you have done for me. Thank you for all the sacrifices you have made so that I can get the best possible education and pursue my dreams. I know it wasn't always easy for you. Thank you for your unconditional love, encouragement, faith, prayers and support. I am very blessed to have a mom like you. I love you mom!

My fiancé, or shall I say very soon to be husband, Francois: I know it wasn't always easy for you, but thank you for your patience, support and constant encouragement. I am very grateful to have you in my life, and can't wait for our future together. Love you lots!

My dad, Pieter, brothers, Francois and Jacques, and grandma, Mercy: Thank you for your prayers. I am sorry for all the times I had to let you down because I had to work.

Charles Bernhardt: Thank you for helping me out financially.

My laboratory friends Carmen Langeveldt, Nicky Verhoog, Koch Visser, Dewald Noeth: Thank you for always listening to my moans and groans about experiments not working, for encouraging me in the bad times, brainstorming for new ideas and coffee/lunch breaks. A special thanks to Carmen for maintaining my cells, and teaching me tissue culture. You have all became very good friends of mine, and I hope that you will always be part of my life.

To all the other members of the laboratory, Prof Ann Louw, Easter Ndlovu, Legh Wilkinson, Craig Andrews and Morne Mortimer, and the eager volunteer student Baby: Thank you for all the support, help and laughs in the lab. A special thank you to Easter for always being willing to help me out with a few experimental work.

My co-supervisor, Prof Janet Hapgood: Thank you for your invaluable suggestions, input and support. I really appreciated it.

And most importantly, my Saviour and Father in heaven Jesus Christ: Thank you for taking care of me, financially, physically and emotionally, and for giving me strength and wisdom. Without You this would not have been possible.

Thank you also to the University of Stellenbosch, National Research Foundation of South Africa and the Medical Research Council, for providing research funding.

ALPHABETICAL LIST OF ABBREVIATIONS

AF-1	activation factor-1
AF-2	activation factor-2
AF-3	activation factor-3
Ald	aldosterone
AP-1	activator protein-1
ANOVA	analysis of variance
AR	androgen receptor
ARE(s)	androgen response element(s)
ATCC	American Type Culture Collection
β-Gal	β-galactosidase
bp	base pair
BSA	bovine serum albumin
CBG	corticosteroid-binding globulin
cDNA	complementary deoxyribonucleic acid
C/EBPβ	CCAAT enhancer binding protein beta
CEE / MPA	conjugated equine estrogen / medroxyprogesterone acetate
ChIP	chromatin immunoprecipitation
Co-IP	co-immunoprecipitation
CP	crossing point
C-terminal	carboxy-(COOH-) terminal

DBD	DNA binding domain
DEPC	diethylpyrocarbonate
Dex	Dexamethasone
DHT	dihydrotestosterone
DMEM	Dulbeco's Modified Eagle's Medium
DMPA	depot medroxyprogesterone acetate
DNA	deoxyribonucleic acid
E_2	17β-estradiol
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EDTA	ethylenediaminetetra-acetic acid
ER	estrogen receptor
ERE(s)	estrogen response element(s)
EtOH	ethanol
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
FSH	follicle stimulating factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gilz	GRE-containing glucocorticoid-induced leucine zipper
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
GRE(s)	Glucocorticoid response element(s)

hGR	human glucocorticoid receptor
HIV-1	human immunodeficiency virus type 1
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HPV	human papilloma virus
HRT	hormone replacement therapy
Hsp	heat shock protein
HSV-1	herpes simplex virus type-1
HSV-2	herpes simplex virus type-2
IL-2	interleukin 2
IL-4	interleukin 4
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
IL-12	interleukin 12
IgG	immunoglobulin G
KSFM	keratinocyte serum-free medium
LBD	ligand binding domain
LH	luteinizing hormone
Luc	luciferase
MAPK	mitogen-activated protein kinase
Mib	mibolerone

MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
MRE(s)	mineralocorticoid response element(s)
mRNA	messenger ribonucleic acid
MWS	million women study
N-CoR	nuclear receptor co-repressor
NET	norethindrone / norethisterone
NET-A	norethisterone-acetate
NET-EN	norethisterone-enanthate
NFκB	nuclear factor kappa-B
NK	natural killer
NSC	non-silencing control
nGRE(s)	negative glucocorticoid response element(s)
PAGE	polyacrylamide gel electrophoresis
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PHA	phytohemaglutinin
PCR	polymerase chain reaction
PR	progesterone receptor
PRE(s)	progesterone receptor element(s)
Prog	progesterone
qPCR	quantitative polymerase chain reaction

RANTES	Regulated-upon-Activation, Normal T cell Expressed and Secreted
RLU	relative light units
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
R5020	promegestone
RU486	mifepristone
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SHBG	sex hormone binding globulin
SHIV	simian-human immunodeficiency virus
siRNA	short interfering ribonucleic acid
Sp1	Specific protein 1
SRE	steroid receptor response element
SRC-1	steroid receptor co-activator-1
STAT	signal transducer and activator of transcription
STI(s)	sexually transmitted infection(s)
TBS	TRIS buffered saline
TE	TRIS-EDTA
Tm	melting temperature
TNF-α	tumour necrosis factor-α
UNG	Uracil-DNA N-glycosylase

WHI	Women's Health Initiative
WHO	World Health Organization

THESIS OUTLINE

This thesis consists of five chapters. Chapters 3 and 4 include a brief introduction of the particular study, and report and discuss the results obtained. The references for all the chapters are included in one section following Chapter 5.

1. Chapter 1: **Literature review**. This chapter provides an overview of the relevant knowledge currently available in the literature, with a particular focus on the molecular mechanisms of action of medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN)/norethisterone acetate (NET-A) and mucosal immunity in the lower female genital tract.
2. Chapter 2: **Materials and Methods.** This chapter provides a detailed description of the experimental protocols and materials used to obtain the results presented in Chapters 3 and 4.
3. Chapter 3: **The molecular mechanism of progestin-induced regulation of interleukin (IL)-12p40 and IL-10 in a human ectocervical epithelial cell line.** This chapter contains the results of a study investigating the regulation of the endogenous cytokine genes, IL-12p40 and IL-10, by progesterone (Prog), MPA and NET-A, and underlying mechanism, in an Ect1/E6E7 (human ectocervical) cell line.
4. Chapter 4: **Modulation of cytokine gene expression in a human ectocervical epithelial cell line by the HIV-1 accessory viral protein R (Vpr)**. This chapter reports on the findings of a study investigating whether HIV-1 Vpr can modulate the effects of Prog, MPA and NET-A on the expression of IL-12p40, IL-12p35 and IL-10 in the Ect1/E6E7 cell line.

5. Chapter 5: **Concluding discussion.** The results of the overall study are discussed and conclusions drawn in this final chapter.

Addendums A and B at the back of the thesis include data not shown, but referred to in Chapters 2-4, additional results not included in the chapters, as well as experimental optimisations. Addendum C includes the publication to which the candidate contributed.

Although the collective terms “we” and “our” are sometimes used in the thesis, all the experimental work was performed by the candidate, except for the maintenance of the Ect1/E6E7 cell line, which was performed by Carmen Langeveldt.

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Africander, D., **Louw, R.**, Verhoog, N., Noeth, D., Hapgood, J.P. Differential regulation of endogenous pro-inflammatory cytokine genes by MPA and NET-A in cell lines of the female genital tract. Contraception, 2011. 84 (4): 423-435.

- My contribution to the article was figure 4A, figure 4B (determining the ER levels in the ectocervical epithelial cell line using the whole cell binding assay) and figure 7.

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Synthetic progestins are used clinically in a wide variety of applications, such as contraception, hormone replacement therapy (HRT) and certain gynaecological disorders. These progestins were designed to mimic the actions of the endogenous hormone progesterone (Prog) and to elicit their biological effects via the progesterone receptor (PR). However, the clinical use of some synthetic progestins has been reported to cause undesirable side-effects, most likely due to binding to other members of the steroid receptor family such as the androgen- (AR), mineralocorticoid- (MR) and glucocorticoid receptor (GR) (reviewed in (Africander et al., 2011b). Medroxyprogesterone acetate (MPA) and norethisterone/norethindrone enanthate (NET-EN) are examples of synthetic progestins used as progestin-only injectable contraception. Side-effects associated with the use of MPA and oral contraceptive pills include an increased risk of genital tract infections due to the regulation of a variety of components of the immune system (reviewed in (Kaushic, 2009, Gravitt and Ghanem, 2010, Kaushic et al., 2010))(Brabin, 2002, Morrison et al., 2004). Hormonal contraceptives have been shown to affect mucosal immunity in the female genital tract, which may increase susceptibility to genital tract infections like human papilloma virus (HPV), herpes simplex virus (HSV), human immunodeficiency virus (HIV) and *Chlamydia trichomatis* and *Candida albicans* (UNAIDS, 2007, Ochiel et al., 2008, Gravitt and Ghanem, 2010). It is thus important to understand the role of factors such as hormonal contraceptives that may affect local immune responses in the female genital tract, as this my shed light on the molecular events occurring during infections. The epithelial cells lining the ectocervix play a protective role when the female genital tract is exposed to pathogens (Wira et al., 2005a, Gravitt and Ghanem, 2010, Wira et al., 2010, Wira et al., 2011). In addition to providing a physical barrier against sexually transmitted infections these epithelial cells are capable of eliciting both innate and acquired local immune responses at the mucosal surface against invading pathogens, enabling them to fight infections (Shrier et al., 2003, Ochiel et al.,

2008, Dubicke et al., 2010). This chapter provides a brief overview of some of the therapeutic applications and diverse biological activities of MPA, also known as Depo-Provera®, and NET-EN, also known as Nuristerate®, extensively used as injectable contraceptives in South Africa (Smit et al., 2001, Medical Research Council, 2003, Kleinschmidt et al., 2007, Morrison et al., 2012). Furthermore, the effect of MPA and/or norethisterone/norethindrone (NET) on genital tract immunity and the association between the use of these contraceptives and risk of susceptibility to genital tract infections, specifically HIV, will be reviewed.

1.2. The progestogens: Natural Prog and the synthetic progestins, MPA and NET

The term progestogens refer to compounds that exhibit progestational activity, and include both the endogenous Prog and the synthetic progestins designed to mimic its actions. The endogenous ovarian hormone, Prog, is the first biologically active compound synthesised from cholesterol (Hu et al., 2010) and serves as the precursor for the synthesis of androgens, estrogens, glucocorticoids and mineralocorticoids (Gellersen et al., 2009, Skouby and Jespersen, 2009, Hu et al., 2010). Prog plays an important, yet diverse, role in the regulation of female reproduction (Ottesen and Pedersen, 1996, Graham and Clarke, 1997, Schindler et al., 2003, Sitruk-Ware, 2004a, Sasha et al., 2008, Gellersen et al., 2009). For example, in the uterus and ovaries of mammalians Prog is responsible for the release of mature oocytes, facilitates implantation of the fertilised ovum, prevents contraction of the myometrium, maintains pregnancy via its anti-estrogenic activity, and inhibits further ovulation through its anti-gonadotropic activity (Graham and Clarke, 1997, Sitruk-Ware, 2004a). During pregnancy, Prog stimulates the growth and development of the mammary gland for milk production, storage and secretion (Graham and Clarke, 1997). There is also evidence that Prog plays a role in cancer (Graham and Clarke, 1997). Furthermore, Prog has been shown to stimulate

the γ-aminobutyric acid (GABA) signalling pathways in specific areas of the brain, thereby playing a role in neuroprotection (Graham and Clarke, 1997). Interestingly, changes in the concentration of Prog due to reproductive processes such as pregnancy and menstruation can affect the local immune system (Bouman et al., 2005, Wira et al., 2005a, Gravitt and Ghanem, 2010). For example, high Prog concentrations during the luteal phase of the human menstrual cycle and pregnancy, the maternal immune system changes from a pro-inflammatory to an anti-inflammatory state thereby decreasing inflammation. As inflammation contributes to preterm birth (Romero et al., 1994), this decrease in the inflammatory response provides protection for the developing fetus (Fleischman and Fessler, 2010).

Low Prog production in females has been associated with miscarriages, preterm labor, infertility, endometriosis, abnormal menstrual bleeding, breast and endometrial cancer, depression and anxiety (Gellersen et al., 2009). As a result, synthetic progestins were designed to mimic the actions of Prog, and have been used in a number of therapeutic or clinical applications (Speroff, 1996, Howell et al., 1998, Kimmick and Muss, 1998, Li et al., 2000, Schweppe, 2001, Stahlberg et al., 2004). These synthetic progestins can be used either on their own or in combination with estrogen (Solter, 1999, Greydanus et al., 2001, Truitt et al., 2003, Mansour, 2005, Archer et al., 2011). Combined oral contraceptives are available in different forms such as pills, transdermal patches or vaginal rings, whereas the progestin-only contraceptives can be taken in the form of pills (orally), intramuscular injections, implants or intrauterine devices (Greydanus et al., 2001, Mansour, 2005, Simon, 2007).

MPA is a 17α-hydroxyprogesterone derivative, while NET is a 19-nortestosterone derivative (Schindler et al., 2003, Stanczyk, 2003, Sitruk-Ware, 2004b). The chemical structures of Prog, MPA and NET are indicated in figure 1.1.

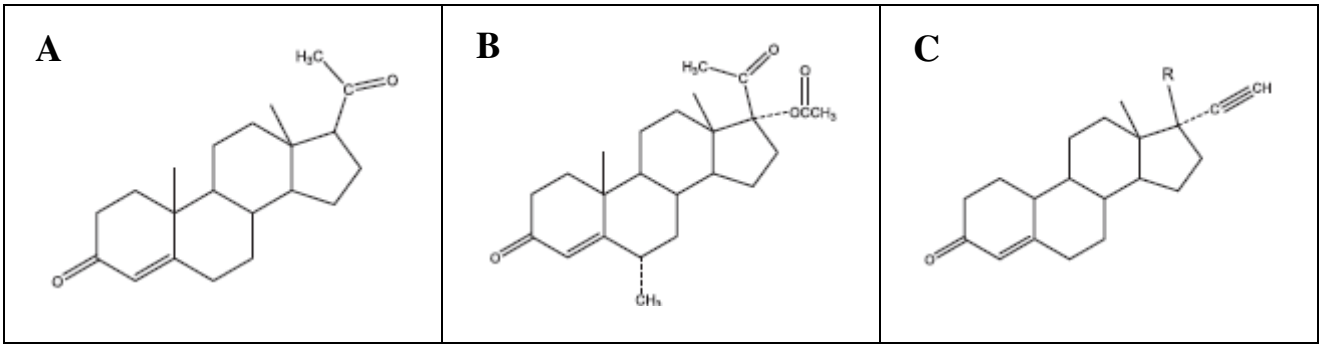

Figure 1.1. The chemical structures of (A) Prog, (B) MPA and (C) NET (R = OH), NET-A (R = $OCOCH_3$) and norethisterone enanthate (NET-EN) [R = $OCO(CH_2)_5CH_3$] (Taken from Africander et al., 2011b).

1.3. Therapeutic applications

1.3.1. Contraception

For injectable contraceptive use in women, MPA and NET-EN are administered by intramuscular injections (Westhoff, 2003, Sitruk-Ware, 2004b, Bakry et al., 2008). MPA is administered as an aqueous suspension at a dose of 150 mg every three months (Mishell, 1996, Kaunitz, 1998, Bakry et al., 2008), while NET-EN is administered as an oily suspension at a dose of 200 mg every two months (Howard et al., 1975, Fotherby et al., 1978, Goebelsmann et al., 1979). MPA and NET-EN are known to be long-acting progestins as they are slowly released from the muscle (Mishell, 1996). Both MPA (Schindler et al., 2003, Bakry et al., 2008) and NET-EN (Stanczyk and Roy, 1990, Schindler et al., 2003) are metabolised in the liver. However, MPA itself is known to be the major progestogenic compound, while NET-EN and NET-A are hydrolysed to NET, which together with its metabolites form the active contraceptive agents (Stanczyk and Roy, 1990). NET-A is used in oral contraception and HRT.

It has been reported that women using the intramuscular injection of 150 mg MPA have serum concentrations ranging between 2.6 and 3.9 nM (Mathrubutham and Fotherby, 1981, Mishell, 1996), while women receiving the intramuscular administration of 200 mg NET-EN have serum

concentrations ranging between 1.5 and 59 nM (Fotherby et al., 1983). However, serum concentrations of these parenterally administered progestins vary depending on the time after administration (Stanczyk, 2003). For example, the intramuscular injection of 150 mg MPA has been reported to reach peak plasma concentrations of 1.75 - 9 ng/ml (4.5 - 23.3 nM), 1 - 7 ng/ml (2.6 – 18.1 nM) and 0.68 – 2.6 ng/ml (1.8 – 6.7 nM) one, three and eleven weeks, respectively, after administration, followed by a gradual decrease to undetectable levels (<100 pg/ml or 0.26 nM) after 17 to 29 weeks (Depo-Provera contraceptive injection, 2006, New York, Pharmacia and Upjohn Company, Pfizer Inc.)(Shrimanker et al., 1978). In contrast, NET has been shown to reach a peak plasma concentration of 50 nM one week after intramuscular administration of 200 mg NET-EN, and then gradually decreases to ~ 13 nM, which is maintained for one to four months (Howard et al., 1975, Fotherby et al., 1978, Goebelsmann et al., 1979).

The primary mode of action by which MPA and NET exert their contraceptive action is by inhibiting ovulation. This is achieved by the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary being suppressed, or by decreasing the pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Mishell, 1996, Greydanus et al., 2001, Mansour, 2005, Bakry et al., 2008, Beijerink et al., 2008). In addition, MPA and NET limit sperm penetration by thickening the cervical mucus (Greydanus et al., 2001, Bakry et al., 2008), as well as causing atrophy of the endometrium, thus preventing the implantation of fertilised ova (Bakry et al., 2008). Moreover, MPA and NET-EN are under investigation as male contraceptives, since MPA or NET-EN combined with testosterone, suppress spermatogenesis, by synergistically suppressing the secretion of gonadotropic hormones (Kamischke et al., 2000a, Kamischke et al., 2000b); reviewed in Nieschlag et al., 2003; Gu, 2004; reviewed in Amory, 2008).

1.3.2. Hormone replacement therapy (HRT)

HRT is commonly prescribed to postmenopausal women to alleviate symptoms such as hot flushes, vaginal atrophy and dryness, as well as bone loss. These symptoms are as a consequence of decreased estrogen levels. Estrogen combined with progestins such as MPA or NET is administered to menopausal women with an intact uterus, so as to prevent estrogen-induced endometrial cancer, while women without a uterus are administered estrogen alone (Whitehead et al., 1979, Gambrell et al., 1980, Taitel and Kafrissen, 1995, Greendale et al., 1999, Sitruk-ware, 2006). The combined HRT is administered either continuously (daily) or sequentially (part of a month) (Kuhl, 2005). The typical daily dosages of the progestins used in HRT range between 2.5 and 10 mg for MPA and 0.5 – 1 mg for NET-A (Kuhl, 2005, Kuhl, 2011).

The serum levels of MPA have been reported to range between 0.01 – 0.1 ng/ml (0.02 and 0.2 nM) (Ghatge et al., 2005). In addition, it has been shown that postmenopausal women using 2.5 mg/day and 5 mg/day MPA have serum concentrations ranging between 0.3 – 0.45 ng/ml (0.776 – 1.16 nM) and 0.6 – 0.9 ng/ml (1.55 – 2.33 nM), respectively (Jarvinen et al., 2004). For the 10 mg MPA/day, peak serum levels ranged between 3 -5 ng/ml (8 - 13 nM) (Hiroi et al., 1975). The reported serum levels of NET seem to be higher than those of MPA. When administered 0.5 mg NET-A in combination with 1 mg estradiol, NET serum levels range between 5 - 7 ng/ml (14.7 and 20.6 nM) (Stadberg et al., 1999, Zdravkovic et al., 2001) and 1.2 - 6.0 ng/ml (3.64 – 17.7 nM) (Activelle package insert reg. no. 33/21.8.2/0532, Novo Nordisk Inc.) Similarly, peak serum levels of 5 – 10 ng/ml (14.7 – 29.4 nM) was obtained after the oral administration of 1 mg NET-A (Kuhl, 2011).

1.3.3. Other applications

MPA and NET/NET-A/NET-EN are also used for other therapeutic applications. Both MPA (Kaunitz, 1998, Harrison and Barry-Kinsella, 2000, Irahara et al., 2001) and NET (Muneyyirci-Delale and Karacan, 1998, Vercellini et al., 2003, Williams and Creighton, 2012) are used for the treatment of ovulatory pain, endometriosis and menstrual disorders such as dysmenorrhea and menorrhagia, while MPA can also be used in cancer therapy (Etienne et al., 1992, Yamashita et al., 1996). Depending on the treatment the administered dosage varies. For example, both MPA and NET are used for the treatment of endometriosis at a dosage of 50 – 100 mg/day (Harrison and Barry-Kinsella, 2000; Telimaa et al., 1989), while for cancer therapy the dosages range between 500 and 1500 mg/day for approximately 12 weeks (Blossey et al., 2006, a revised edition of Blossey et al., 1984; (Kim et al., 2012).

1.4. Molecular mechanism of action via steroid receptors

Following the administration of MPA and NET, these steroids enter the bloodstream where they interact with several serum-binding proteins (reviewed in Africander et al., 2011b). MPA binds to plasma proteins such as albumin (± 90%), but does not bind to sex-hormone-binding globulin (SHBG) or corticosteroid-binding globulin (CBG) (Schindler et al., 2003, Bakry et al., 2008). Thus < 10% of MPA is free in the blood. In contrast, 36% of NET binds to SHBG, while 61% is bound to albumin and 3% is free (unbound) (Hammond et al., 1982, Schindler et al., 2003). The free steroid is biologically active and can readily diffuse from the blood into the cells of target tissues, while the bound steroids are unavailable to tissues. Once the free MPA and NET enters the cell, they will elicit their effects on target genes by binding to intracellular steroid receptors, such as the PR, AR, MR, possibly the estrogen- (ER), or the GR (reviewed in (Africander et al., 2011b). In the sections

below, the general mechanism of action of the above-mentioned steroid receptors, as well as known effects of MPA and NET via these receptors, are briefly described.

1.4.1. General mechanism of action of steroid receptors

The PR, AR, MR, ER and GR are ligand activated transcription factors which belong to the nuclear hormone receptor family (Evans, 1988, Mangelsdorf et al., 1995, Griekspoor et al., 2007, Huang et al., 2010, Sladek, 2010, Aagaard et al., 2011, Ahmad and Kumar, 2011, Bagamasbad and Denver, 2011). In general, these receptors all contain three functional domains: a variable N-terminal transactivation domain, a central highly conserved DNA-binding domain (DBD), a hinge region and a moderately conserved ligand-binding domain (LBD) found at the C-terminal (figure 1.2) (Mangelsdorf et al., 1995, Griekspoor et al., 2007, McEwan, 2009, Aagaard et al., 2011); reviewed in (Africander et al., 2011b). The N-terminal transactivation domain contains an activation function (AF)-1 domain, which has been reported to be responsible for protein-protein interactions between general transcription factors and is required for optimal transcriptional activity (reviewed in (Aagaard et al., 2011, Africander et al., 2011b). The DBD, known to be highly homologous between steroid receptors, plays an important role in DNA-binding specificity, receptor dimerization and interaction with co-factors (Griekspoor et al., 2007); reviewed in Aagaard et al., 2011 and Africander et al., 2001b), while the LBD is involved in protein-protein interactions with chaperone proteins (McEwan et al., 1997, Moras and Gronemeyer, 1998, Beato and Klug, 2000). Furthermore, the signal for nuclear localisation is embedded in the DBD and LBD (Tang et al., 1998, Griekspoor et al., 2007). A second activation function domain (AF-2), found within the LBD, is present and is important for the induction of transcriptional activity of the receptor. This AF-2 domain also contains a LXXLL motif to which co-factors bind (Weatherman et al., 1999); reviewed in Aagaard et al., 2011 and Africander et al., 2001b).

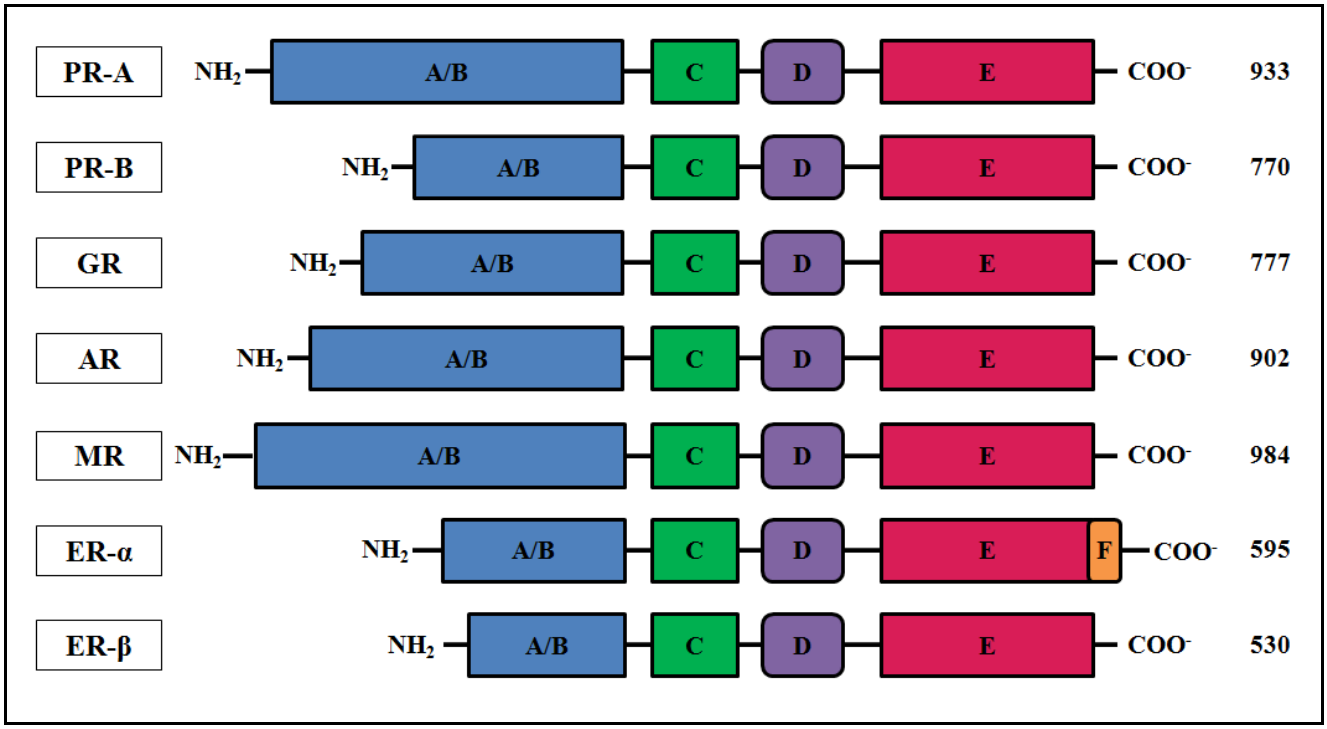

Figure 1.2. A schematic illustration of the general structure and organisation of the functional domains of steroid receptors. In general, steroid receptors consist of various functional domains: region A/B denotes the variable N-terminal domain containing the AF-1 transactivation domain, region C denotes the highly conserved DBD, region D denotes the hinge region between the DBD and LBD, and region E refers to the moderately conserved LBD, which contains the second transactivation domain AF-2. The ER-α is unique in that it also contains an additional carboxy-terminal F domain. The numbers represent the length of the steroid receptors in amino acids. Figure redrawn from Africander et al., 2011b.

The lipohilic nature of steroid hormones enables them to readily diffuse across the cell membrane into the target cell (Hammes and Levin, 2007, Sladek, 2010). Once inside the target cell, the hormone binds to the intracellular steroid receptor at its LBD. In the absence of ligand, the AR, MR, GR and PR-B isoform are found mainly in the cytoplasm, whereas the PR-A isoform and ER are found predominantly in the nucleus (Lim et al., 1999, Griekspoor et al., 2007). The unliganded receptor is bound to chaperone proteins such as heat shock protein 90 (hsp 90), hsp 70, hsp 56 and hsp 26, immunophilin p59 and phosphoprotein p23 (Pratt and Toft, 1997, Griekspoor et al., 2007). Upon ligand binding, the receptor undergoes a conformational change, allowing it to dissociate from the multimeric protein complex, and the liganded receptor subsequentially translocates to the

nucleus (Zhou and Cidlowski, 2005, Griekspoor et al., 2007). The ligand bound receptor then binds to specific regulatory elements within the promoter region of target genes, to either activate (transactivation) or repress (transrepression) transcription of target genes (reviewed in (Huang et al., 2010, Africander et al., 2011b).

1.4.1.1. Transactivation

Ligand-activated steroid receptors bind as a dimer to palindromic DNA sequences known as steroid responsive elements (SREs) (Webster and Cidlowski, 1999) in the promoter area of target genes (figure 1.3). Dimerisation of the GR has also been suggested to occur via the cooperative association of two GR monomers to half-GRE sites (Dahlman-Wright et al., 1990, Holmstrom et al., 2008). The consensus palindromic SREs consist of two hexanucleotide sequences separated by any three nucleotides, and in humans has the sequence GGTACAnnnTGTTCT (Newton, 2000); reviewed in (McEwan, 2009). Due to the highly conserved DBD of steroid receptors (reviewed in (Beato, 1989, Africander et al., 2011b), most receptors bind to the same SRE. For example, the PR binds to the progesterone response element (PRE), which can also serve as a response element for the AR, MR and GR, and is then called the androgen response element (ARE), mineralocorticoid response element (MRE) and glucocorticoid response element (GRE), respectively. The ER differs from the other steroid receptors in that it binds specifically to estrogen response elements (EREs), which consist of the DNA sequence GGTCAnnnTGACC (Klinge, 2001). Following DNA binding, multi-protein complexes including chromatin-remodelling proteins, co-activators and components of the basal transcription machinery are recruited to the promoter of target genes (Beato and Klug, 2000, Lu et al., 2006, Griekspoor et al., 2007, Africander et al., 2011b). The recruitment of co-activators to the liganded steroid receptors on the promoter of target genes stimulates histone

acetylation, which results in the opening of the chromatin and recruitment of the basal transcription machinery (figure 1.4A), to positively regulate transcription (reviewed in (Griekspoor et al., 2007).

1.4.1.2. Transrepression

Inhibition of transcription or transrepression can occur when steroid receptors bind as a monomer to negative-SREs (nSREs), or more frequently to another transcription factor such as nuclear factor kappa-B (NFκB), activator protein (AP)-1 and CCAAT enhancer binding protein (C/EBP) (figure 3) (Cato and Wade, 1996, Almawi and Melemedjian, 2002, Zhou and Cidlowski, 2005, Gross and Cidlowski, 2008, De Bosscher and Haegeman, 2009). The latter mechanism of protein-protein interaction is known as tethering (Newton, 2000, De Bosscher and Haegeman, 2009), and is best-studied and characterised for the GR through its interaction with AP-1 or NFκB (Cato and Wade, 1996, Almawi and Melemedjian, 2002, De Bosscher et al., 2003, Kassel and Herrlich, 2007). Although much less is known about the mechanism of transrepression via other members of the steroid receptor family such as the PR, AR, MR and ER, there is evidence that these receptors interact with NFκB to repress the expression of genes (Kalkhoven et al., 1996, Palvimo et al., 1996, De Bosscher et al., 2003, Kobayashi et al., 2010). In contrast to transactivation, co-repressors are recruited to the promoter of target genes to negatively regulate transcription (Heinlein and Chang, 2002). The recruitment of co-repressors results in histone deacetylation, thereby producing compact chromatin (figure 1.4B) (reviewed in (Griekspoor et al., 2007).

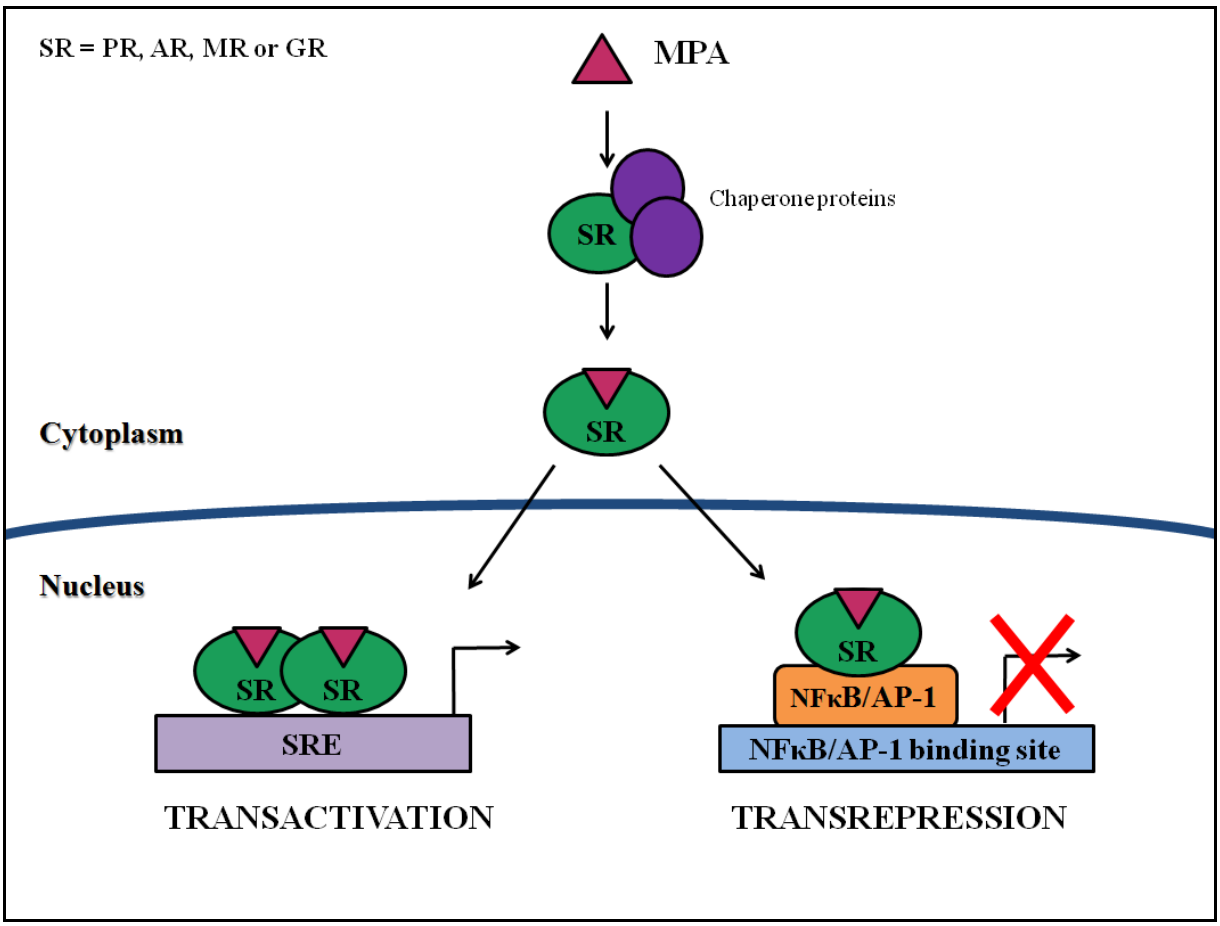

Figure 1.3. General molecular mechanism of action of steroid receptors. A lipohilic steroid hormone such as MPA diffuses across the cell membrane and binds to an intracellular steroid receptor (depicted as SR). The ligand-activated steroid receptor binds either as a dimer to SREs in the promoter of a target gene to positively regulate transcription (transactivation) or as a monomer to other DNA bound transcription factors (NFκB or AP-1) to negatively regulate transcription (transrepression).

In addition, it has been established that tethering of the steroid receptors to other DNA-bound transcription factors can also enhance target gene expression. For example, glucocorticoids have been shown to positively regulate transcription of the MAPK-phosphatase gene in human epithelial lung carcinoma cells via a tethering mechanism between the glucocorticoid-bound GR and the transcription factor C/EBPβ (Johansson-Haque et al., 2008). The PR has also been shown to tether to the transcription factors Sp1 and C/EBPβ to increase target gene transcription (Faivre et al., 2008, Goldhar et al., 2011). Furthermore, the transcriptional response of a gene also depends on

whether the ligand bound to the steroid receptor is an agonist or antagonist (reviewed in (Africander et al., 2011b)). In general, an agonist-bound steroid receptor induces a conformational change that facilitates the binding of a co-activator to increase transcription, whereas an antagonist-bound receptor recruits a co-repressor to decrease transcription (figure 1.4) (McKenna et al., 1999, Nettles and Greene, 2005, McPaul, 2008).

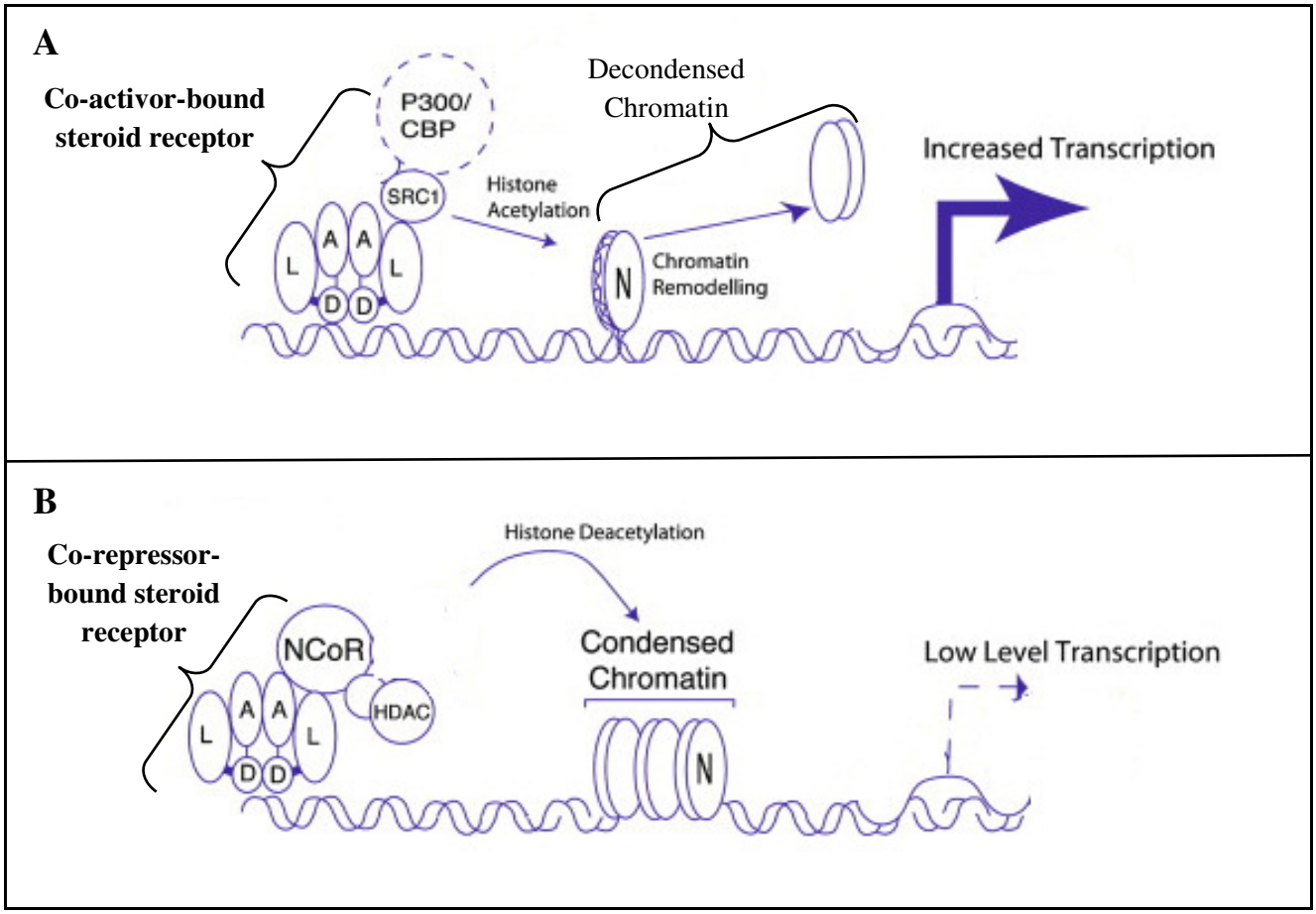

Figure 1.4. A general model showing the role of co-regulators in the transcriptional regulation of target genes by steroid receptors. (A) Binding of co-activators e.g. steroid receptor (SRC-1) and p300/CREB-binding protein (CBP) to steroid receptors stimulates histone acetylation. The latter causes remodelling of the chromatin structure, i.e. opening of the chromatin to make it more accessible for the basal transcription machinery, thereby increasing transcription. **(B)** Binding of co-repressors, e.g. nuclear receptor co-repressor (NCoR) to steroid receptors stimulates histone deacetylation. As a result the chromatin is more condensed and less accessible to proteins of the basal transcriptional machinery, which leads to a decrease in transcription. Abbreviations: A: N-terminal activation domain of steroid receptor; D: DBD and L: LBD of the steroid receptor; N: nucleosome; HDAC: histone deacetyltransferase. (Redrawn form McPaul, 2008).

1.4.2. Biological activities via the PR, AR, MR, ER and GR

1.4.2.1. PR

MPA and NET-A, like other synthetic progestins, were designed to mimic the biological actions of the endogenous PR ligand Prog by binding to the PR. Human PR is expressed in the female genital tract, mammary gland, pancreas, brain and the pituitary gland (reviewed in Africander et al., 2011b). Two PR-isoforms exist, PR-A and PR-B, which are transcribed from two promoters of a single gene (Kastner et al., 1990). PR-B is known to be transcriptionally more active that PR-A and differs from PR-A in that it contains an additional 164 amino acids at the N-terminal domain encoding for an activation function (AF)-3 domain (Kastner et al., 1990, Edwards et al., 1995, Keightley, 1998, Leonhardt and Edwards, 2002, Lange, 2008). The differences in the transcriptional activity of PR-A and PR-B has been reported to be cell- and promoter-specific (Lim et al., 1999). Interestingly, the human PR-A has been shown to act as a strong repressor of human PR-B, as well as the AR, ER, MR and GR (Vegeto et al., 1993, McDonnell and Goldman, 1994, McDonnell et al., 1994). Although the PR-isoforms are normally expressed at similar levels in female reproductive tissues, there is evidence that the ratio of PR-A and PR-B can vary extensively during the menstrual cycle, due to changing hormonal levels, as well as in breast, endometrial, cervical and ovarian cancers (Graham et al., 1996, Conneely et al., 2002, Mote et al., 2002, Richer et al., 2002, Scarpin et al., 2009). In addition to the differential expression of the PR-isoforms, these isoforms also have differential physiological functions in various tissues. For example, the biological effects of Prog are predominantly mediated by PR-A in the ovaries and uterus, while in the mammary gland it is primarily mediated via PR-B (Buser et al., 2007). Thus, it is possible that MPA and NET-A also exhibit differential biological activities, as well as different relative binding affinities (RBAs) via PR-A and PR-B, depending on the type of tissue (reviewed in Africander et al., 2011b). Indeed, a

recent study using recombinant *in vitro* binding showed that NET-A has a ~ two-fold higher RBA for human PR-B than PR-A (Attardi et al., 2010).

Numerous studies have shown that the synthetic progestins MPA and NET bind to the rat, rabbit and human PR, and that both these progestins have higher RBAs for the PR than Prog (Table 1), and are full PR agonists (reviewed in Africander et al., 2011b). Furthermore, MPA and NET-A have been shown to regulate the expression of several genes in the PR-positive T47D breast cancer cell line. In human endometrial stromal cells, a role for the PR in the MPA-induced inhibition of the chemokine Regulated-upon-Activation, Normal T Cell Expressed and Secreted (RANTES) transcriptional activity have been found (Zhao et al., 2002). In addition, the gene expression of two vascular inflammation markers, intracellular molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, are upregulated by MPA and NET-A in part via the PR (Tatsumi et al., 2002).

1.4.2.2. AR

The AR is expressed in various tissues, such as the mammary gland, vagina, prostate, testes, muscle, skin and bone marrow (Lu et al., 2006). In addition to playing an important role in male reproduction, the AR also has a role in the development of the ovaries and the breast in females. Both MPA and NET-A have been shown to bind to endogenous AR in human breast cancer cell lines, as well as to the human AR overexpressed in the COS-1 (monkey kidney fibroblast) cell line (Table 1) (Bergink et al., 1983, Bentel et al., 1999, Kemppainen et al., 1999a, Deckers et al., 2000, Schoonen et al., 2000, Africander, 2010). In the latter model, high RBAs were reported for Prog (K_i = 36.6 nM), MPA (K_i = 19.4 nM) and NET-A (K_i = 21.9 nM) compared to the natural androgen dihydrotestosterone (DHT) (K_i = 29.4 nM) (Africander, 2010), indicating that these progestogens can elicit its effects via the AR. Some studies have shown that Prog is a weak partial agonist for the

AR, others have reported that Prog acts as an AR antagonist (reviewed in Africander et al., 2011b; (Fuhrmann et al., 1996, Bentel et al., 1999, Sasagawa et al., 2008, Africander, 2010), while both MPA and NET-A are reported to be AR agonists (Africander, 2010). In addition, using NFκB and AP-1 promoter-reporter constructs, both MPA and NET-A, but not Prog, have been shown to act as agonists for transrepression via the AR (Africander, 2010). Thus, these progestins may exert inhibitory effects of inflammatory response genes via the AR. MPA has been shown to regulate the mRNA expression of a number of genes in human breast cell lines via the AR (Ghatge et al., 2005, Moore et al., 2012). In addition, a recent study in the human ectocervical epithelial cell line, Ect1/E6E7, showed a role for the AR in mediating the MPA induced downregulation of the pro-inflammatory cytokine RANTES. Using the cervical epithelial cell line, Hela, NET has also been shown to have AR-mediated transcriptional activation (Garcia-Becerra et al., 2004).

1.4.2.3. MR

The MR is expressed in epithelial tissues such as the kidney and colon, and nonepithelial cells such as the central nervous system, the heart and adipocytes (Krozowski and Funder, 1983, de Kloet et al., 2000, Funder, 2004). Both MPA and NET-A have been shown to bind to overexpressed MR in the COS-1 cell line with similar weak affinity to each other, but with much lower affinity than that of Prog (Table 1) (Africander, 2010). When comparing the RBAs of MPA and NET-A as percentages relative to aldosterone (Ald) (100%), the values obtained by the latter study for MPA (0.08%; Ki = 197 nM) and NET-A (0.07%; Ki = 229 nM) (Africander, 2010), are 40-fold lower than the previously reported values of 3.1% and 2.7%, respectively (Philibert et al., 1999, Winneker et al., 2003). Furthermore, Prog exhibits weak partial agonist and potent antagonist activity for the MR (Wambach and Higgins, 1978, Quinkler et al., 2002, Sasagawa et al., 2008, Africander, 2010), while MPA and NET-A have no agonist activity and weak MR antagonist activity (Sasagawa et al.,

2008, Africander, 2010). Using a rat cardiomyocyte cell line transiently transfected with the human MR, 1 µM NET-A, but not 1 µM MPA, displayed similar antagonistic activity to 1 µM Prog on the endogenous MRE-containing genes, orosomucoid-1 (Orm-1) and plasminogen activator inhibitor-1 (PAI-1). The anti-mineralocorticoid activity of NET-A may exert beneficial effects on blood pressure and have cardiovascular protective benefits. Moreover, unlike MPA, Prog and NET-A are agonists for transrepression via the MR (Africander, 2010). Although the concentration of MPA and NET-A used in this study is beyond the physiologically relevant concentrations, there is a possibility that *in vivo,* lower concentrations of NET-A may have the same effects.

1.4.2.4. ER

The ER has diverse physiological roles and is expressed in the female reproductive system (mammary glands, uterus, vagina and ovaries), male reproductive system (testis, epididymis and prostate), as well as the brain, lung and heart (Osborne et al., 2000, Mertens et al., 2001); reviewed in Africander et al., 2011b). For example, the ER plays a role in sexual development, reproduction and cardiovascular disease (Grese and Dodge, 1998, Fan et al., 2010). Two ER-isoforms, ERα (66 kDa) and ERβ (56 kDa), have been identified which are transcribed from two different genes and differ mainly in the AF-1 domain located in the A/B N-terminal domain (Griekspoor et al., 2007, Bai and Gust, 2009). ERα also differs from ERβ, and other steroid receptors, in that it contains an additional F-domain at the C-terminal (Kong et al., 2003). Several studies have shown that unlike the endogenous ER ligand, estradiol, MPA and NET do not bind to the ER (Teulings et al., 1980, Bergink et al., 1983, Schoonen et al., 2000, Lemus et al., 2009), while a single study showed that both MPA and NET bind to the ER (Di Carlo et al., 1983). However, whether it is MPA or NET themselves, or in fact their metabolites that bind to the ER is not clear. Indeed it has been shown that metabolites of NET bind weakly to the ER (Larrea et al., 1987, Schoonen et al., 2000).

It has been shown that NET and its A-ring reduced metabolites display weak agonist activity via the ER (Oropeza et al., Markiewicz and Gurpide, 1994, Schoonen et al., 2000, Schindler et al., 2003), while other studies have reported no estrogenic activity for MPA or NET via the ER (Markiewicz and Gurpide, 1994, Schindler et al., 2003). Whether these effects are via ERα or ERβ is unknown. Interestingly, Sasagawa and co-workers showed that NET displayed more agonist activity towards ERα than ERβ (Sasagawa et al., 2008). Furthermore, a 5α-reduced metabolite of NET (3β, 5α-NET) showed ERα selective activity at low concentrations, while ERβ agonistic activity was observed at high concentrations (Larrea et al., 1987). Some of the observerd effects of progestogens on gene expression may occur due to indirect genomic effects via the PR or another steroid receptor. For example, progestogens elicit indirect estrogenic activity via the PR. Binding of the progestogens to the PR then causes upregulation of 17β-hydroxysteroid dehydrogenase (HSD) type 2 gene expression, which inactivates estradiol by converting it to estrone (Kuhl, 2005). Progestogens can also exert indirect anti-estrogenic effects in the endometrium by binding to the PR, which then suppresses gene expression of the ER (Kuhl, 2005).

1.4.2.5. GR

The GR has diverse roles and although ubiquitously expressed, its levels are regulated in a tissue- and cell-specific manner (Lu et al., 2006). Thus, the levels of GR expressed vary substantially between tissues. For example, the GR has been shown to be expressed at a concentration of 4.1 *f*mol GR per mg protein in peripheral blood mononuclear cells (PBMCs) (Chriguer et al., 2005) and 893 *f*mol GR per mg protein in the skin (Guo et al., 1996). Furthermore, in certain disease states such as cancers, the concentration of the GR changes within the same tissue type of an individual. For example, the levels of the GR has been reported to increase by 5-fold in healthy vs cancerous brain tissue biopsies (30 *f*mol GR per mg protein vs 142 *f*mol GR per mg protein) (Yu et

al., 1981) and 2-fold in healthy vs cancerous lung tissue biopsies (30.3 – 84.9 *f*mol GR per mg protein vs 56.5 – 87.8 *f*mol GR per mg protein) (Beattie et al., 1985). Moreover, expression of the GR was significantly higher in the tumour tissue within the skin of AIDS patients with Kaposi's sarcoma compared with the noncancerous skin of the same patients (4663 vs 2777 *f*mol/mg protein) (Guo et al., 1996). GR expression levels (density) can affect the response of a ligand. Zhao and co-workers have previously shown that, depending on the GR density MPA displays full agonist, partial agonist/antagonist or antagonist activity for transrepression (Zhao et al., 2003).

Numerous studies have indicated that MPA binds the GR with a high RBA (Table 1) (reviewed in Africander et al., 2001b). One study in human mononuclear cells showed that MPA has a higher RBA for the GR than the natural glucocorticoid cortisol (42% vs 25%, respectively, relative to 100% binding of the synthetic glucocorticoid dexamethasone (Dex)) (Kontula et al., 1983). In contrast, MPA has been shown to have a similar RBA to that of Dex via the GR in the human lung carcinoma cell line (A549), COS-1 cell line and rats (Winneker and Parsons, 1981, Koubovec et al., 2005, Ronacher et al., 2009). In contrast, Prog and NET have been shown to bind with very low affinity to the human GR (Koubovec et al., 2005, Ronacher et al., 2009) (Table 1).

The evidence in the literature suggests that Prog and MPA act as GR agonists for transactivation and transrepression, while NET-A acts as a weak GR antagonist for transactivation and very weak partial GR agonist for transrepression (Koubovec et al., 2005, Ronacher et al., 2009, Africander, 2010, Courtin et al., 2011). Interestingly, Bamberger and co-workers have previously indicated that MPA exerts dissociative glucocorticoid-like properties in normal human lymphocytes (Bamberger et al., 1999), as MPA significantly repressed interleukin (IL)-2 promoter-reporter activity, while transactivation of a GRE promoter in the same cell system was minimal. They also showed that

MPA dose-dependently suppressed the protein levels of IL-6 and IL-2 in normal human lymphocytes, most probably via the GR (Bamberger et al., 1999). These effects of MPA were assumed to be mediated via the GR, as this cell line does not express the PR or AR. Consistent with the transrepression abilities observed by Bamberger et al., other studies have also shown that MPA elicits anti-inflammatory activity via the GR (Bamberger et al., 1999, Kurebayashi et al., 2003, Koubovec et al., 2004, Koubovec et al., 2005). However, in contrast to Bamberger et al., most of these studies observed significant transactivation of a GRE promoter by MPA. Kurebayashi and co-workers show that MPA, in a dose dependent manner, decreases the secretion and mRNA expression of the pro-inflammatory cytokine IL-6 in the human thyroid cancer cell line (KTC-2) via the GR (Kurebayashi et al., 2003). Furthermore, a role for the GR in mediating the MPA induced repression of the IL-6 protein and IL-6 and IL-8 promoter reporter activity at the level of transcription was demonstrated in the murine fibrosarcoma L929sA cell line (Koubovec et al., 2004). In contrast, MPA and other GR ligands such as Dex and cortisol have been reported to also elicit pro-inflammatory responses (Visser et al., 1998, Enomoto et al., 2007, Verhoog, 2010, Lannan et al., 2012). For example, the GR endogenously expressed in human endocervical epithelial cell line (End1/E6E7) was shown to be involved in the upregulation of IL-6 mRNA expression by Prog, MPA and NET-A (Verhoog, 2010). Interestingly, NET has previously been shown to display no GR transactivational activity (Schoonen et al., 2000, Koubovec et al., 2005).

Taken together, MPA, unlike NET-A and Prog has high affinity for the GR, and displays greater GR agonist activity for transactivation and transrepression that NET-A and Prog. Moreover, these ligands can exert both anti- and pro-inflammatory activity via the GR. In addition, the fact that GR levels determine whether MPA acts as a GR agonist or antagonist, may have implications for the physiological effect of MPA, and maybe even NET-A, in different systems. Although they were

designed to have similar biological activities via the PR, these collective studies described in section 1.4.2.2-1.4.2.5 indicate that these progestins also exert off-target biological effects via the AR, MR, GR and possibly the ER. It is thought that these off-target effects of MPA and NET contribute to the undesirable side-effects associated with their clinical use. To summarise, the mechanism of action of MPA and NET via steroid receptors, it is clear that many unanswered questions remain.

Table 1. Relative binding affinities (RBAs) and biological activities of natural Prog and the synthetic progestins, MPA and NET/NET-A, via steroid receptors

Progestogen	PR		AR			GR		MR		ER		
	RBA %	Progestogenic	RBA %	Agonistic	Anti-androgenic	RBA %	Glucocorticoid	RBA %	Anti-mineralocorticoid	RBA %	Estrogenic	Anti-estrogenic
Prog	100[ha]; 50[d]; 30[i]; 12[h]	+	80[b]; 62[b]; 3[a]; 1[b]; 0[d]	±	(+)	35[a]; 6[b]; 2[c]; 0.84[g]	±	1000[a]; 100[bf]; 9[b]	+	0.5[a]; 0[d]	-	+
MPA	298[a]; 224[e]; 131[h]; 112[e]; 84[f]; 65[e]; 25[h]; 9.4[f]	++	151[b]; 75[b]; 53[f]; 36[a]; 8[f]; 2[b]	±	-	79[b]; 58[a]; 42[h]; 39[c]; 21.6[h]	+	160[d]; 3.1[a]; 0.08[b]	-	<0.02[a]; 0[d]	-	+
NET	134[a]; 75[d]	+	55[a]; 15[d]; 3.2[h]	+	-	1.4[a]; 0[d]	-	2.7[a]; 0[d]	-	0.15[a]; 0[d]	+	+
NET-A	43[a]; 27[a]	++	134[b]; 1.7[b]	+	-	1.6[c]; 0.88[b]	-	0.07[b]	-	0		

[a] In vitro human steroid receptor (Philibert et al., 1999, Krattenmacher, 2000, Attardi et al., 2010).
[b] hAR (Kemppainen et al., 1999b, Africander, 2010), hGR (Koubovec et al., 2005) or hMR (Fuhrmann et al., 1995, Africander, 2010) expressed in the COS-1 cell line.
[c] The A549 cell line expressing endogenous GR (Koubovec et al., 2005).
[d] Values compiled by cross comparisons from several studies that used different methods and were taken from (Kuhl, 2005). Most of the data are from animal tissues or cell lines expressing several receptors.
[e] Rabbit uterine cytosol (Zhang et al., 2000, Shields-Botella et al., 2003).
[f] Different rat tissues (Phillips et al., 1990, Pollow et al., 1992, Zhang et al., 2000, Shields-Botella et al., 2003)
[g] Dog liver cytosol (Selman et al., 1996).
[h] T47D or MCF-7 cells lines (Bergink et al., 1983, Deckers et al., 2000, Zhang et al., 2000, Shields-Botella et al., 2003).
[i] Human uterine tissue (Pollow et al., 1992, Stanczyk, 2003).

-, (+), +, ++ and ± denotes the different levels of activity: -, not effective; (+), weakly effective; +, effective; ++, strongly effective; ±, literature inconsistent. (Taken from Africander et al., 2011b and Stanczyk et al., 2012).

1.5. Side-effects associated with the therapeutic usage of MPA and NET

A number of undesirable side-effects, some more severe than others, have been associated with the clinical use of MPA and NET. Examples of these side-effects include amenorrhea, irregular bleeding, weight gain, headaches, nausea, acne, fatigue, depression, insomnia, abdominal pain or discomfort, breast tenderness, hot flushes, reduced libido, vaginal itchiness as wells as decreased bone mineral density (Darney, 1995, Kaunitz, 2000, Li et al., 2000, Greydanus et al., 2001, Haider and Darney, 2007, Ojule et al., 2010, Williams and Creighton, 2012). Furthermore, clinical evidence from the Women's Health Initiative (WHI) study indicated that MPA significantly increased the risk of invasive breast cancer, coronary heart diseases, strokes and venous thromboembolism (Rossouw et al., 2002, Anderson et al., 2004, Warren, 2004) in post-menopausal women using MPA and conjugated equine estrogens (CEE) in HRT, while the Million Women Study indicated that both MPA and NET increased the risk of breast cancer in long-term HRT users (Beral et al., 2003).

There is clinical evidence in the literature to suggest that the use of MPA as a contraceptive increase the risk of acquiring genital tract infections such as HSV-2 (Mostad et al., 1997, Mostad et al., 2000), *Chlamydia* (Morrison et al., 2004), *Gonorrhoea* (Morrison et al., 2004) and HIV-1 (Hel et al., 2010, Morrison et al., 2010, Heffron et al., 2012, Morrison et al., 2012). In addition, mice treated with contraceptive doses of MPA showed a 100-fold increased susceptibility to genital HSV-2 infection compared to untreated mice (Kaushic et al., 2003). Similarly, it has been shown that MPA increased susceptibility to vaginal simian-HIV (SHIV) in rhesus macaques. In the latter study, the increase in susceptibility was ascribed to an immune- rather than transmission-based mechanism (Trunova et al., 2006). Contraceptive doses of MPA have also been shown to modulate local immune capacity in the human vaginal epithelium by altering its thickness and increasing the

number of intraepithelial immune cells (Ildgruben et al., 2003). Surprisingly little research has investigated the effects of MPA and NET-A on immune function at the cellular level. A recent study using human ectocervical and vaginal epithelial cell lines as *in vitro* models for mucosal immunity, showed that Prog, MPA and NET-A differentially regulate the tumor necrosis factor (TNF)-α induced gene expression of IL-6, IL-8 and RANTES in a ligand-, promoter- and cell-specific manner (Africander et al., 2011a). MPA has also been reported to decrease the lipopolysaccharide (LPS)-induced mRNA expression of cytokines/chemokines in the cervix of mice and include interferon (IFN)-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13 and IL-15 (Elovitz and Gonzalez, 2008). Considering the above information, it is clear that more research is needed on the effects of MPA and NET-A on target genes involved in immune function, particularly in cells relevant to viral infections.

In the next section, the evidence in the literature for an association between hormonal contraceptive use and HIV-1 pathogenesis will further be discussed.

1.6. Hormonal contraception and HIV-1

Numerous epidemiological studies have assessed the link between the use contraception and susceptibility to HIV-1, the disease progression to AIDS, as well as HIV viral shedding. However, whether hormonal contraception is indeed a risk factor for HIV-1 acquisition remains controversial. Re-analysis of published data (Morrison et al., 2007) by Morrison and co-workers showed that the use of DMPA, but not combined oral contraceptives containing estrogen and the progestin levonorgestrel, is associated with an increase in HIV acquisition (Morrison et al., 2010). Similarly, another study showed evidence for a modest increase in HIV acquisition risk among young DMPA users in South African women (Morrison et al., 2012). Heffron and co-workers reported that

injectable contraceptive use in seven African nations including South Africa, and of which DMPA was the most commonly used method, significantly increased the risk of HIV-1 acquisition in uninfected women (Heffron et al., 2012). In contrast, some studies report no association between use of DMPA or combined oral contraceptives and HIV-1 acquisition (Kleinschmidt et al., 2007, Myer et al., 2007). Limited data currently available for NET-EN, shows no association between contraceptive use of NET-EN and the risk of acquiring HIV infection (Kleinschmidt et al., 2007, Myer et al., 2007, Morrison et al., 2012).

Whether hormonal contraception is been linked to HIV-1 disease progression to AIDS (Beaten et al., 2007, Hel et al., 2010), is also controversial. While some studies suggest that women infected with HIV-1 and using DMPA or oral contraceptives have higher viral loads, and more rapid loss of $CD4^+$ T cells, compared to women not using any contraception, other studies show no relationship between hormonal contraception and HIV-1 disease progression (Cejtin et al., 2003, Richardson et al., 2007). There is also evidence in the literature that MPA and other hormonal contraceptives, increase cervicovaginal shedding of HIV-1 DNA (a marker of HIV-1 infected cells), in HIV-infected women (Clemetson et al., 1993, Mostad et al., 1997, Wang, 2004, Stringer and Antonsen, 2008, Hel et al., 2010). Furthermore, a recent study showed an increased risk of HIV-1 transmission from HIV-1 infected women using MPA to uninfected men (Heffron et al., 2012). The authors suggested that the increased levels of HIV-1 RNA, detected in the endocervical secretions of these women, could explain the increased risk of HIV-1 transmission.

Nonetheless, the majority of studies seem to suggest that there is indeed an association between the use of MPA and increased susceptibility to HIV-1 infection. However, the exact mechanism whereby contraception increases susceptibility to HIV-1 is not clear. Some studies have suggested

that it may be due to the fact that sex steroid hormones affect the thickness and permeability of the vaginal mucosal epithelium in women as well as female macaques (Hel et al., 2010). For example, high estrogen and low Prog levels induce thickening of the vaginal stratified squamous epithelium (figure 1.5A), whereas high Prog and low estrogen levels are associated with thinning of the vaginal epithelium and vaginal atrophy (figure 1.5B). The latter can result in an increase in the susceptibility of the lower female genital tract to various sexual transmitted infections. As MPA and NET-EN are thought to act similar to Prog, it has been suggested that they should also cause thinning of the vaginal epithelium (Blish and Baeten, 2011). However, no studies have addressed this for NET-EN, while the effect of MPA on vaginal wall thickness appears to be controversial. Some studies have shown a vaginal thinning (Mauck et al., 1999), others have shown an increase in vaginal thickness (Ildgruben et al., 2003), while others show no effect (Cejtin et al., 2003, Richardson et al., 2007, Morrison et al., 2009) on vaginal epithelium thickness. There is evidence in the literature to suggest that genital tract inflammation may be a potential mechanism whereby hormonal contraceptives increase HIV-1 susceptibility (reviewed in (Stringer and Antonsen, 2008, Blish and Beaten, 2011, Roberts et al., 2012a). An increase in the cervical production of the pro-inflammatory cytokines TNF-α, IL-1, IL-6 and IL-8 has been associated with increased levels of HIV-1 shedding in the female genital tract (Gumbi et al., 2008), suggesting that upregulation of pro-inflammatory cytokines in the genital tract, and hence an increase in inflammation, may promote HIV-1 replication and shedding. Whether or not MPA and NET cause increase inflammation, and whether an increase in pro-inflammatory cytokines/chemokines increases HIV-1 infectivity, remains to be determined.

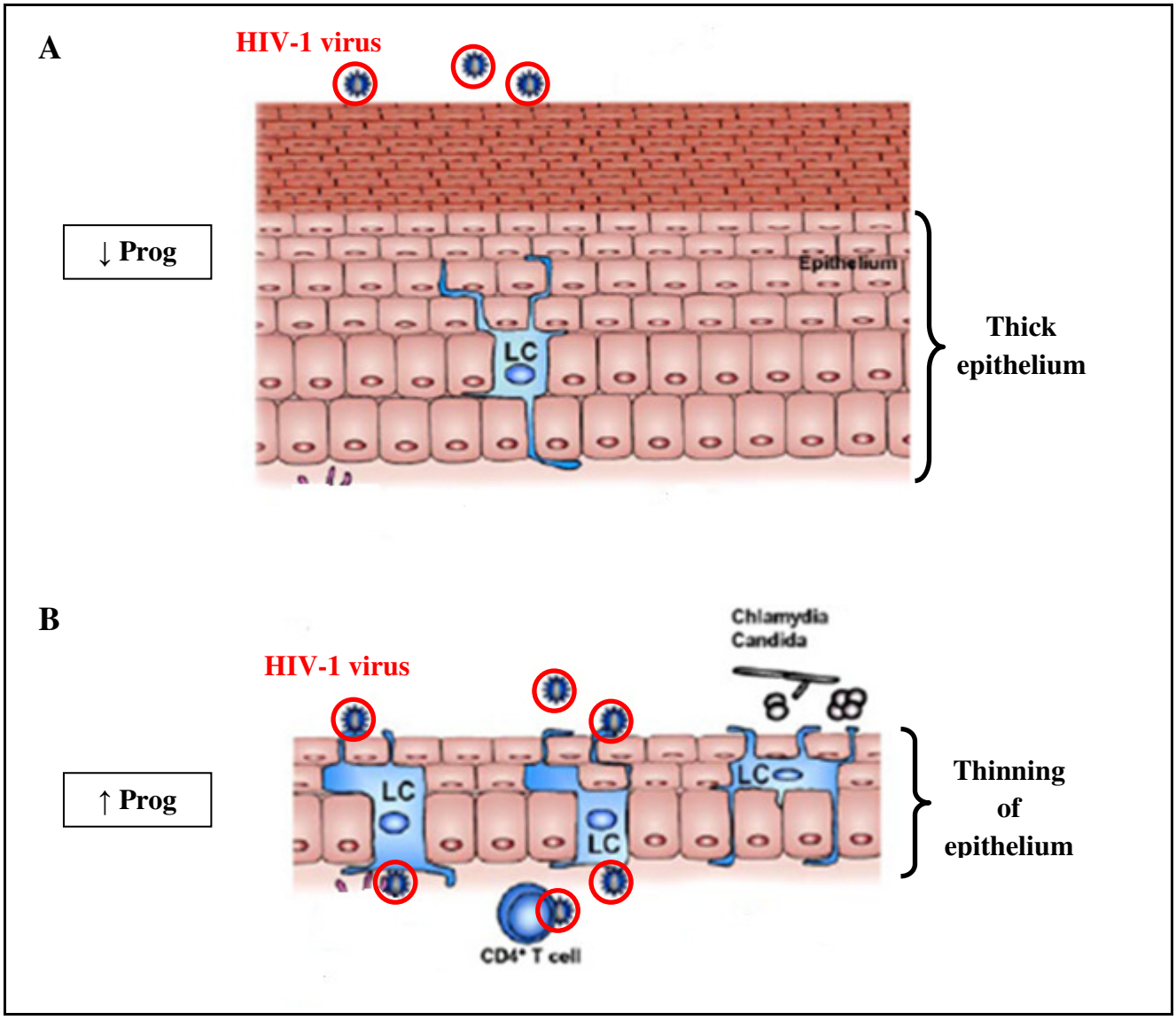

Figure 1.5. Prog affects the thickness and permeability of the vaginal mucosal epithelium. (A) At low Prog concentrations, a thick vaginal epithelium is shown. **(B)** Prog triggers thinning of the vaginal epithelium, and thereby increase susceptibility to infections such as HIV-1, candida and Chlamydia. Adapted from Hel et al., 2010.

1.7. Mucosal immunity in the female genital tract

The epithelial cells lining the lower female genital tract, as part of the mucosal immune system, are the first line of defence against invading pathogens (Wira et al., 2005a, Wira et al., 2010). These cells provide a physical barrier against sexual transmitted infections to protect women without disrupting reproductive functions (Wira et al., 2005a, Wira et al., 2005b). The lower female genital

tract is made-up of three anatomical sites: the endocervix, ectocervix and vagina. The endocervix is lined with simple columnar epithelial cells, while the ectocervix and the vagina consist of multiple layers of stratified squamous epithelial cells (figure 1.6) (Fichorova et al., 1997, Fichorova and Anderson, 1999, Quayle, 2002, Wira et al., 2005a, Wira et al., 2005b).

The majority of women infected with HIV-1 acquire the virus via the female genital tract, which is the primary route for heterosexual transmission of HIV-1 (Cummins and Dezzutti, 2000, Pope and Haase, 2003, Hladik and Hope, 2009, Kaushic et al., 2010). The ectocervix and vagina, as part of the lower female genital tract, is a non-sterile environment and the first portal of entry for pathogens (Fichorova and Anderson, 1999, Quayle, 2002, Wira et al., 2005a, Wira et al., 2005b, Ochiel et al., 2008). In response to invading pathogens, the epithelial cells of the lower female genital tract are capable of evoking a local immune response via the production of cytokines and chemokines which activate and recruit immune cells such as macrophages, natural killer (NK) cells, neutrophils, B-cells and T-cells (Fichorova and Anderson, 1999, Wira et al., 2005b, Wira et al., 2010, Hickey et al., 2011). These cells are also capable of secreting antimicrobial peptides, for example human α- and β-defensins, to provide protection against invading microbes and assist in innate immunity (Wira et al., 2010, Wira et al., 2011). Moreoever, these epithelial cells have been shown to express a variety of toll-like receptors (TLRs) (Fichorova et al., 2002, Wira et al., 2005b, Kaushic et al., 2010) that recognize a wide range of microbial ligands and release intracellular signals which lead to the production of cytokines and chemokines (Fichorova et al., 2002, Zarember and Godowski, 2002, Kaushic et al., 2010).

A number of factors have been shown to influence the immune response in the female genital tract. For example, it has been shown that variations in the levels of sex steroid hormones influence the

function and distribution of various immune cells, such as T cells, B cells, natural killer (NK) cells, monocytes and macrophages (Ansar Ahmed and Talal, 1990, Bouman et al., 2005, Gomez et al., 2009), antigen presentation, the production of antimicrobial peptides such as human beta defensins (HBD), as well as the production of cytokines and chemokines (Wira et al., 2005a, Fahey et al., 2006, Ochiel et al., 2008, Gravitt and Ghanem, 2010, Wira et al., 2010, Wira et al., 2011). Other factors, such as the use of antibiotics, corticosteroids, oral contraceptives and HRT have been shown to increase the susceptibility of women to fungal vaginal infections (Spinillo et al., 1995, Geiger and Foxman, 1996, Magliani et al., 2002).

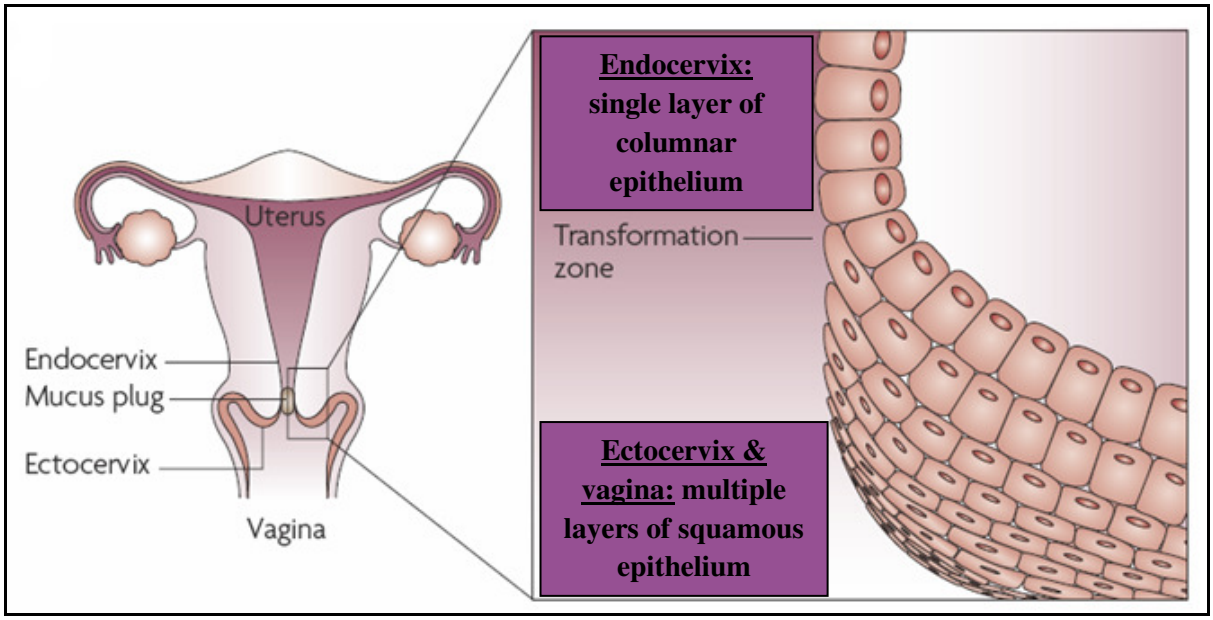

Figure 1.6. An illustration of the human lower female genital tract. The cervix is divided into two parts, the endocervix and the ectocervix, which is separated by a mucus plug. The endocervix forms a sterile canal into the upper genital tract, whereas the ectocervix together with the vagina are non-sterile environments and the portal of entry for pathogens. The type of epithelial cells in each anatomical site is highlighted. (Taken from Hladik and McElrath, 2008).

1.7.1. Cytokine and chemokine milieu in the lower female genital tract

A number of studies have reported that cytokines and chemokines are constitutively secreted by primary epithelial cells of the female genital tract (Shrier et al., 2003, Ochiel et al., 2008, Dubicke

et al., 2010). These include IL-1, IL-2, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), TNF-α and macrophage inflammatory protein (MIP)-1β. Pro-inflammatory cytokines and chemokines such as IL-6, IL-8, IL-12 and RANTES, which stimulate and recruit immune cells to the site of infection, are pivotal for the propagation and progression of inflammation (Altman et al., 1999, Dinarello, 2000, Fernandez and Lolis, 2002, Kayisli et al., 2002, Melchjorsen et al., 2003, Fahey et al., 2005, Wira et al., 2005b, Ochiel et al., 2008). Conversely, to control the course of the inflammatory process, anti-inflammatory cytokines such as IL-10 are released (Opal and DePalo, 2000). In this way homeostasis is maintained to avoid the onset of unfavourable chronic inflammation (Pascual and Glass, 2006).

The cytokine milieu in the vaginal and ectocervical mucosa is a vital determinant of inflammation and most likely susceptibility to infections such as HIV-1 (Gravitt and Ghanem, 2010, Kaushic et al., 2010). As pro- and anti-inflammatory cytokines are suggested to play opposing roles in the pathogenesis, transmission, susceptibility and resistance of HIV-1 (Fichorova, 2004), it is proposed that maintenance of a balance between pro- and anti-inflammatory mediators in the female genital tract is important. Acute inflammation in the female genital tract is considered a beneficial process to provide protection against intracellular pathogens (Hasko and Szabo, 1999, Barousse et al., 2007, Hamza et al., 2010), while chronic inflammation due to enhanced pro-inflammatory cytokine production by the epithelial cells of the female genital tract, and the consequent increase in the recruitment of HIV-1 target cells (e.g. $CD4^+$ T cells and macrophages) into the site of infection, has been suggested to increase HIV-1 susceptibility (Bebell et al., 2008, Gumbi et al., 2008, Nkwanyana et al., 2009, Kaushic et al., 2010, Nazli et al., 2010, Mian and Ashkor, 2011, Roberts et al., 2012a). The pro-inflammatory cytokine, IL-12, and anti-inflammatory cytokine, IL-10, are

known to be key role players during various types of infections, including HIV-1, and regulate the balance between pro- and anti-inflammatory immune responses (Trinchieri and Scott, 1995b, Trinchieri and Gerosa, 1996, Trinchieri, 1998, Schottelius et al., 1999, Leifeld et al., 2002, Pestka et al., 2004, Nemeth et al., 2005, Couper et al., 2008).

1.7.1.1. IL-12

IL-12 is produced during the early events of the immune response in response to pathogens, mainly by immune cells such as monocytes, macrophages, neutrophils and dendritic cells (Trinchieri and Scott, 1995b, Becker et al., 2001, Trinchieri, 2003, Watford et al., 2003, Del Vecchio et al., 2007). However, IL-12 can also be produced by non-immune cells such as epithelial and endothelial cells, infected-keratinocytes and osteoblasts (Aragane et al., 1994, Bost et al., 1999). Secretion of IL-12 induces the activation of NK and cytotoxic T cells, which subsequently induce the production of interferon (IFN)-γ and other pro-inflammatory cytokines, stimulate proliferation of the activated NK and T cells, as well as enhancing the cytotoxic functions of the activated NK and T cells (figure 7) (Kobayashi et al., 1989, D'Andrea et al., 1992, Brunda, 1994, Trinchieri, 2003). In addition to its pro-inflammatory function, IL-12 also acts as an immunoregulator (Trinchieri and Scott, 1995b, Trinchieri and Gerosa, 1996, Trinchieri, 1998).

IL-12 is a 70 kDa heterodimeric glycoprotein consisting of two disulfide-linked subunits, a heavy chain of 40 kDa (IL-12p40) and a light chain of 35 kDa (IL-12p35) (Kobayashi et al., 1989, Stern et al., 1990, Aragane et al., 1994, Trinchieri and Scott, 1995b, Chizzonite et al., 1998, Trinchieri, 2003, Byrnes et al., 2008), which are encoded by two separate genes, located on different chromosomes (Sieburth et al., 1992). Co-expression of the covalently linked IL-12p40 and IL-12p35 in the same cell is required to form the biologically active heterodimer IL-12p70 (Kobayashi

et al., 1989, Hasko and Szabo, 1999). The IL-12p40 gene is regulated at the level of transcription, whereas the IL-12p35 gene is regulated at both the level of transcription and translation (Watford et al., 2003).

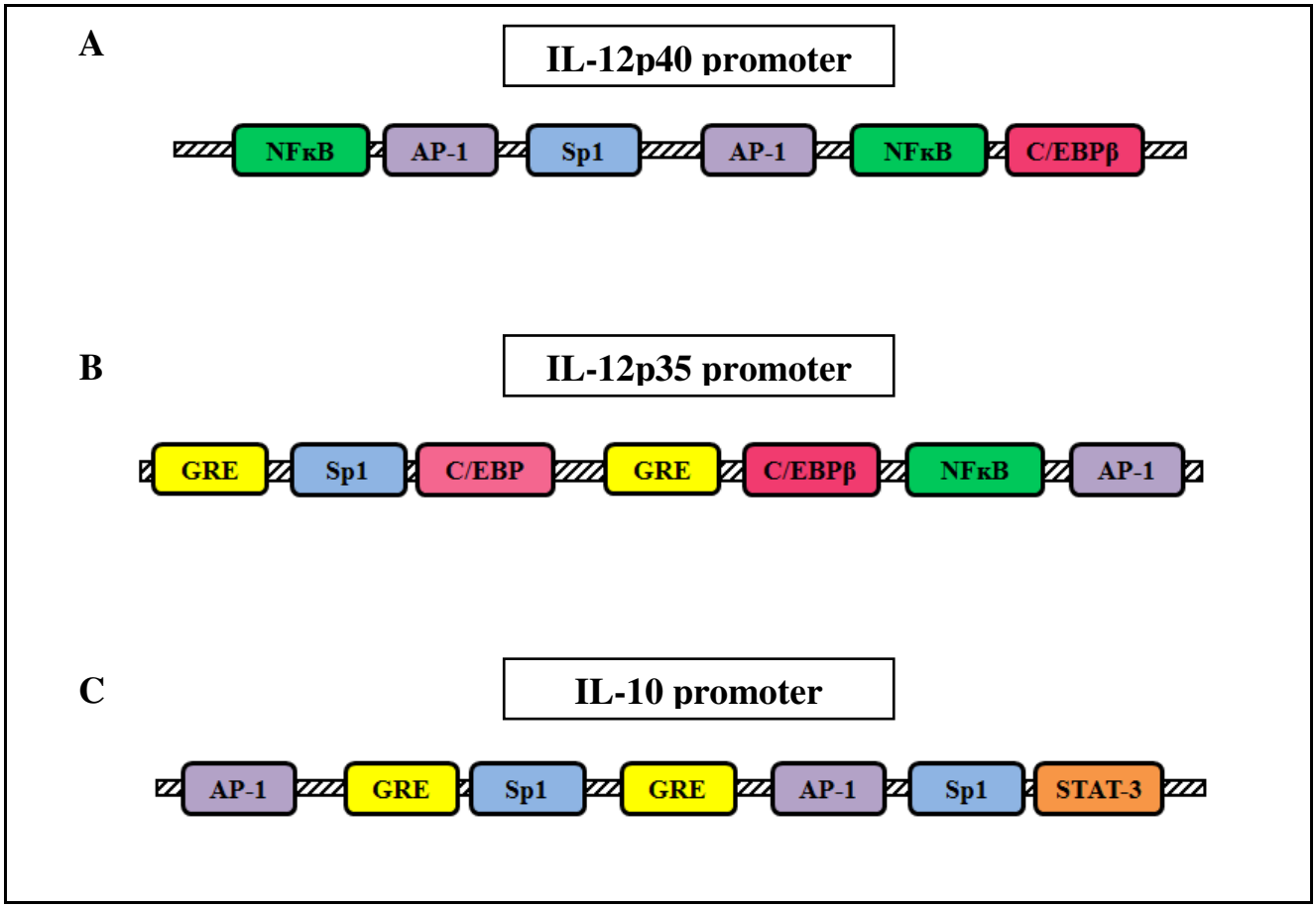

Figure 1.7. Schematic representation of *cis*-regulatory elements found in the promoters of human (A) IL-12p40, (B) IL-12p35 and (C) IL-10.

Human IL-12p40 gene expression can be regulated via several inducible transcription factors binding to specific regulatory elements within the IL-12p40 promoter. These regulatory elements are situated in close proximity to each other (figure 1.7A), and include a C/EBPβ binding site, a NFκB half-site, an AP-1 binding site and specific protein (Sp)-1 sites (Jones and Tjian, 1985, Grimm and Baeuerle, 1993, Murphy et al., 1995, Plevy et al., 1997, Gri et al., 1998, Becker et al.,

2001, Cao et al., 2002, Chambers et al., 2004, Ma et al., 2004). In contrast to the human IL-12p40 promoter, not much is known about the human IL-12p35 promoter. Transcriptional regulation of the human IL-12p35 gene is poorly characterized and appears to be quite complex (Hayes et al., 1998, Goriely et al., 2003). It is speculated that the proximal promoter of the human IL-12p35 gene contains IFN responsive elements, several GREs as well as elements for NFκB, AP-1, Sp1 and C/EBP (figure 1.7B) (Kollet et al., 2001, Mirani et al., 2002).

Despite its protective role, overproduction of IL-12 causes damage to normal tissues (Trinchieri, 1997, Suzuki et al., 2002). To suppress the prolonged or excessive release of IL-12, anti-inflammatory cytokines such as IL-10, IL-4, IL-13, transforming growth factor (TGF)-β, IFN-α, IFN-β and prostaglandin E2 (PGE_2) are produced (D'Andrea et al., 1993, Kubin et al., 1994b, Trinchieri, 1998, Opal and DePalo, 2000, Watford et al., 2003, Del Vecchio et al., 2007, Couper et al., 2008).

1.7.1.2. IL-10

IL-10, originally known as cytokine synthesis inhibiting factor (CSIF) (Fiorentino et al., 1989), is produced by several immune cells such as $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, dendritic cells, lymphocytes, monocytes and macrophages, as well as keratinocytes and epithelial cells (Fiorentino et al., 1989, Ma et al., 2001, Moore et al., 2001, Asadullah et al., 2003, Mocellin et al., 2003). IL-10 functions as a key immunoregulator during infections due to its potent anti-inflammatory and immunosuppressive activities (Schottelius et al., 1999, Pestka et al., 2004, Nemeth et al., 2005, Couper et al., 2008). It exerts its anti-inflammatory effects by inhibiting the activity of Th cells, NK cells and macrophages (Moore et al., 2001, Couper et al., 2008), thereby suppressing the production of pro-inflammatory cytokines, for example IL-6, IL-12, TNF-α and

IFN-γ, as well as chemokines, such as RANTES and IL-8. In addition, IL-10 promotes the release of other anti-inflammatory cytokines (Selzman et al., 1998).

Human IL-10 is a homodimeric protein (de Waal Malefyt et al., 1992, Moore et al., 1993, Pretolani, 1999, Asadullah et al., 2003), which is regulated at the level of transcription by the binding of various inducible transcription factors to regulatory elements within IL-10 promoter (Kube et al., 1995, Benkhart et al., 2000, Mosser and Zhang, 2008, Szalmas et al., 2008). These regulatory elements include (figure 1.7C), Sp1 binding sites, two putative signal transducer and activator of transcription (STAT) binding sites (STAT-1 and STAT-3), a C/EBPβ site, two AP-1 binding sites and GREs (Kube et al., 1995, Ma et al., 2001, Ziegler-Heitbrock et al., 2003, Kremer et al., 2007, Mosser and Zhang, 2008, Unterberger et al., 2008).

1.7.2. HIV-1 entry through the female genital tract epithelium

For HIV-1 to be successfully transmitted during heterosexual intercourse, the virus in the semen has to cross the mucosal barrier of the genital tract in order to infect HIV-1 target cells such as $CD4^+$ T cells, macrophages and/or dendritic cells (reviewed in Hladik and McElrath, 2010; (Bobardt et al., 2007, Kaushic et al., 2010). Evidence in the literature indicate that HIV-1 can bind and infect the stratified squamous epithelial cells of the ectocervix, and subsequently release the virus to infect susceptible leukocytes and $CD4^+$ T cells in the mucosal stoma (Howell et al., 1997, Cummins and Dezzutti, 2000, Maher et al., 2005, Bobardt et al., 2007, Hladik and McElrath, 2008, Gali et al., 2010, Haase, 2010). It has been suggested that local genital tract infection, due to HIV-1 infection of the cervicovaginal epithelium, can lead to the systemic spread of the virus (figure 1.8) (reviewed in Hladik and McElrath, 2008; Haase, 2010; Cummins and Dezzutti, 2000; (Bebell et al., 2008).

However, there has been no conclusive evidence of HIV-1 infection of the genital epithelium *in vivo* (reviewed in Shacklett, 2009 and Haase, 2010).

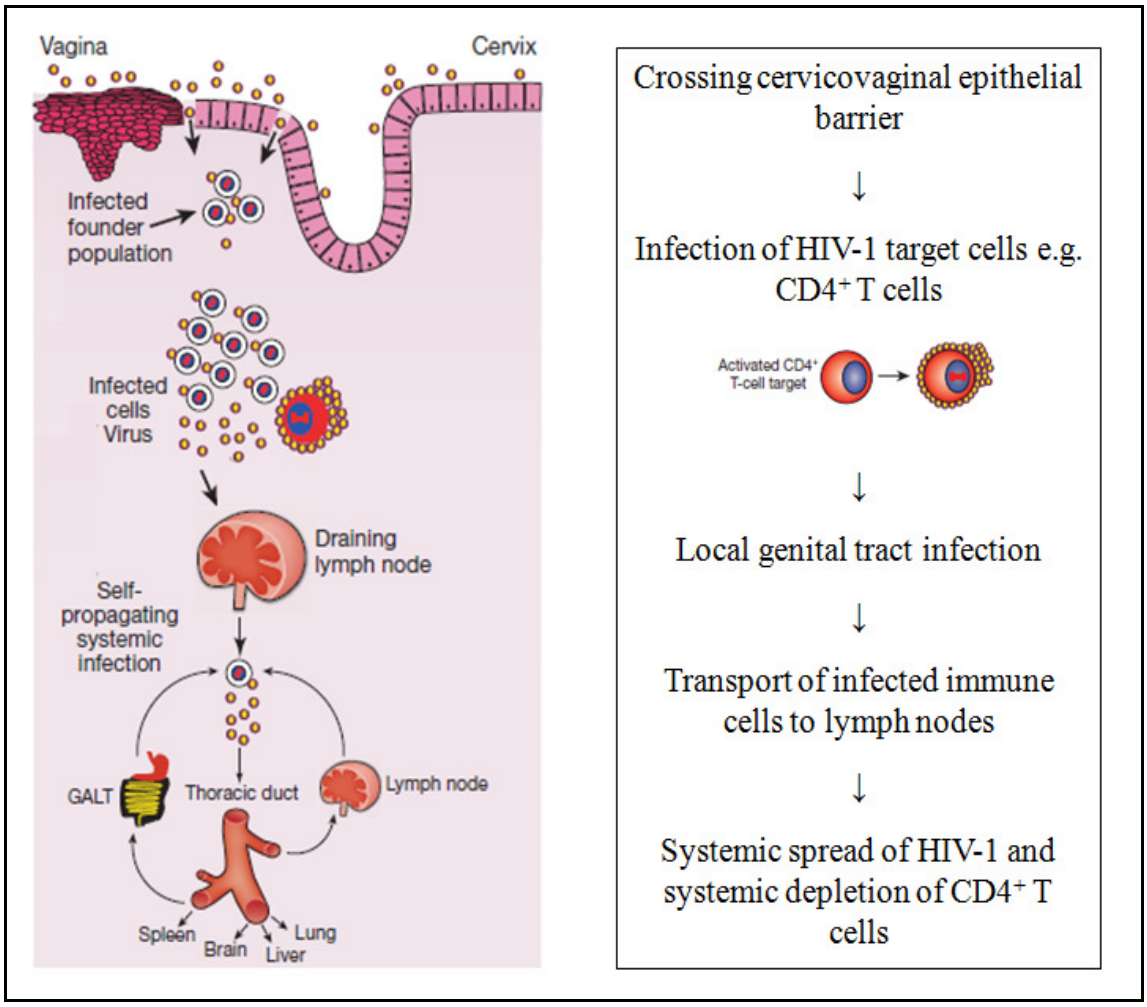

Figure 1.8. Mucosal transmission of HIV-1 via the female genital tract can lead to the systemic CD4$^+$ T cell depletion. Local genital tract infection of HIV-1 has been shown to cause systemic HIV-1 infection. Figure adapted from Haase, 2010.

There are several lines of evidence to suggest that the HIV virus could exploit the host GR for successful pathogenesis and modulation of the host immune function. For example, AIDS patients have decreased innate and T-helper cell-directed immunity and muscle wasting (Kino et al., 2003), which could be explained by glucocorticoid-induced suppression of cytokines regulating the innate and T-helper-driven cellular immune responses (Mirani et al., 2002). One of the key markers of

AIDS is the decrease in the expression of the pro-inflammatory cytokine gene IL-12 in peripheral blood mononuclear cells (PBMCs) (Chehimi and Trinchieri, 1994, Ma and Montaner, 2000, Byrnes et al., 2008). In contrast, IL-10 levels have been reported to increase during HIV-1 infection (Trinchieri and Scott, 1995a, Li and Lau, 2007). Elevated IL-10 protein levels in the female genital tract have been proposed to enhance HIV-1 acquisition (Cohen et al., 1999). On the other hand, it has been suggested that an increase in the production of IL-10 may reduce susceptibility to HIV-1 infection and protect against disease progression (Naicker et al., 2009). A decrease in the IL-12/IL-10 ratio in the epithelial cells of the vagina and cervix could lead to the inability to mount a Th1 immune response, thereby weakening the defense mechanisms of the host against infections (Kelly et al., 1997, Stringer and Antonsen, 2008).

The HIV-1 accessory viral protein R (Vpr) has been shown to affect the transcription of IL-12p35, but not IL-12p40 or IL-10 in human PBMCs (Mirani et al., 2002). Vpr, a small 14 kDa protein consisting of 96 amino acids, is one of fifteen viral proteins encoded by the HIV-1 retrovirus RNA genome (reviewed in Li et al., 2005; (Emerman, 1996b, Le Rouzic and Benichou, 2005, Morellet et al., 2009, Romani and Engelbrecht, 2009) and has been recognised as a vital pathogenic determinant of HIV-1 (Andersen and Planelles, 2005). Deletion of the Vpr gene has been found to yield HIV-1 strains that are not capable of maintaining successful infection in monocytes, thereby dramatically reducing the virulence of HIV-1 (Hattori et al., 1990, Westervelt et al., 1992, Levy et al., 1995). Consistent with this, inhibition of Vpr expression produces HIV-1 strains that decrease HIV-1 replication in monocytes (Balotta et al., 1993).

Exogenous Vpr can invade various cells such as monocytes, macrophages, dendritic cells, resting lymphocytes (Tristem et al., 1992, Heinzinger et al., 1994, Cohen et al., 1996, Emerman, 1996a,

Andersen and Planelles, 2005, Li et al., 2010). This protein has the ability to penetrate cell membranes and could potentially also exert its effects on epithelial cells (Hapgood and Tomasicchio, 2010). In addition to being found inside virions in cells, Vpr has also been detected in the nucleus of infected cells (Andersen and Planelles, 2005), as well as in blood plasma and cerebrospinal fluid of infected individuals (Paxton et al., 1993, Mirani et al., 2002, Le Rouzic and Benichou, 2005, Li et al., 2005). This protein has been shown to have a wide range of effects on the host (reviewed in Romani and Engelbrecht, 2009; (Ayyavoo et al., 1997, Le Rouzic and Benichou, 2005, Li et al., 2005, Varin et al., 2005, Romani and Engelbrecht, 2009, Li et al., 2010). For example, it enhances viral replication and affects transcriptional regulation of several viral promoters, including the HIV-1 LTR promoter, and/or target genes of the host. In addition, Vpr facilitates nuclear translocation of viral DNA as a component of the HIV-1 pre-integration complex, induces host cell arrest in the G2/M phase of the cell cycle, induces apoptosis of infected cells, as well as decreases the antigen-mediated $CD4^+$ and $CD8^+$ response thereby promoting replication of the HIV-1 virus within the host (Kino et al., 1999, Sherman et al., 2000, Ayyavoo et al., 2002, Kino et al., 2002, Mirani et al., 2002, Le Rouzic and Benichou, 2005).

Several studies using *in vitro* cell culture models have described an interaction between Vpr and the human GR (Refaeli et al., 1995, Kino et al., 1999, Muthumani et al., 2006); reviewed in Hapgood and Tomasicchio, 2010). As both Vpr and the host GR have been reported to affect viral replication, as well as transcriptional regulation of host genes (Kino et al., 1999, Sherman et al., 2000, Mirani et al., 2002, Muthumani et al., 2004), it has been suggested that the host GR is used by HIV-1 Vpr to achieve successful pathogenesis (reviewed in Hapgood and Tomasicchio, 2010).

Kino and co-workers have shown that Vpr acts as a co-activator for the GR in the presence of the synthetic glucocorticoid Dex, via binding to the GR co-activator motif LXXLL. Furthermore, Vpr

associates with the co-factors p300/CBP and SRC-1, as well as the transcription factors TFIIB, TFIID and TFIIH to increase the transcriptional activity of the GR (Kino et al., 1999, Kino et al., 2002). In addition, Muthumani and co-workers showed that a GR-Vpr complex is required for Vpr-mediated transcriptional suppression of NFκB (Muthumani et al., 2006). A role for the GR was suggested in mediating some of the effects of Vpr (Schafer et al., 2006), as the GR antagonist mifepristone (RU486) blocked the Vpr-repressed cytokine production and T-cell proliferation.

1.8. Conclusion

The synthetic progestins MPA, NET-EN and NET-A are used by millions of women in contraception, HRT and other therapeutic applications. Although a number of side-effects are associated with the clinical use of these progestins, not much is known about their mechanism of action at the cellular level, particularly with respect to their effects on infections such as HIV. The importance of investigating these mechanisms is highlighted by the clinical evidence showing that the contraceptive use of MPA increases susceptibility to genital tract infections such as HSV-2 and HIV-1 (Clemetson et al., 1993, Mostad et al., 1997, Mostad et al., 2000, Kaushic et al., 2003, Wang, 2004, Morrison et al., 2010, Heffron et al., 2012). Epithelial cells of the female genital tract may serve as the first targets for the initial infection by sexually transmitted HIV-1 (Howell et al., 1997, Cummins and Dezzutti, 2000, Maher et al., 2005, Bobardt et al., 2007, Hladik and McElrath, 2008). Understanding the effects of MPA and NET on local immune function in this environment is likely to play a critical role in understanding the effects of MPA and/or NET on sexually transmitted infections. However, surprisingly little research has been done to investigate the effects of MPA and NET-A on immune function in the female genital tract. Our recent study using human ectocervical and vaginal epithelial cell lines as *in vitro* models for mucosal immunity, showed that Prog, MPA and NET-A differentially regulate the TNF-α induced gene expression of IL-6, IL-8 and

RANTES in a ligand-, promoter- and cell-specific manner (Africander et al., 2011a). Clearly more research is needed to investigate the relative effects of MPA and NET-A on immune mediators in the female genital tract, in particular target genes involved in the inflammatory process. This may shed light on the molecular events occurring during infections and may assist with the design of new progestins with fewer side-effects.

1.9. Hypothesis and Aims of study

Little is known about the molecular mechanism of the synthetic progestins MPA and NET-A in the lower female genital tract, specifically their role on local ectocervical epithelial immune function and risk of susceptibility to infections. However, previous studies have indicated differential regulation of pro-inflammatory genes by MPA and NET-A. To this end, the primary hypothesis of this thesis was that these progestogens would differentially regulate the cytokine gene expression of IL-12 and IL-10 in the cervical mucosa as part of the lower female genital tract. Furthermore, it was hypothesised that the HIV-1 accessory Vpr would modulate the effects of MPA and/or NET-A on IL-12 and IL-10 gene expression in the female genital tract.

Using an immortalised human ectocervical epithelial (Ect1/E6E7) cell line from the ectocervix, shown to constitutively express a wide variety of pro- and anti-inflammatory mediators which are upregulated in response to TNF-α (Fichorova and Anderson, 1999, Steele and Fidel, 2002), as an *in vitro* model system for mucosal immunity, the aims of this project were three-fold:

Firstly, the regulation of the TNFα-induced pro-inflammatory cytokines IL-12p40 and IL-12p35, as well as the anti-inflammatory cytokine, IL-10, in response to the endogenous hormone Prog, and the synthetic progestins, MPA and NET-A, was investigated. Considering that the GR is known to play a role in inflammation and that Prog, MPA and NET-A can bind the GR, we next investigated

a role for the GR in mediating the effects of these ligands by re-evaluating the regulation of IL-12p40 and IL-10 gene expression when the endogenous GR protein levels were reduced.

Secondly, the mechanism by which the GR mediates the effects of Prog, MPA and NET-A on IL-12p40 and IL-10 gene expression was further examined by investigating which *cis*-regulatory elements within these promoters are required for the responses, as well as the transcription factor(s) involved.

Thirdly, the possible modulation of the progestogen-induced regulation of IL-12p40, IL-12p35 and IL-10 gene expression by HIV-1 accessory protein Vpr was investigated. Furthermore, a putative role of the GR was investigated as it has previously been suggested that the host GR can be exploited by HIV-1 Vpr to achieve successful pathogenesis.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell culture

The human ectocervical epithelial cell line (Ect1/E6E7) immortalised with the E6 and E7 genes of the human papilloma virus 16 (HPV-16) was purchased from American Type Culture Collection (ATCC, USA). This cell line was maintained in keratinocyte serum-free medium (KSFM) (GibcoBRL, Paisley, UK or Sigma-Aldrich, South Africa) supplemented with 50 µg/ml bovine pituitary extract (BPE) (GibcoBRL, Paisley, UK or Sigma-Aldrich, South Africa), 0.1 ng/ml human epidermal growth factor (EGF) (GibcoBRL, Paisley, UK or Sigma-Aldrich, South Africa), $CaCl_2$ to a final concentration of 0.4 mM, 100 IU/ml penicillin and 100 µg/ml streptomycin (penicillin-streptomycin) (Gibco-BRL Life Technologies, United Kingdom). These cells were maintained at 37°C in 75 cm^2 culture flasks (Greiner Bio-One International, Austria), at 90% humidity and 5% CO_2. These cells were passaged when a confluency of ~60 % was reached, and detached from the flask surface by the addition of trypsin (Highveld Biologicals, South Africa) for 10 minutes. The proteolytic activity was inhibited by adding 5 ml neutralisation medium (Dulbecco's Modified Eagle's Medium (DMEM)/F-12 nutrient mixture (Ham) (1:1) (Gibco-BRL Life Technologies, United Kingdom), 10% (v/v) fetal calf serum (FCS) (Sigma-Aldrich, South Africa) and 1% penicillin-streptomycin. Thereafter the cells were pelleted by centrifugation at 1 000 rpm for 5 minutes. The pellet was subsequently resuspended in supplemented KSFM (see above) and maintained in 75 cm^2 culture flasks as described above. Ect1/E6E7 cells were regularly tested for Mycoplasma infection by means of Hoechst staining (Freshney RI, 1987) and only Mycoplasma-negative cells were used in experiments. A representative photograph of mycoplasma-negative ect1/E6E7 cells are shown in addendum A (figure A.1).

2.2. Test compounds

Prog (4-pregnene-3, 20-dione), MPA (6α-methyl-17α-hydroxy-progesterone acetate), NET-A (17α-ethynyl-19-nortesterone 17β-acetate), Dex (11β, 16α-9-fluoro-11, 17, 21-trihydroxy-16-methylpregna-1, 4-diene-3, 20-dione), cortisol (11β, 17α, 21-trihydroxy-4-pregnene-3, 20-dione), dihydrotestosterone (DHT; 5α-androstan-17β-ol-3-one), and β-estradiol (E_2; 17β-estra-1, 3, 5 (10)-triene-3, 17-diol) were purchased from Sigma-Aldrich, South Africa. NET-A, the acetate, was used as it is soluble in water compared to the insoluble ester, NET-EN. *In vivo*, both NET-A and NET-EN are hydrolysed to NET and its metabolites (Stanczyk and Roy, 1990). Mibolerone (Mib; 17β-hydroxy-7α, 17α-dimethylestr-4-en-3-one) was purchased from PerkinElmer Life and Analytical Science, South Africa. Stock solutions of all the test compounds were prepared in 100% ethanol and stored in light-protective screw cap glass vials at -20°C. These compounds were added to serum-free culturing medium to obtain a final ethanol concentration of 0.1%. The cells were incubated with 0.1% ethanol to serve as control. [^{3}H]-Dex (82.8 Ci/mmol) and [^{3}H]-E_2 (110 Ci/mmol) were purchased from AEC-Amersham (South Africa). Tumor necrosis factor-alpha (TNF-α) was purchased from Sigma-Aldrich, South Africa and used at a final concentration of 0.02 µg/ml.

2.3. Plasmids

The plasmid expressing the human GR, pRS-hGRα, was received as a gift from Prof. R. Evans (Havard Hughes Medical Institute, La Jolla, United States of America). Plasmids expressing the human AR, pSV-ARo (previously described by Brinkmann *et al.* 1989), as well as the human PR isoforms A and B, pSG5-hPRA and pSG5-hPRB respectively (previously described by Kastner *et al.* 1990), were a kind gift from Prof. Frank Claessens (University of Leuven, Leuven, Belgium). The plasmids expressing the human ERα (pSG5-ERα) and β (pSG5-ERα) isoforms, respectively, were received from Prof. Frank Gannon (European Molecular Biology Laboratory, Germany),

while the pCMV4-3HA.Vpr plasmid, previously described by Sherman *et al.,* 2000, was received from Dr Warner Greene (University of California, San Francisco). The following promoter-reporter constructs were used: the plasmid pTAT-2xGRE-E1b-luciferase, driven by the E1b promoter and containing two copies of the rat TAT-GRE (described in (Sui et al., 1999) was a kind gift from Dr. G. Jenster (Erasmus University of Rotterdam, Netherlands) and 5x NFκB-luciferase was purchased from Strategene (Houston, Texas, USA). Depending on which steroid receptor was transfected, two different β-galactosidase (β-gal) plasmids were used to correct for the transfection efficiencies: the cytomegalovirus (CMV)-driven β-gal (received from Prof Guy Haegeman) and pSV-driven β-gal (Promega, Madison, USA). The pGL2basic vector (Promega, Madison, USA) was used as a filler plasmid. Plasmid DNA was purified using either the EndoFree® Plasmid Maxi Kit (Qiagen GmbH, Germany) or the PureYield™ Plasmid Maxiprep System kit (Promega, Madison, USA) according to the manufacturer's instructions. Except for the pRS-hGRα and pCMV4-3HA.Vpr plasmids, all other plasmids were only used in Addendum C.

2.4. Promoter-reporter assays

For transactivation assays, the Ect1/E6E7 cell line was maintained as above and seeded into 24-well tissue culture plates (Greiner Bio-One International, Austria) at 5 x 10^4 cells per well. On day 2, the cells were transiently transfected with 300 ng of the promoter-reporter construct pTAT-2xGRE-E1b-luc, 30 ng of the expression vector for the hGR, hAR or the filler plasmid pGL2basic, and 30 ng β-gal, using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions. pSV-β-gal was co-transfected with the hGR, while pCMV-β-gal was co-transfected with the hAR. On day 3, the cells were incubated with 0.1% ethanol (control), 10 μM Dex (for GR) or Mib (for AR), respectively.

For transrepression assays, the human Ect1/E6E7 cell line was maintained as above and seeded into 24-well tissue culture plates at 5 x 10^4 cells per well. On day 2, the cells were transiently transfected using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions as follows: For the GR: 100 ng NFκB-luc, 50 ng pGL2basic and 25 ng pSV-βgal. For the AR: 400 ng NFκB-luc, 70 ng pGL2basic and 70 ng pCMV-βgal. On day 3, the cells were incubated with 0.02 µg/ml TNF-α in the absence (0.1% ethanol) and presence of 10 µM Dex (GR) or Mib (AR), respectively, for 24 hours.

The cells were subsequently washed once with 1x PBS, harvested in 50 µl passive lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) TRIS-phosphate-EDTA and 1.44 mM EDTA) per well and then incubated for 15 minutes at room temperature, while shaking. The lysed cells were subsequently thawed and 10 µl of each lysate was used to determine the luciferase and β-galactosidase activities in black 96-well cliniplates (Thermo Scientific, Finland). The luciferase (Promega Luciferase Assay System, Madison, USA) as well as the β-gal assays (Galacto-Star™ assay system, Applied Biosystems, USA) were performed in a Veritas microplate luminometer. The amount of luciferase and β-gal present in each sample are expressed as relative light units (RLU). To correct for variances in transfection efficiencies the luciferase activity was normalised to the constitutively expressed β-gal activity. See Addendum C for promoter-reporter assay results.

2.5. Whole cell binding assays

Competitive whole cell binding assays were performed essentially as described by Bamberger et al. (1995), with the following modifications (Koubovec et al., 2005). To confirm binding of the test compounds used in this study to the endogenously expressed GR in the human Ect/E6E7 cell line, cells were seeded into 24-well tissue culture plates (Greiner Bio-One International, Austria) at a

density of 1 x 10^5 cells per well. On day 3, the cells were washed three times with 1xPBS, pre-warmed to 37 °C, and incubated for 6 hours at 37°C with 10 nM ^{3}[H]-Dex (82.8 Ci/mmol) (AEC-Amersham, South Africa), in the absence (total binding) and presence of 1 µM unlabelled (non-specific binding) Dex, Prog, MPA, NET-A or cortisol, respectively. To determine the endogenous ER levels in the human Ect1/E6E7 cell line, cells were seeded into 12-well tissue culture plates (Greiner Bio-One International, Austria) at a density of 2 x 10^5 cells per well. On day 3, the cells were washed three times with 1x PBS, pre-warmed to 37°C, and incubated for 2 hours at 37°C with 10 nM ^{3}[H]-E_2 (110 Ci/mmol) (AEC-Amersham, South Africa), in the absence (total binding) and presence of 10 µM unlabelled E_2 (non-specific binding). Working on ice at 4°C, all cells were washed three times, for 15 minutes, with ice-cold 1x PBS containing 0.2% (w/v) bovine serum albumin (BSA) to remove any unbound ligand. For the GR, cells were lysed with 100 µl reporter lysis buffer (Promega, Madison, USA) at room temperature for 15 minutes with agitation, while 200 µl reporter lysis buffer was used for the ER. Thereafter, the lysate was transferred to scintillation vials containing 1.5 ml scintillation fluid (Quickszint FLOW 2, Zinsser Analytic, South Africa) and the total binding measured by scintillation counting as counts per minute (cpm) using a Tri-Carb 2810 scintillation counter. Specific binding (total binding minus non-specific binding) was normalised to the protein concentration, determined by using the Bradford protein assay method (Bradford, 1976). The reported values are averages of three independent assays, with each condition performed in triplicate.

The ER numbers in *f*mol/mg protein were determined as follows:

The specific activity of the 3[H]-E_2 (Ci/mmol) was converted to disintegrations per minute (dpm) per mmol, by multiplying the Ci/mmol by a factor of 2.22 x 10^{12} (1 Curie equals 2.22 x 10^{12} dpm). The dpm/mmol was multiplied by the counting efficiency (40% in our system) to get cpm/mmol,

and then divided by 10^{12} to get specific activity in cpm/$f$mol. The specific binding cpm value was then divided by this value to give the specific binding in $f$mol. To yield the number of binding sites in $f$mol/mg protein, the specific binding ($f$mol) was divided by the protein content of the sample in mg. The ER binding data are shown in Addendum C.

2.6. Western blotting

The human Ect1/E6E7 cell line was maintained as above and plated into either 24-well- (1.5 x 10^5 cells per well), 12-well- (1 x 10^5 cells per well) or 6-well tissue culture plates (Greiner Bio-One International, Austria) (2.5 x 10^5 cells per well). For positive controls, COS-1 cells (1 x 10^5 cells per well in a 12-well plate) were transiently transfected with 250 ng DNA of a particular steroid receptor expression vector, while untransfected COS-1 cells were used as a negative control. The cells were washed with ice cold 1x PBS and lysed with 2x SDS-polyacrylamide gel by electrophoresis (PAGE) loading buffer (100 mM TRIS-HCl, pH6.8, 20% (v/v) glycerol, 5% (w/v) SDS, 0.1% (w/v) bromphenol blue, and 2% (v/v) β-mercaptoethanol) (Sambrook et al., 1989). All lysates were denatured by boiling for 10 minutes at 97°C. Protein samples were separated on a 10% SDS-PAGE at 200 V for 45 minutes using a 1xSDS-PAGE buffer (35 mM SDS, 250 mM TRIS, 1.92 M glycine). Following electrophoresis, the proteins were electroblotted at 180 mA for 1 hour onto an Immobilon®-P transfer polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, USA) using ice-cold 1x transfer buffer (25 mM TRIS, 192 mM glycine, 10% (v/v) methanol). The membranes were then blocked for 90 minutes in 10% (w/v) fat-free milk powder in 1x TRIS buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBST). Thereafter the membranes were probed with the antibodies specific for the GR (H-300), AR (441), PR-A/B (AB-52), PR-B (B-30), MR (H-300), ERα (M-20), ERβ (H-150), C/EBPβ (C-19), STAT-3 (C-20), GAPDH (0411), Hsp90α/β (H-114) (Santa Cruz Biotechnology Inc., Europe), hemagglutinin (HA) protein of human

influenza virus (12CA5, Roche Applied Biotechnology Inc., USA), or β-actin (4967, Cell Signalling Technology, Massachusetts, USA) (Table 2.1) for 16 hours at 4°C. GAPDH, Hsp90 and β-actin were used as loading controls. The membranes were subsequently washed with TBST, once for 15 minutes and three 5 minute washes before incubating with HRP-conjugated secondary antibodies (goat anti-rabbit or anti-mouse) (Santa Cruz Biotechnology Inc., CA, USA) for 90 minutes at room temperature. The membranes were washed again as indicated above, and then visualised using Pierce® ECL Western blotting substrate (Thermo Scientific Inc., USA) and ECL Hyperfilm (GE Healthcare Amersham, USA) or medical X-ray film (Africa X-Ray Industrial and Medical (Pty) LTD., South Africa). All the antibody dilutions used in this study are summarised in Table 2.1. Western Blots showing the expression of steroid receptors in the human ectocervical epithelial cell line, Ect1/E6E7, are shown in Addendum C.

2.7. Isolation of total RNA and complementary DNA (cDNA) synthesis

The human Ect1/E6E7 cell line was plated in 12-well tissue culture plates (Greiner Bio-One International, Austria) at a density of 1 x 10^5 cells per well. Cells were incubated with test compounds as described in the figure legends and the total RNA was isolated from the cells using Tri-reagent (Sigma-Aldrich, South Africa). Briefly, the cells were lysed by adding 400 µl Tri-reagent to the cells, transferring the lysates to 1.5 ml microcentrifuge tubes and incubating for 5 minutes at room temperature. Thereafter, 80 µl chloroform was added and the mixture was vortexed for 1 minute. Samples were incubated for 2-3 minutes at room temperature before centrifugation at 12 200 g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube and an equal volume of ice-cold isopropanol was added. Samples were vortexed for 15 seconds and incubated for 15 minutes at room temperature, followed by centrifugation at 12 200 g for 10 minutes at 4°C to

Table 2.1. Primary and secondary antibody dilutions used for Western blotting.

Primary antibody specific for:	Dilution	HRP-conjugated Secondary Antibody	Dilution
GR	1:4 000	goat anti-rabbit	1:10 000
AR	1:1 000	goat anti-mouse	1:5 000
PR-A/B	1:500	goat anti-mouse	1:2 000
PR-B	1:500	goat anti-mouse	1:2 000
MR	1:200	goat anti-rabbit	1:2 000
ERα	1:200	goat anti-rabbit	1:5 000
ERβ	1:1 000	goat anti-rabbit	1:1 000
HA	1:400	goat anti-mouse	1:1 000
C/EBPβ	1:1 000	goat anti-rabbit	1:2 000
STAT-3	1:1 000	goat anti-rabbit	1:4 000
GAPDH	1:2 000	goat anti-mouse	1:4 000
Hsp90	1:5 000	goat anti-rabbit	1:2 000
β-Actin	1:1 490	goat anti-rabbit	1:10 000

pellet the RNA. The pellets were washed with 500 µl 75% (v/v) ethanol (100% ethanol diluted in diethyl pyrocarbonate (DEPC) water). Samples were vortexed (±1 min) and centrifuged at 8 000 g for 5 min at 4°C. The supernatant was aspirated and the RNA pellets were allowed to air dry for 5-10 minutes on ice. RNA was dissolved in 15 µl DEPC-treated water. The RNA concentration was measured on a NanoDrop (ND-100 Spectrophotometer) and the 260 nm/280 nm ratio was

determined. The integrity of the 28S and 18S ribosomal bands was confirmed on 1% denaturing formaldehyde agarose gels. A representative gel picture is shown in addendum B (figure B.1.1). RNA samples were stored at -80°C.

Total RNA extracted from the Ect1/E6E7 cell line was reverse transcribed using the transcriptor first strand cDNA synthesis Kit (Roche Applied Science, South Africa) according to the manufacturer's instructions. Briefly, 1 µg total RNA, 2.5 µM anchored-oligo$(dT)_{18}$-primer and PCR-grade water, if needed, was added to a final volume of 13 µl. This template-primer mixture was incubated for 10 minutes at 65°C to denature the RNA secondary structures and immediately placed on ice. Then, 1x transcriptor reverse transcriptase reaction buffer, 20 U protector RNase inhibitor, 1 mM deoxynucleotide (dNTP) mix and 10 U transcriptor reverse transcriptase was added. The samples were subsequently incubated for 1 hour at 50°C, after which the reaction was stopped by placing the samples at 85°C for 5 minutes to inactivate the transcriptor reverse transcriptase. The cDNA samples were stored at -20°C until needed.

2.8. Realtime quantitative polymerase chain reaction (qPCR)

Realtime qPCR was performed by using a Light-Cycler, rapid thermal cycler system (Roche Applied Science, South Africa) according to the manufacturer's instructions. Reagents in the LightCycler-FastStart DNA Master$^{non\text{-}plus}$ SYBR Green I system (Roche Diagnostics, South Africa) were used. The PCR reaction mixture was prepared by adding the forward and reverse primers (final concentration of 0.5 µM each), $MgCl_2$ (final concentration of 3 mM for both IL-12p40 and IL-10, 2 mM for IL-12p35 or 4 mM for GAPDH), LightCycler SYBR green I non-plus master mix and PCR-grade water to a final volume of 9 µl per sample. Thereafter, 1 µl cDNA template or PCR water (negative control) was added. All samples were incubated at 95°C for 10 minutes to activate

the Roche FastStart Taq polymerase, followed by denaturation at 95°C for 10 seconds. The annealing temperatures for 10 seconds are indicated in Table 2.2, and this was followed by extension at 72°C for 10 seconds (for all genes except GAPDH). For GAPDH, the thermal profile was denaturation at 95°C for 10 seconds, annealing at 59°C (Table 2.2) for 10 seconds and extension at 72°C for 12 seconds. Typically 45 repeats of the three PCR steps were used. Agarose gel electrophoresis and melting curve analysis were performed to confirm the presence of a single amplicon, as well as the amplicon size. A cDNA dilution series was used to generate a standard curve so as to determine the amplification efficiency of each primer set (Table 2.2). The relative transcript levels of the target genes were calculated using the mathematical model described by Pfaffl, 2001, and normalised to the relative transcript levels of GAPDH.

Table 2.2. Primer sequences and annealing temperatures for all genes investigated.

Gene	Primers (5' - 3')	Strand	Length of amplicon (bp)	Annealing temperature (°C)	Primer efficiency	Reference
IL-12p40	CCACATTCCTACTTCTCCC CTTGTCCGTGAAGACTCTAT	Forward Reverse	87	51	2.39	Designed by Roche Diagnostics, South Africa
IL-12p35	TGATGAGCTGATGCAGGC ATCCGGTTCTTCAAGGGAG	Forward Reverse	73	56	2.80	Designed by Roche Diagnostics, South Africa
IL-10	AAAGGCATCTACAAAGCCA TTGTCATGTAGGCTTCTATGTAGT	Forward Reverse	67	55	2.27	Designed by Roche Diagnostics, South Africa
GAPDH	TGAACGGGAAGCTCACTGG TCCACCACCCTGTTGCTGTA	Forward Reverse	307	59	1.86	Ishibashi et al. (2003)

2.9. Small interference RNA (siRNA) transfection

The human Ect1/E6E7 cell line was plated in 12-well tissue culture plates at a density of 1 x 10^5 cells per well. On day 2, the cells were transfected with 10 nM validated non-silencing scrambled sequence control (NSC) siRNA (Qiagen, USA) or GR HS_NR3C1_6 siRNA (cat # S102654764, Qiagen, USA) or GR_HS_NR3C1_5 siRNA (cat# S102654757, Qiagen, USA) directed against human GR, or C/EBPβ siRNA (sc-29229; Santa Cruz Biotechnology, USA) directed against human C/EBPβ, or STAT-3 siRNA (sc-29493; Santa Cruz Biotechnology, USA) directed against human STAT-3, using HiPerfect transfection reagent (Qiagen, USA) according to the manufacturer's instructions. Briefly, GR, C/EBPβ, STAT-3 or NSC siRNA was diluted in pre-warmed Optimem medium with GlutaMAX^{TM} (GibcoBRL, Paisley, UK), to which 2.95 µl transfection reagent was added. The transfection mixture was incubated at room temperature for 10 minutes and then added drop-wise directly to the cells. After 24 hours, the cells were treated with 0.02 µg/ml TNF-α and either 0.1% ethanol (control) or 1 µM test compound for 6 hours. For experiments determining the role of the GR in mediating effects of Vpr, cells were transiently transfected with 1 µg pCMV4-3HA.Vpr, 24 hours later, using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, South Africa) according to the manufacturer's instructions. RNA was harvested, cDNA synthesised and IL-12p40, IL-12p35 and IL-10 gene expression was quantified by realtime qPCR as described in Sections 2.7 and 2.8, respectively. Reduction in the protein levels were confirmed by Western blot analysis, as described in Section 2.6.

2.10. Multiplex bead array and ELISA

The human Ect1/E6E7 cell line was plated in 12-well tissue culture plates (Greiner Bio-One International, Austria) at a density of 1 x 10^5 cells per well for the Multiplex bead array, and in 6-well tissue culture plates at a density of 2.5 x 10^5 cells per well for the ELISA. The Ect1/E6E7 cells

were transfected with 10 nM GR6 or NSC siRNA, respectively, and after 24 hours either left untreated (–Vpr) or transiently transfected with 1 µg pCMV4-3HA.Vpr (+Vpr), and incubated for a further 24 hours. The cells were subsequently treated with 0.02 µg/ml TNF-α and 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 24, 48 or 96 hours. The supernatants were collected, and protease inhibitors (1x Complete Mini Protease Inhibitor Cocktail; Roche Applied Science, South Africa) were added.

The human 8-plex luminex assay (Millipore, Missouri, USA) was used to simultaneously quantify the protein levels of IL-6, IL-8, IL-10, IL-12p40, IL-12p70, macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES in the Ect1/E6E7 cell culture supernatants according to the manufacturer's instructions. All samples, as well as two quality controls included in the kit, were analysed in duplicate. The levels of all the analytes in the quality controls were within the expected ranges. A standard curve ranging from 3.2 pg/ml to 10 000 pg/ml was used for all the cytokines, and Bio-Plex Manager Software, version 4.1.1., was used to analyse the data.

The protein levels of IL-12p40 and IL-10 in the cell culture supernatants were quantified by ELISA, using the human IL-12/IL-23 (p40) ELISA Max Deluxe set (Biolegend, USA) and human IL-10 ready-set-go kit (eBioscience, USA), respectively, according to the manufacturer's instructions. All samples were analysed in duplicate. The standard curve ranged from 62.5 pg/ml to 4 000 pg/ml for IL-12p40, and 2 pg/ml to 300 pg/ml for IL-10. All standards were analysed in triplicate.

2.11. Co-immunoprecipitation (Co-IP) assay

Co-IP assays were performed as described by Avenant et al. (2010), with the following modifications. The human Ect1/E6E7 cell line was plated in 10 cm^2 tissue culture dishes at a

density of 2 x 10^6 cells. After 48 hours, the cells were incubated with 0.02 µg/ml TNF-α and 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 1 hour. The cells were washed twice with cold PBS and harvested in 500 µl radio-immunoprecipitation (RIPA) buffer (50 mM TRIS-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing protease inhibitors (1x Complete Mini Protease Inhibitor Cocktail; Roche Applied Science, South Africa). After two cycles of freeze-thaw, the cells were collected, centrifuged at 16 000 g for 10 minutes at 4°C, and the supernatants collected. The input samples were prepared by adding 10 µl 2xSDS-PAGE loading buffer (Sambrook et al., 1989) to 20 µl of the supernatant, and samples stored at – 20°C. The remaining lysate was pre-blocked to reduce the non-specific binding of proteins to the protein A/G PLUS beads. This was done by adding 5 µg anti-IgG and 20 µl 50:50 (v/v) pre-blocked protein A/G PLUS beads (sc-2003, Santa Cruz Biotechnology, USA) to the supernatant and incubating for 1 hour at 4°C on a rotating wheel. These pre-blocked protein A/G PLUS beads were prepared by incubating 500 µl of the beads with 2 ml IP dilution buffer (see above), salmon sperm DNA (final concentration 0.2 mg/ml) and bovine serum albumin (BSA) (final concentration 1 mg/ml) for 1 hour at 4°C on a rotating wheel. This was followed by centrifugation at 1 500 g for 5 minutes at 4°C, and the beads were subsequently resuspended as a 50% slurry in IP dilution buffer containing protease inhibitors (see above). The pre-blocked samples were centrifuged at 14 000 g for 10 minutes at 4°C and the supernatant collected for immunoprecipitation. The Co-IP samples were prepared by adding 2 µg anti-GR antibody (H-300, Santa Cruz Biotechnology Inc., USA) to the pre-cleared supernatants, followed by incubation overnight at 4°C on a rotating wheel. The following day, 20 µl 50:50 (v/v) pre-blocked protein A/G PLUS beads were added to the mixture and incubated for 2 hours at 4°C on a rotating wheel. The samples were then centrifuged at 1 000 g for 1 minute at 4°C and the beads were washed twice with cold PBS. Thereafter, 30 µl 2xSDS-PAGE loading buffer was added to the beads, and samples

boiled for 5 minutes, before centrifugation at 16 000 g for 10 minutes at room temperature to collect the supernatant. The immuneprecipitates were subjected to electrophoresis on a 10% SDS polyacrylamide gel Western blot analysis was performed as described in Section 2.6.

2.12. Chromatin immunoprecipitation (ChIP) and re-ChIP assays

2.12.1. Treatment of Ect1/E6E7 cells

To determine whether the GR is recruited to the endogenous IL-12p40 and IL-10 promoters in the human Ect1/E6E7 cell line, the basic method described by Ma and co-workers (2003) was followed with a few modifications (Avenant et al., 2010a, Verhoog et al., 2011). The human Ect1/E6E7 cell line was seeded in 14 cm^2 tissue culture dishes at a density of 1 x 10^7 cells per dish. After 72 hours, the supplemented KSFM was aspirated and replaced with unsupplemented KSFM. Following 24 hours, the cells were treated with 0.02 µg/ml TNF-α and 0.1% ethanol (control) or 1 µM Prog, MPA, NET-A or cortisol for 2 hours.

2.12.2. Formaldehyde cross-linking, lysing of cells and DNA fragmentation

Following the 2 hour incubation period, 37% formaldehyde was added directly to the medium to a final concentration of 1%, and incubated for 10 minutes at 37°C to allow for cross-linking of the proteins and the chromatin. To stop the cross-link, glycine to a final concentration of 0.125 M was added to the mixture, while shaking and incubated, for 5 minutes at room temperature. The cells were subsequently washed twice with ice-cold PBS, scraped and harvested in PBS containing protease inhibitors (1x Complete Mini Protease Inhibitor Cocktail tablet; Roche Applied Science, South Africa), followed by centrifugation at 1 200 g for 10 minutes at 4°C. The cell pellets were resuspended in 500 µl nuclear lysis buffer (1% (w/v) SDS, 50 mM TRIS-HCl (pH 8.0), 10 mM EDTA and 1 tablet 1x Complete Mini Protease Inhibitor Cocktail per 10 ml). Using the Misonix

sonicator (Qsonica, South Africa), the cell lysates were sonicated on 75% power for 50 cycles at 30 seconds per cycle, with 30 second intervals between pulses, causing fragmentation of the DNA to sizes of between 100 and 500 bp. A representative gel picture is shown in addendum A (figure B.2.1). The sonicated chromatin was centrifuged at 15 000 g for 10 min at 4°C to pellet the cell debris. The supernatant was transferred to a clean microcentrifuge tube and the amount of A_{260} units/µl in the sonicated lysate was measured on a NanoDrop (ND-100 Spectrophotometer). Nuclear lysis buffer was used to dilute samples to equal chromatin concentration. For the input samples, ~30 µg of the diluted sonicated chromatin was made up to a volume of 30 µl with nuclear lysis buffer, and further diluted with 90 µl IP dilution buffer (0.01% (w/v) SDS, 20 mM TRIS-HCl (pH 8.0), 1.1% (v/v) Triton-X-100, 167 mM NaCl, 1.2 mM EDTA and 1x Complete Mini Protease Inhibitor Cocktail tablet per 10 ml). The input samples were stored at -80°C, whilst remaining sonicated chromatin was immediately prepared for immunoprecipitation.

2.12.3. Preparation of the sonicated cell lysate for immunoprecipitation

A total of ~100 µg sonicated chromatin in 100 µl nuclear lysis buffer containing protease inhibitors (1x Complete Mini Protease Inhibitor Cocktail tablet per 10 ml) was diluted with 900 µl IP dilution buffer containing protease inhibitors (see above). To reduce the non-specific binding, the chromatin was pre-blocked by adding 20 µl 50:50 (v/v) pre-blocked protein A/G PLUS beads (sc-2003, Santa Cruz Biotechnology, USA) and incubating for 1 hour at 4°C on a rotating wheel. These pre-blocked protein A/G PLUS beads were prepared as described above. The pre-cleared chromatin was centrifuged at 15 000 g for 10 minutes at 4°C, and the supernatants were transferred to a new microcentrifuge tube, and 5 µg primary antibody (anti-GR or anti-IgG) added. This mixture was incubated overnight at 4°C on a rotating wheel. The following day, 40 µl pre-cleared protein A/G PLUS beads were added and the mixture was incubated for 6 hours at 4°C on a rotating wheel,

before centrifugation at 5 000 g for 1 minute at 4°C. To remove DNA and proteins non-specifically associated with the protein A/G PLUS beads, the pellet was washed sequentially with 1 ml of each of the wash buffers I, II and III: wash buffer I (0.1% SDS, 1% (v/v) Triton-X-100, 2 mM EDTA, 20 mM TRIS-HCl (pH 8.0) and 150 mM NaCl), wash buffer II (0.1% SDS, 1% (v/v) Triton-X-100, 2 mM EDTA, 20 mM TRIS-HCl (pH 8.0) and 500 mM NaCl) and wash buffer III (1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 500 mM LiCl, 1 mM EDTA and 10 mM TRIS-HCl (pH 8.0). This was followed by a further three washes with 1 ml TE buffer (10 mM TRIS-HCl (pH 8.0) and 0.1 mM EDTA). The immunoprecipitated DNA-protein complexes were eluted from the protein A/G PLUS beads twice with 150 µl elution buffer (1% (w/v) SDS and 100 mM $NaHCO_3$) at room temperature, while on a rotating wheel for 15 minutes.

2.12.4. Isolation and purification of DNA associated with the immunoprecipitated protein

To reverse the cross-linking, NaCl to a final concentration of 300 nM, was added to the eluted DNA-protein complexes, as well as the input samples, and incubated overnight at 65°C. The next day, EDTA (final concentration of 15 nM), TRIS-HCl (final concentration of 125 nM) and 60 ng/ml proteinase K (Roche Applied Science, South Africa) were added to the samples, prior to a further incubation for 1 hour at 45°C to digest the proteins. Both immunoprecipitated- and input DNA were purified using the NucleoSpin® Extract II kit (Thermo Scientific Inc., USA) according to the manufacturer's instructions.

2.12.5. Analysis of DNA coupled with the immunoprecipitated protein

The purified immunoprecipitated- and input DNA were analysed using realtime qPCR using primers specific for *cis*-elements of the IL-12p40 and IL-10 promoters (see Table 2.3). For the IL-12p40 promoter we investigated the following regulatory elements: Sp1, AP-1 and NFkB-C/EBPβ,

while AP-1, Sp1 and STAT-3 regulatory elements were investigated for the IL-10 promoter. It is important to note that some primers span more than one regulatory element, as certain *cis*-elements are located in close proximity to each other. All primers used for the ChIP and re-ChIP assays were designed by the candidate. As a positive control, to verify that the GR ChIP assay is working the recruitment of the GR to the GRE-containing glucocorticoid-induced leucine zipper (GILZ) promoter using the following primer set: (forward) 5'-AGTTAAGCTCCTGATTTAAGAAG-3', (reverse) 5'- CCCGAT CTCAGGACATTC-3' (Avenant et al., 2010a) was also investigated.

Table 2.3. Primers specific for *cis*-elements of the IL-12p40 and IL-10 promoters used in ChIP and re-ChIP assays.

Cis-element	Position relative to transcription start site	Primers (5' - 3')	Strand	Length of amplicon (bp)	Annealing temperature (°C)
HUMAN IL-12p40 PROMOTER					
Sp1	-743 to -674	TGCCTCCCTGAGGGTATTTCACTT ATCCTGTGCTCATAGGTACTCGCT	Forward Reverse	70	60
AP-1	-250 to -162	GCATCTCCATCTCCTTCCTTATTC CGAGGAGGGAACATAGACATC	Forward Reverse	89	57
NFκB-C/EBPβ	-179 to +11	GTCTATGTTCCCTCCTCGTT GTTTCTTCTGCTGCTGTTGC	Forward Reverse	190	53
HUMAN IL-10 PROMOTER					
GRE / Sp1	-717 to -570	GGGACAGCTGAAGAGGTGGA CCTCAAAGTTCCCAAGCAGC	Forward Reverse	148	57
Sp1 / STAT-3	-183 to -100	TAGAGAAGGAGGAGCTCTAAGGAG AGGGAGGCCTCTTCATTCA	Forward Reverse	84	60

All samples were incubated at 95°C for 10 minutes to activate the Roche FastStart Taq polymerase, followed by the denaturation at 95°C for 10 seconds, annealing for 10 seconds (see temperature in Table 2.3), followed by extension at 72°C for 10 seconds. Typically 40 repeats of the three PCR steps were used for all primer pairs. Agarose gel electrophoresis and melting curve analysis were performed to confirm the presence of a single amplicon, as well as the amplicon size. The relative protein recruitment was calculated by the method described by Pfaffl with slight modifications (Pfaffl, 2001) as the primer efficiency was assumed to be 2 and normalised relative to input, which was set as one.

For the chromatin reimmunoprecipitation (re-ChIP) assay (a few modifications were made to the protocol received from Prof Susanne Mandrup, Department of Biochemistry and Molecular Biology, University of Southern Denmark), the immunoprecipitated DNA-protein complexes were eluted at 37°C by incubation for 30 minutes in 100 µl elution buffer (1% (w/v) SDS and 10 mM dithiothreitol (DTT)), while on a rotating wheel. The supernatants were diluted 10 times with the ChIP dilution buffer without SDS (20 mM TRIS-HCl (pH 8.0), 1.1% (v/v) Triton-X-100, 167 mM NaCl, 1.2 mM EDTA and 1x Complete Mini Protease Inhibitor Cocktail tablet per 10 ml), and 100 µl of the diluted chromatin was stored at -80°C as control for the first immunoprecipitation. The remaining sonicated chromatin was diluted with 1 ml IP dilution buffer and reimmunoprecipitated with an antibody specific for either C/EBPβ or STAT-3 before analysing as above.

2.13. Data manipulation and statistical analysis

GraphPad Prism® version 5 (GraphPad Software, Inc., San Diego, CA) was used for data manipulation, graphical presentations and statistical analysis. Unless otherwise stated in the figure legends, one-way ANOVA analysis of variance, with Dunnett's (compares all columns vs. control column) and Bonferroni's (compares all pairs of columns) post-tests were used for ungrouped

column graphs. For grouped analysis (response is affect by two factors), two-way ANOVA analysis of variance with Bonferroni's (compares all pairs of columns) post–test was used. Statistically significant differences are indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively, whereas no statistical significance ($p>0.05$) is indicated by ns. The letters a,b,c etc. are also used to denote statistically significant differences, where all those values which differ significantly from others, are assigned a different letter. Unless otherwise indicated, the error bars represent the standard error of the mean (SEM) of three independent experiments.

CHAPTER 3

BRIEF INTRODUCTION, RESULTS AND DISCUSSION

THE MOLECULAR MECHANISM OF PROGESTIN-INDUCED REGULATION OF INTERLEUKIN (IL)-12p40 AND IL-10 IN A HUMAN ECTOCERVICAL EPITHELIAL CELL LINE

3.1. Brief Introduction

The female genital tract (cervicovaginal environment) consist of the vagina and ectocervix, which are both considered to be non-sterile environments, as well as the endocervix, a more sterile environment. Epithelial cells lining the female genital tract are capable of eliciting both innate and acquired local immune responses at the mucosal surface against invading pathogens. These epithelial cells have properties enabling them to fight infections, such as the production of cytokines and chemokines (Shrier et al., 2003, Ochiel et al., 2008, Dubicke et al., 2010). Although the vagina is the first compartment exposed to potential pathogens, the ectocervix is proposed to be the first target site to become infected with sexually transmitted HIV-1 (Howell et al., 2005, Hladik and Hope, 2009, Kaushic et al., 2010). It is suggested that this is mainly due to HIV-1 target cells such as $CD4^+$ T cells and macrophages being prevalent in this region (Pudney et al., 2005).

Contraceptive use of MPA has been shown associated with increased cervical shedding of HIV DNA (Clemetson et al., 1993, Mostad et al., 1997, Wang, 2004). Although the precise mechanism is not known, it has been suggested that it may be due to altered physiology of the local genital tract or modulating cervical immunity. Whether NET-A has similar effects is not known. Thus, investigating the effects of MPA, as well as NET-A on immune responses in the female genital tract, particularly the mechanisms of immune gene regulation in the cervicovaginal environment may further our understanding of the factors affecting mucosal immunity and hence shed light on determinants of susceptibility. In light of the above, this study investigated the effects, and the underlying molecular mechanisms, of MPA and NET-A, relative to Prog, on mucosal immunity, by comparing their regulation of the endogenous cytokine genes, IL-12p40 and IL-10. The human Ect1/E6E7 epithelial cell line, generated from normal human ectocervical cells and immortalized by expression of the E6 and E7 genes of HPV 16 (Fichorova et al., 1997), was used as an *in vitro* cell culture model system of the female ectocervical environment.

3.2. Results

3.2.1. Prog, MPA and NET-A upregulate IL-12p40 mRNA expression, while IL-10 mRNA expression is downregulated

The human Ect1/E6E7 cell line was treated with 0.02 µg/ml TNF-α and 0.1% EtOH (control) or 1 µM Prog, MPA or NET-A for 6 hours, followed by qPCR analysis for the mRNA expression of IL-12p40 and IL-10, respectively. Figures 3.1A and 3.1B shows the TNF-α induction of IL-12p40 and IL-10 mRNA expression, while figures 3.1C and 3.1D show the effects of the ligands on the mRNA expression of the cytokine genes. Surprisingly, Prog, MPA and NET-A significantly ($p<0.001$) upregulated IL-12p40 gene expression (figure 3.1C). Conversely, all the ligands significantly ($p<0.001$) downregulated the mRNA expression of the IL-10 gene (figure 3.1D). Interestingly, no significant differences were detected between any of the ligands in the regulation of IL-12p40 or IL-10. The effects of Prog, MPA and NET-A on IL-12p40 and IL-10 protein levels were also investigated, using a combination of Multiplex bead array and ELISA. The Ect1/E6E7 cell line was treated with 0.02 µg/ml TNF-α and 0.1% EtOH (control) or 1 µM Prog, MPA or NET-A for 24, 48 and 96 hours, respectively. No conclusions could be drawn from these results, as negligible IL-12p40 and IL-10 protein levels were detected in the cell culture supernatant (Addendum A, figure A3).

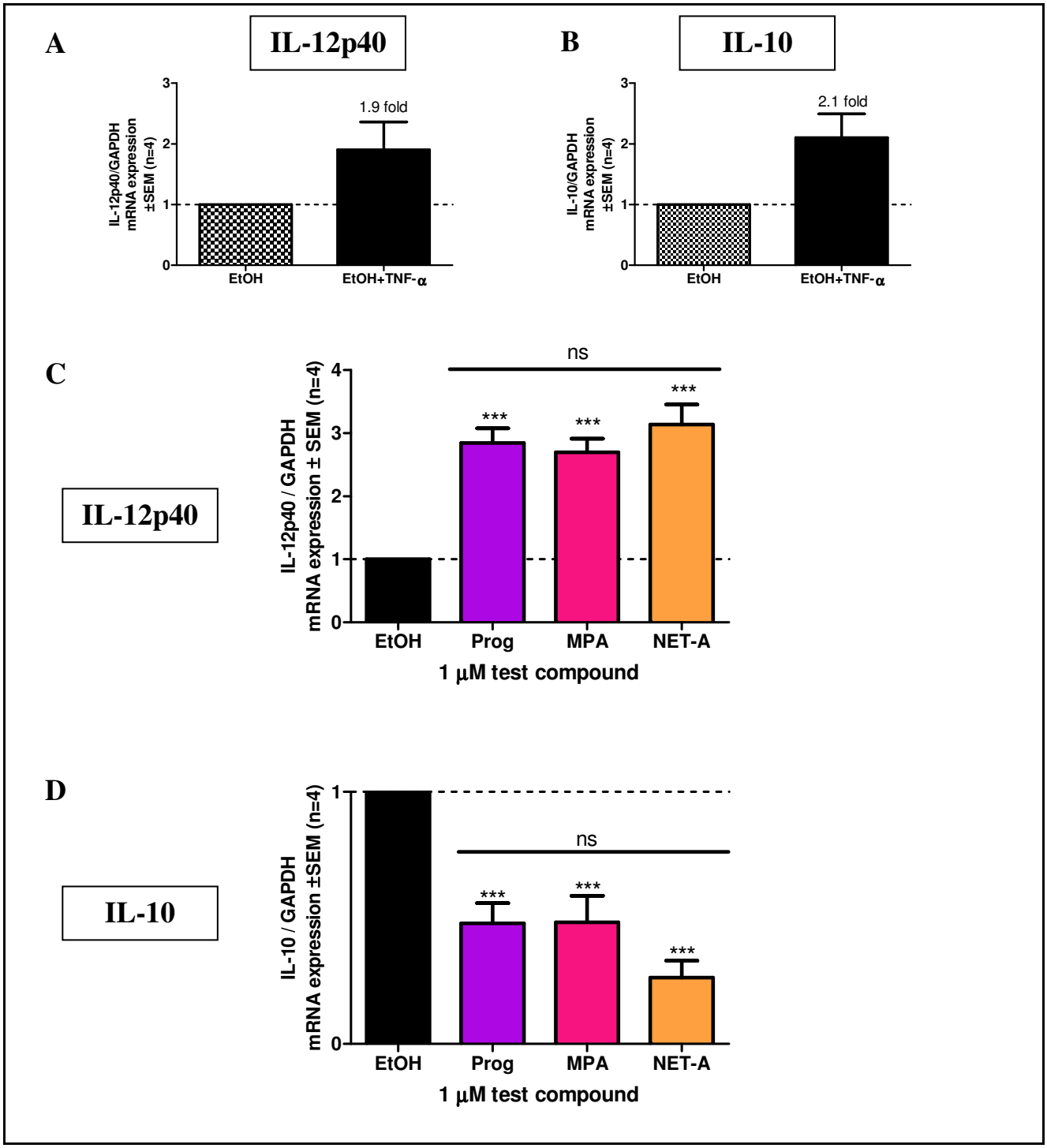

Figure 3.1. Effect of Prog, MPA and NET-A on the TNF-α induced expression of IL-12p40 and IL-10 in the human ectocervical cell line. The human Ect1/E6E7 cell line was incubated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA or NET-A for 6 hours. Total RNA was isolated and reversed transcribed to cDNA. Realtime qPCR was performed to determine the mRNA expression levels of IL-12p40 **(A and C)** and IL-10 **(B and D)**, using GAPDH as the internal standard. Results shown are the averages (± SEM) of at least four independent experiments. Relative IL-12p40 and IL-10 mRNA expression of treated samples were calculated relative to vehicle control (EtOH), which was set as one. One-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis.

3.2.2. A role for the GR in the regulation of IL-12p40 and IL-10 mRNA expression by Prog, MPA and NET-A

As we have previously shown that the endogenously expressed GR in the Ect1/E6E7 cell line is the only endogenous steroid receptor transcriptionally active in both a transactivation and transrepression assay (Africander et al., 2011a), and it is known that both MPA and NET-A bind to the GR (Koubovec et al., 2005), we hypothesised that the regulation of the IL-12p40 and IL-10 genes by Prog, MPA and NET-A is mediated via the GR. Using competitive whole cell binding assays we confirmed binding of Prog, MPA and NET-A to the endogenously expressed GR in the human Ect1/E6E7 cell line (figure 3.2A). We show that at 1 μM, there is no statistical difference in the total specific binding of the synthetic glucocorticoid Dex, the natural glucocorticoid cortisol, Prog or MPA to the endogenous GR. In agreement with a previous study, NET-A has a lower binding affinity for the GR, than Prog and MPA. Next, we used Western blotting to determine whether Prog, MPA and NET-A, in the presence of TNF-α, have an effect on the endogenous GR protein levels. Ect1/E6E7 cells treated with the ligands in the presence of TNF-α for 6 hours were analysed by means of Western blotting using antibodies specific for the GR and GAPDH (loading control). The results showed that none of the ligands had any effect on the GR protein levels after 6 hours (figure 3.2B and 3.2C).

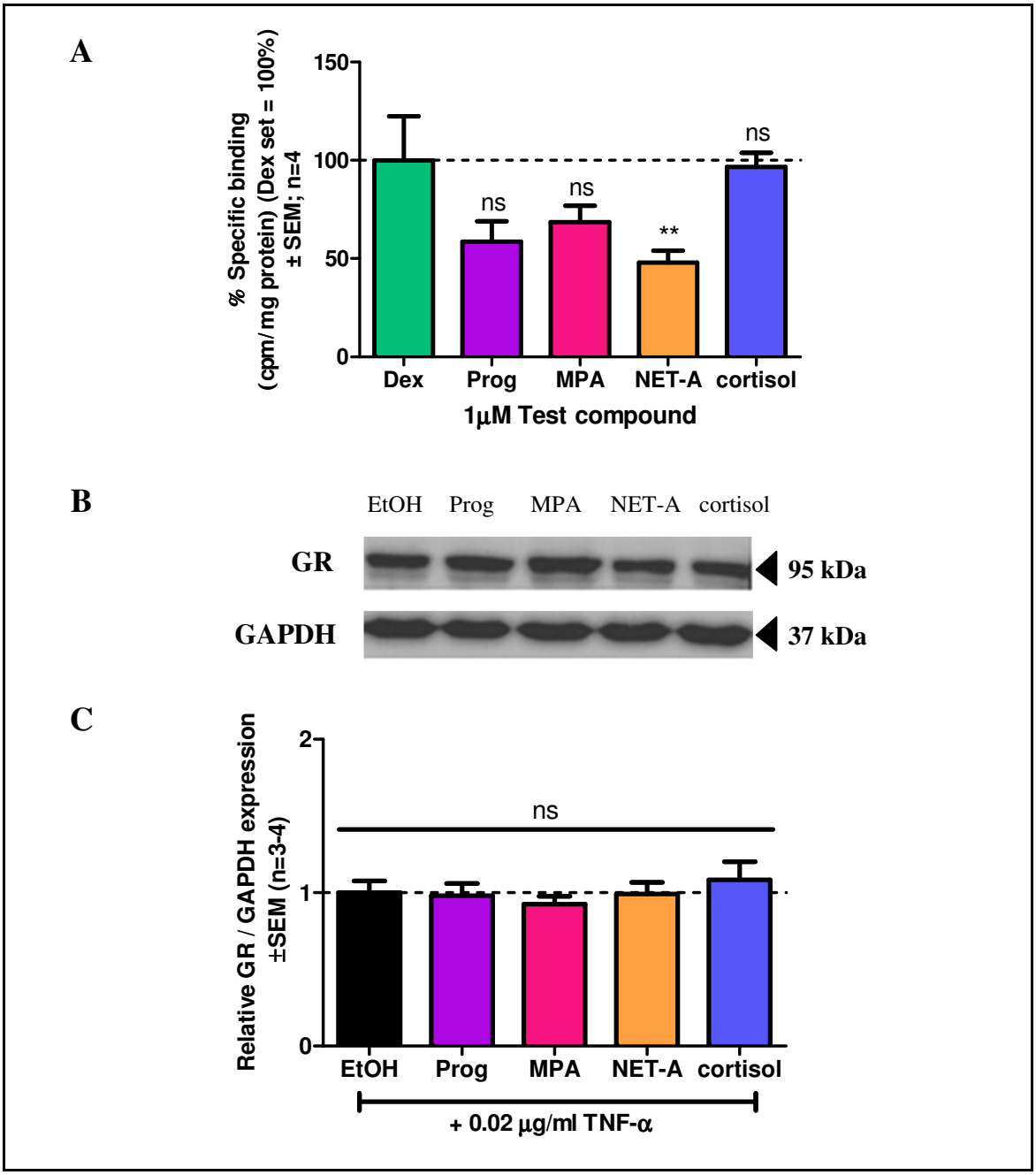

Figure 3.2. Prog, MPA and NET-A bind to the endogenous GR in the Ect1/E6E7 cell line, but do not modulate GR protein levels. (A) The human Ect1/E6E7 cell line was incubated with 10 nM [^{3}H]-Dex in the absence (total binding) and presence of 1 µM unlabelled (non-specific binding) Dex, Prog, MPA, NET-A or cortisol for 6 hours. Percentage specific binding (total binding minus non-specific binding) is plotted. Binding of the test compounds to the GR is shown relative to binding of Dex set as 100%. One-way ANOVA analysis of variance and Dunnett (compare all columns versus control (Dex) column) was performed as post-test **(B)** Whole cell extracts were prepared from the human Ect1/E6E7 cell line treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH or 1 µM Prog, MPA, NET-A or cortisol for 6 hours to harvest total protein. Protein lysates were subjected to electrophoresis on a 10% SDS polyacrylamide gel. The GR protein levels were analysed using a GR-specific antibody. GAPDH was used as a loading control. A single representative gel of at least three independent experiments is shown. **(C)** GR protein levels were quantified using UN-SCAN-IT and normalised to the quantified GAPDH levels. The effect of the test compounds on the endogenous GR protein levels is shown as fold induction relative to EtOH set as 1.

To further investigate the role of the GR in mediating the regulation of Prog, MPA and NET-A on the IL-12p40 and IL-10 genes the GR was silenced using siRNA transfections. The Ect1/E6E7 cell line was transfected with validated GR-specific siRNA oligonucleotides or non-silencing RNA oligonucleotides (control) for 48 hours, followed by treatment with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. To confirm GR knockdown, Western blot analysis confirmed reduction of GR protein levels of approximately 83% (figure 3.3A and 3.3B). Similar results were obtained when transfecting the GR-specific GR5 siRNA oligonucleotides. Gene expression analysis by qPCR showed that, as expected, cortisol induced upregulation of IL-12p40 and downregulation of IL-10, was significantly reduced by the decrease in GR protein levels (figure 3.3C and 3.3D). Similarly, the decreased GR levels significantly inhibited the upregulation of IL-12p40 and downregulation of IL-10 by Prog, MPA and NET-A, indicating that the effect of these ligands on IL-12p40 and IL-10 genes is mediated via the GR.

3.2.3. The GR is recruited to the endogenous IL-12p40 and IL-10 promoters in response to Prog, MPA and NET-A

Having established that the GR is required for the Prog, MPA and NET-A induced regulation of both IL-12p40 and IL-10 gene expression, it was next investigated whether the GR could be recruited to the endogenous IL-12p40 and IL-10 promoters in the Ect1/E6E7 cells. This was done by chromatin immunoprecipitation (ChIP) assays, using a GR specific antibody for immunoprecipitation and primers spanning 190 bp of the IL-12p40 promoter containing both the nuclear factor kappa B (NFκB) and CCAAT enhancer binding protein (C/EBP)-β regulatory

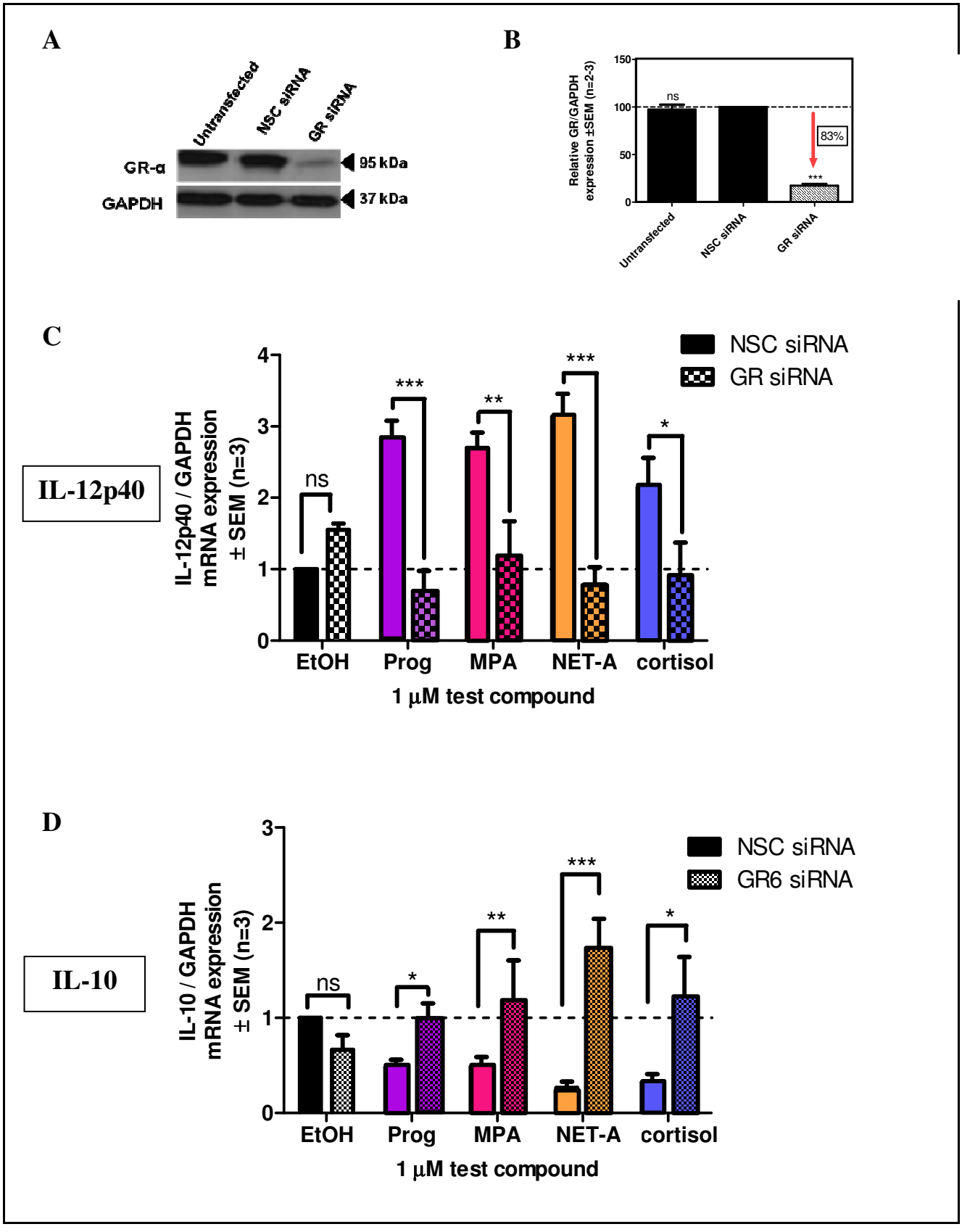

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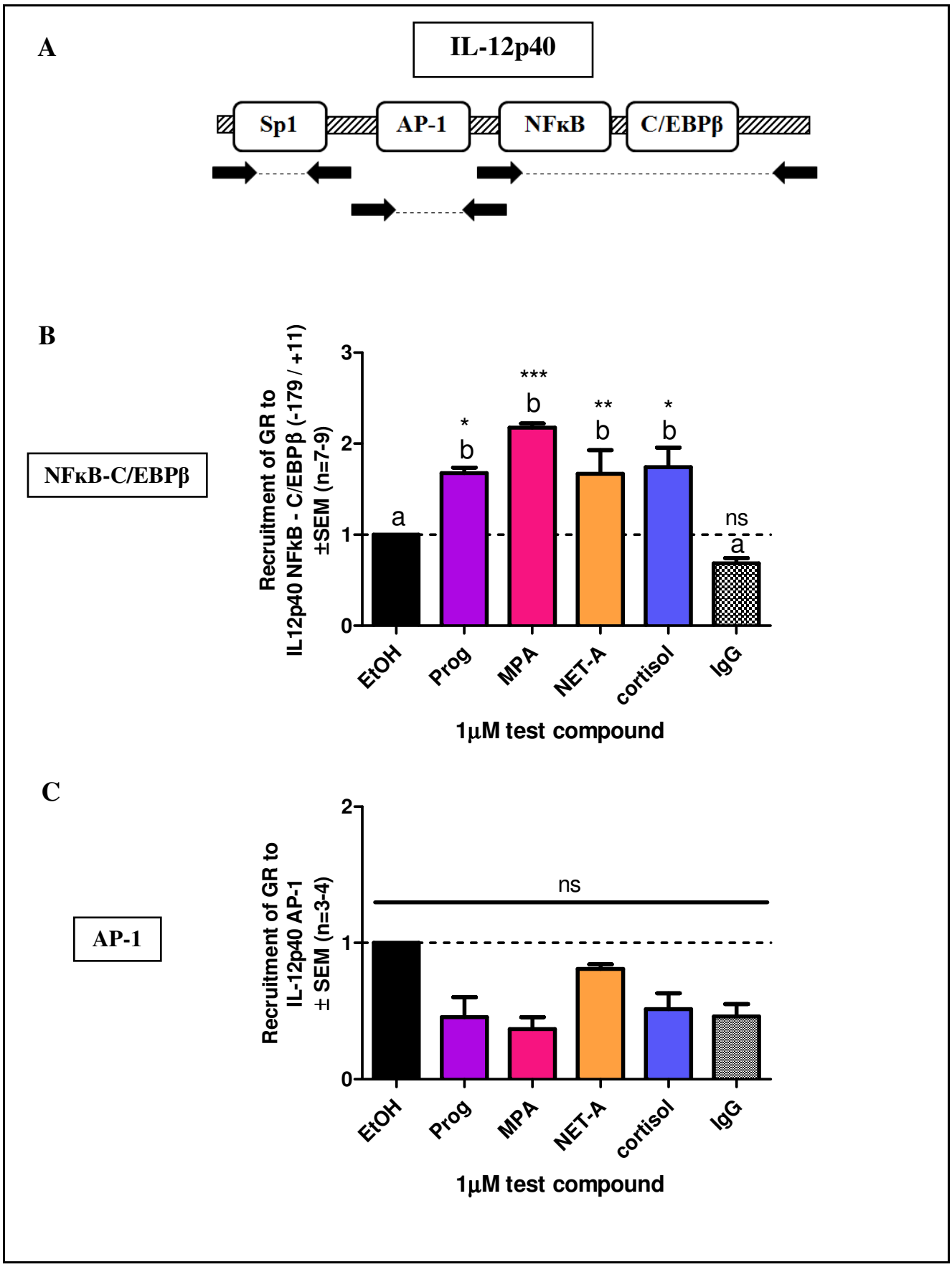
Figure 3.3. Decreasing GR protein levels by siRNA indicates a role for the GR in mediating the effects of Prog, MPA and NET-A on IL-12p40 and IL-10 mRNA expression in the Ect1/E6E7 cell line. Untransfected human Ect1/E6E7 cells, as well as cells transfected with 10 nM NSC or GR siRNA oligonucleotides, were either left untreated or treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. **(A)** For verification of GR knockdown, total protein from the untreated cells were harvested to perform Western blotting, using antibodies specific for the GR and GAPDH. The latter was used as a loading control. A representative blot is shown. **(B)** GR expression levels relative to GAPDH were quantified using UN-SCAN-IT. Western blots of at least two independent experiments were quantified to determine the percentage GR protein knockdown. One-way ANOVA analysis of variance and Dunnett (compares all columns vs. control (NSC siRNA) column) post-test was used for statistical analysis. **(C-D)** Total RNA was isolated and reversed transcribed to cDNA. Thereafter realtime qPCR was performed to determine the mRNA expression levels of (C) IL-12p40 and (D) IL-10, using GAPDH as the internal standard. Relative IL-12p40 and IL-10 gene expression of treated samples was calculated relative to vehicle control (EtOH) of the NSC siRNA, which was set as one. Results shown are the average (± SEM) of at least three independent experiments. Two-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis.

elements, and 84 bp of the IL-10 promoter region containing both a specific protein (Sp)-1 and signal transducer and activator of transcription (STAT)-3 regulatory element.

As there are no GRE sequences defined within the IL-12p40 promoter, other regulatory elements known to regulate IL-12p40 promoter activity were investigated. These include C/EBPβ and Sp1. Similarly, for the IL-10 promoter, *cis*-regulatory elements previously shown to be involved in the downregulation of IL-10 gene expression, such as Sp1 and STAT-3 (Steinke et al., 2004, Lee et al., 2010) were investigated. Intact Ect1/E6E7 cells were treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 2 hours. As shown in figure 3.4B and 3.5B, and as expected, the natural glucocorticoid cortisol caused significant recruitment of the GR to the NFkB-C/EBPβ region of the endogenous IL-12p40 and Sp1/STAT-3 region of the IL-10 promoters, respectively. Similarly, the GR was also recruited to these sites on the endogenous IL-12p40 (figure 3.4B) and IL-10 (figure 3.5B) promoters in the presence of Prog, MPA and NET-A. In contrast, we show no recruitment in the presence of all ligands to an AP-1 or Sp1 regulatory element within the IL-12p40 promoter (figure 3.4C and 3.4D) or the GRE/Sp1 binding site (figure

3.5C) in the IL-10 promoter. Interestingly, significant recruitment of the GR to the Sp1 binding site of the endogenous IL-12p40 promoter was observed with cortisol treatment. The IgG negative control confirmed the specificity of the GR antibody used for immunoprecipitation (figure 3.4 and 3.5). For verification and validation of the ChIP assay, the recruitment of the GR, in response to all ligands, to the endogenous GRE-containing glucocorticoid-induced leucine zipper (GILZ) promoter was investigated in the Ect1/E6E7 cell line (figure 3.5D). We show recruitment of the GR to the GILZ promoter in response to all the ligands, except NET-A. These results suggest that the ligand (cortisol, Prog, MPA or NET-A) bound GR interacts with the NFkB-C/EBPβ *cis*-elements of the IL-12p40 promoter to enhance transcription of this gene, whereas recruitment of the liganded GR to the Sp1/STAT-3 *cis*-elements in the IL-10 promoter results in the inhibition of IL-10 gene transcription.



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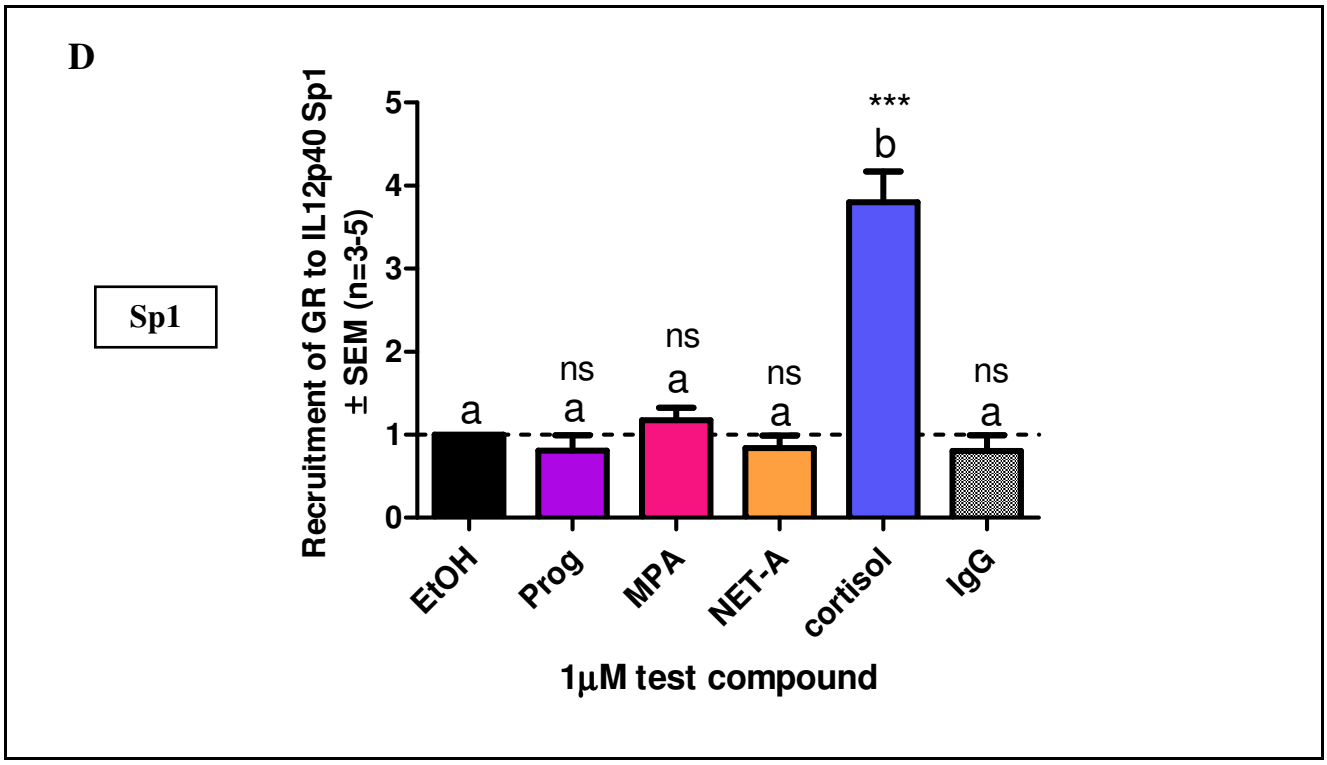
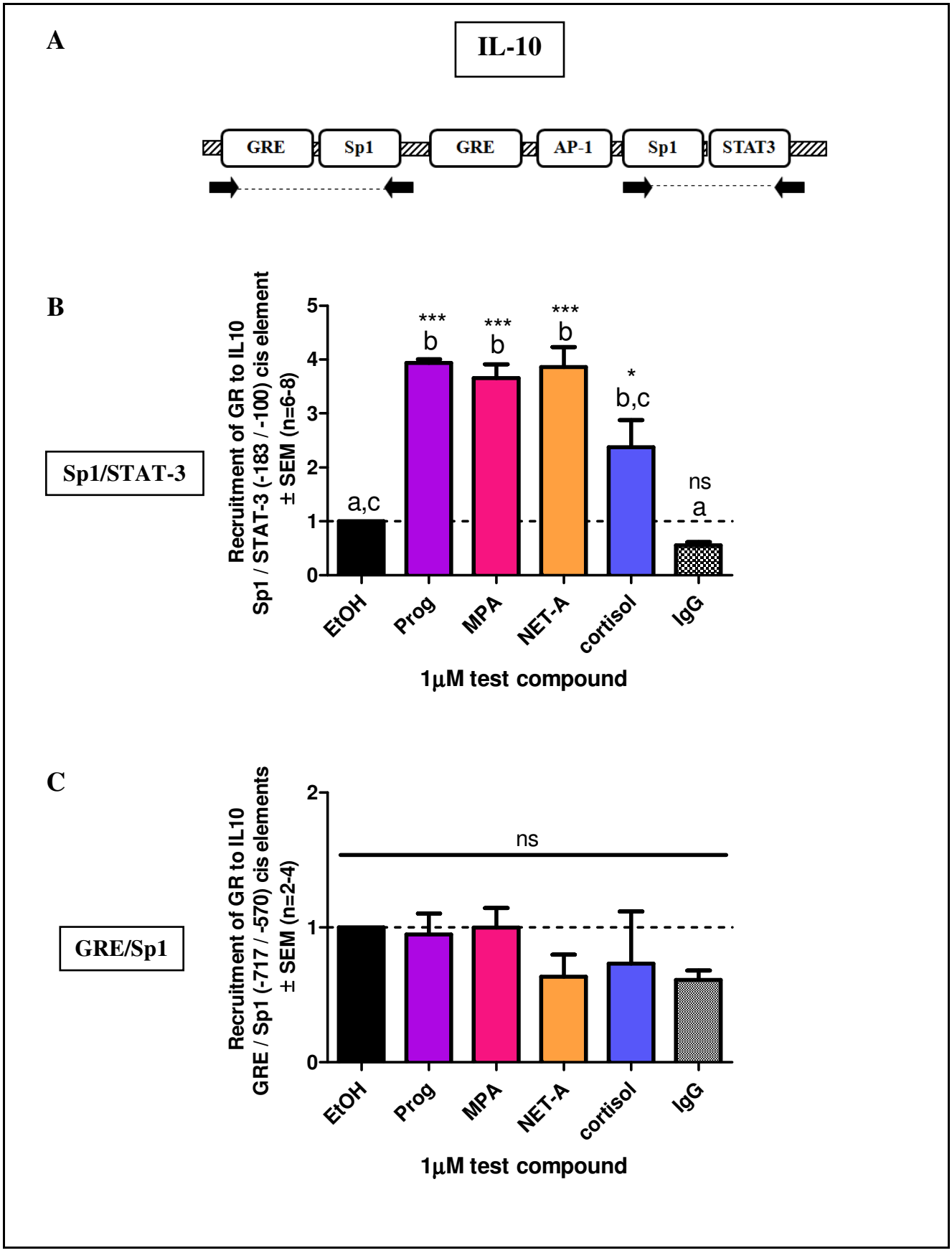


Figure 3.4. The GR is recruited to the NFkB-C/EBPβ region of the IL-12p40 promoter in response to cortisol, Prog, MPA and NET-A. Human Ect1/E6E7 cells were incubated with 0.02 μg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 μM Prog, MPA, NET-A or cortisol for 2 hours, followed by the ChIP assay. **(A)** A schematic illustration of the IL-12p40 promoter indicating the positions of the primer pairs. Immunoprecipitated GR protein bound to the endogenous IL-12p40 promoter was detected using primers encompassing **(B)** the NFkB-C/EBPβ, **(C)** AP-1 and **(D)** Sp1 regulatory elements. Immunoprecipitation with anti-IgG served as negative control. The co-immunoprecipitated DNA fragments and input DNA were analysed by realtime qPCR. Data shown are normalised to input and expressed as the fold-response relative to EtOH (control), which was set as one. Results shown are the average (± SEM) of at least three independent experiments. One-way ANOVA analysis of variance and both Bonferroni (compares all pairs of columns) and Dunnett (compares all columns vs. control (EtOH) column) post-tests were used for statistical analysis.



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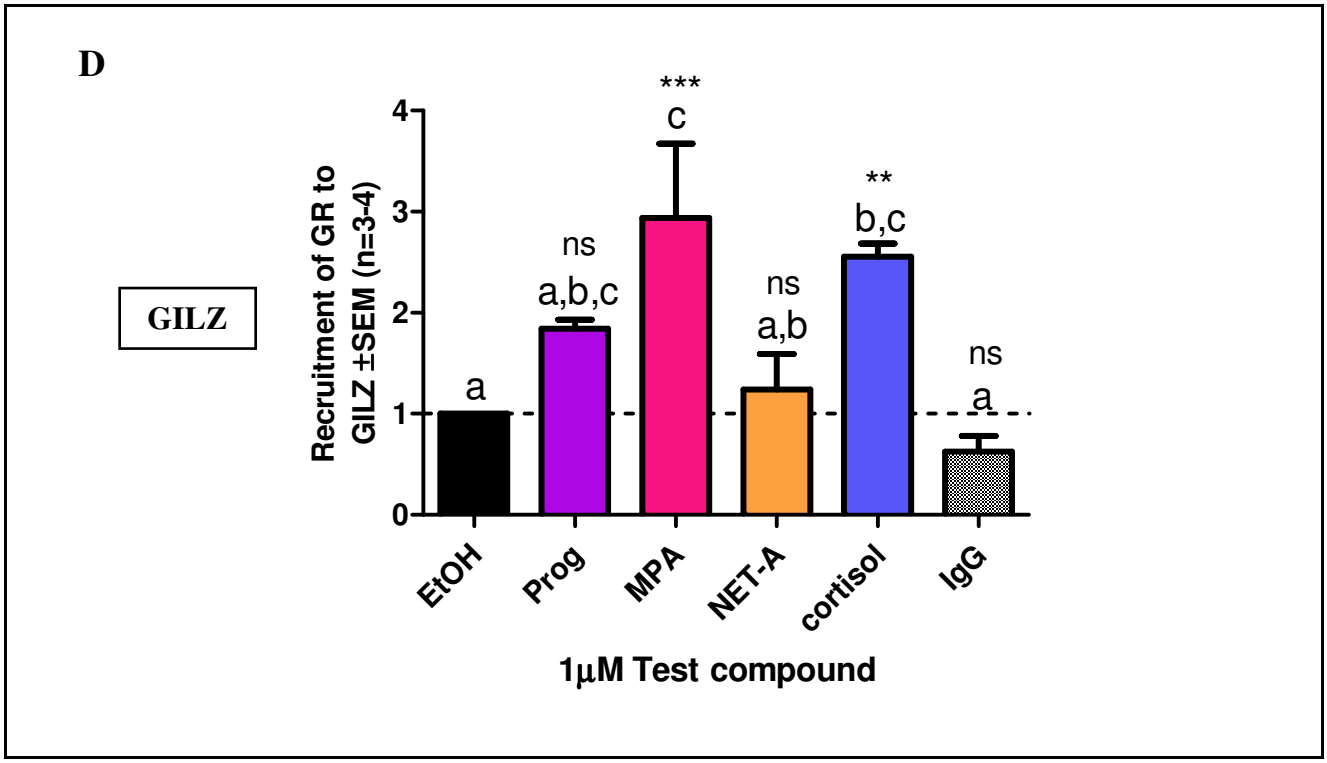

Figure 3.5. The GR is recruited to the Sp1/STAT-3 region of the IL-10 promoter in response to cortisol, Prog, MPA and NET-A. Human Ect1/E6E7 cells were incubated with 0.02 μg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 μM Prog, MPA, NET-A or cortisol for 2 hours, followed by the ChIP assay. **(A)** A schematic illustration of the IL-10 promoter indicating the positions of the primer pairs. Immunoprecipitated GR protein bound to the endogenous IL-10 promoter was detected using primers encompassing **(B)** the Sp1/STAT-3 and **(C)** GRE/Sp1 regulatory elements of IL-10, and **(D)** the GILZ promoter using primers spanning the equivalent of GREs 3-6. Immunoprecipitation with anti-IgG served as negative control. The co-immunoprecipitated DNA fragments and input DNA were analysed by realtime qPCR. Data shown are normalised to input and expressed as the fold-response relative to EtOH (control), which was set as one. Results shown are the average (± SEM) of at least three independent experiments. One-way ANOVA analysis of variance and both Bonferroni (compares all pairs of columns) and Dunnett (compares all columns vs. control (EtOH) column) post-tests were used for statistical analysis.

3.2.4. C/EBPβ is required for the GR-mediated upregulation of the IL-12p40 gene in response to TNF-α and Prog, MPA or NET-A

The finding that the GR is recruited to the region of the endogenous IL-12p40 promoter encompassing the NFkB and C/EBPβ *cis*-elements suggests that C/EBPβ, rather than NFkB, is involved in the transcriptional activation of the IL-12p40 gene, as tethering of the GR to NFkB is generally associated with the downreglation of pro-inflammatory cytokine genes (Smoak and Cidlowski, 2004). Interestingly, C/EBPβ, a transcription factor, has previously been shown to play a

role in the activation of the IL-12p40 promoter (Plevy et al., 1997, Becker et al., 2001). For this reason, the involvement of C/EBPβ in the ligand-dependent GR-mediated upregulation of IL-12p40 mRNA expression was thus further investigated, by siRNA. The Ect1/E6E7 cell line was transfected with 10 nM validated C/EBPβ- or NSC-specific siRNA oligonucleotides, followed by treatment with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. Western blot analysis showed 54% reduction in the expression of endogenous C/EBPβ protein levels as compared to the NSC (figure 3.6A and 3.6B). Reduced C/EBPβ levels significantly reversed the cortisol-induced upregulation of IL-12p40 gene expression (figure 3.6C). Similarly, knockdown of C/EBPβ abrogated the upregulation of IL-12p40 by Prog, MPA and NET-A, indicating that C/EBPβ is required for the GR-mediated upregulation of IL-12p40 gene expression in response to these ligands.

Taken together, these results suggest that C/EBPβ is needed for the upregulation of IL-12p40 gene expression by Prog-, MPA- or NET-A- bound GR, suggesting that the GR and C/EBPβ are co-recruited to the IL-12p40 promoter. Co-immunoprecipitation assays were thus used to investigate whether these two proteins interact with each other. The Ect1/E6E7 cell line was treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for an hour, and immunoprecipitated using a GR-specific antibody. Western Blot analysis confirmed the presence of the GR and C/EBPβ in the input samples (figure 3.7A). Figure 3.7B shows that the GR is present in all the immunoprecipitated samples, independent of the presence of ligand. When the same membrane was stripped to remove the anti-GR antibody, and reprobed with a C/EBPβ-specific antibody, the results show that C/EBPβ co-immunoprecipitated with the GR in the presence of TNF-α, and that the presence of ligand does not disrupt this interaction. These results suggest that the liganded GR and C/EBPβ can occur in a complex in the Ect1/E6E7 cell line. However, due to high signal background observed for the GR Western blot, the co-immunoprecipitation assay

should be repeated by firstly probing with a C/EBPβ-specific antibody, followed by reprobing with a GR-specific antibody.

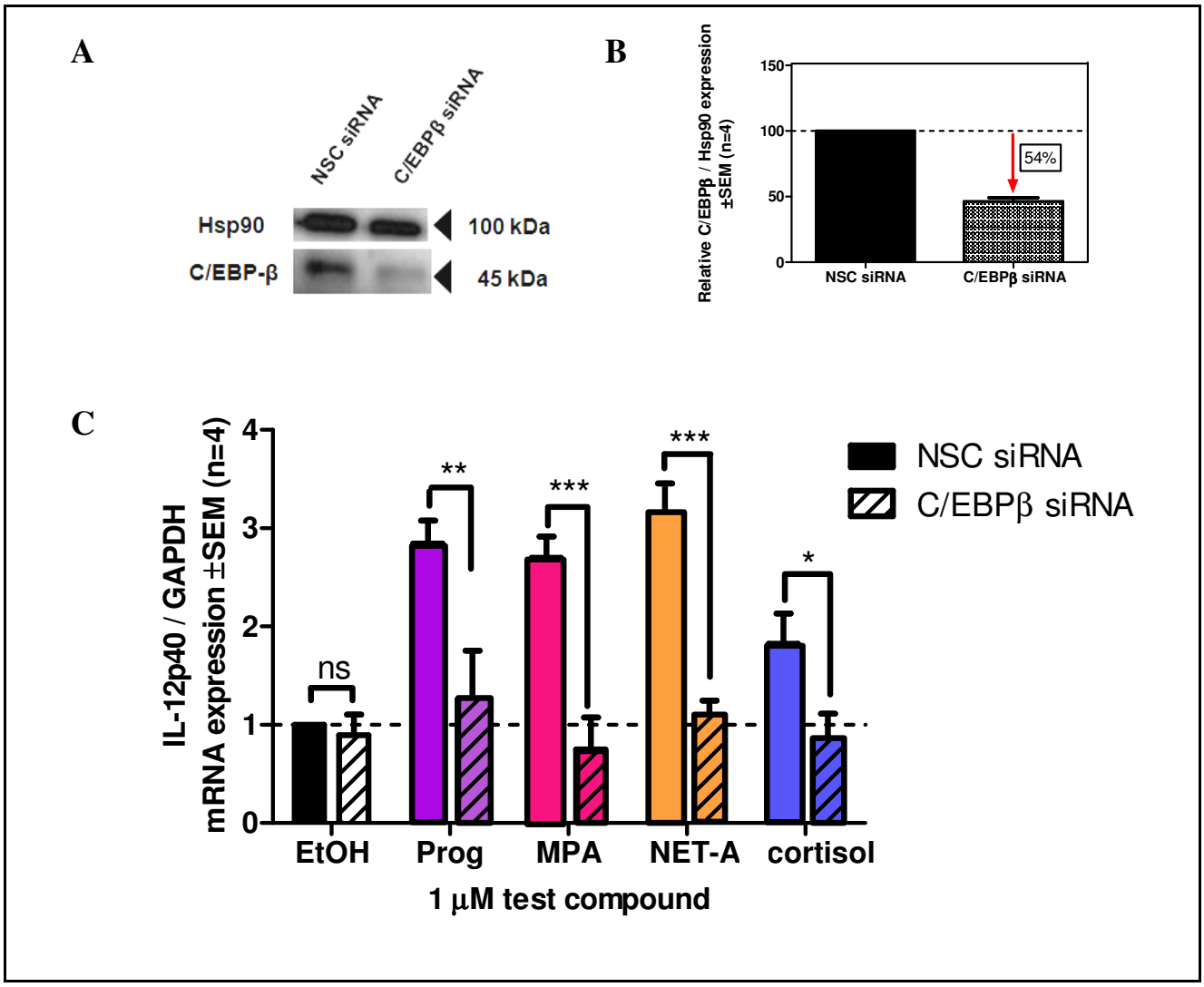

Figure 3.6. Reducing C/EBPβ protein by siRNA indicates a role for C/EBPβ in the GR-mediated upregulation of IL-12p40 gene expression in the Ect1/E6E7 cell line. Human Ect1/E6E7 cells were transfected with 10 nM NSC or C/EBPβ siRNA oligonucleotides and after 24 hours treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. **(A)** For verification of C/EBPβ knockdown, total protein from the untreated cells were harvested to perform Western blotting using antibodies specific for C/EBPβ and Hsp90. The latter was used as a loading control. A representative blot is shown. **(B)** C/EBPβ expression levels relative to Hsp90 were quantified using UN-SCAN-IT. Western blots of at least four independent experiments were quantified to determine the percentage C/EBPβ protein knockdown. **(C)** Total RNA was isolated and reversed transcribed to cDNA. Thereafter realtime qPCR was performed to determine the mRNA expression levels of IL-12p40, using GAPDH as the internal standard. Relative IL-12p40 gene expression of treated samples was calculated relative to vehicle control (EtOH) of the NSC siRNA, which was set as one. Results shown are the average (± SEM) of at least three independent experiments. Two-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis.

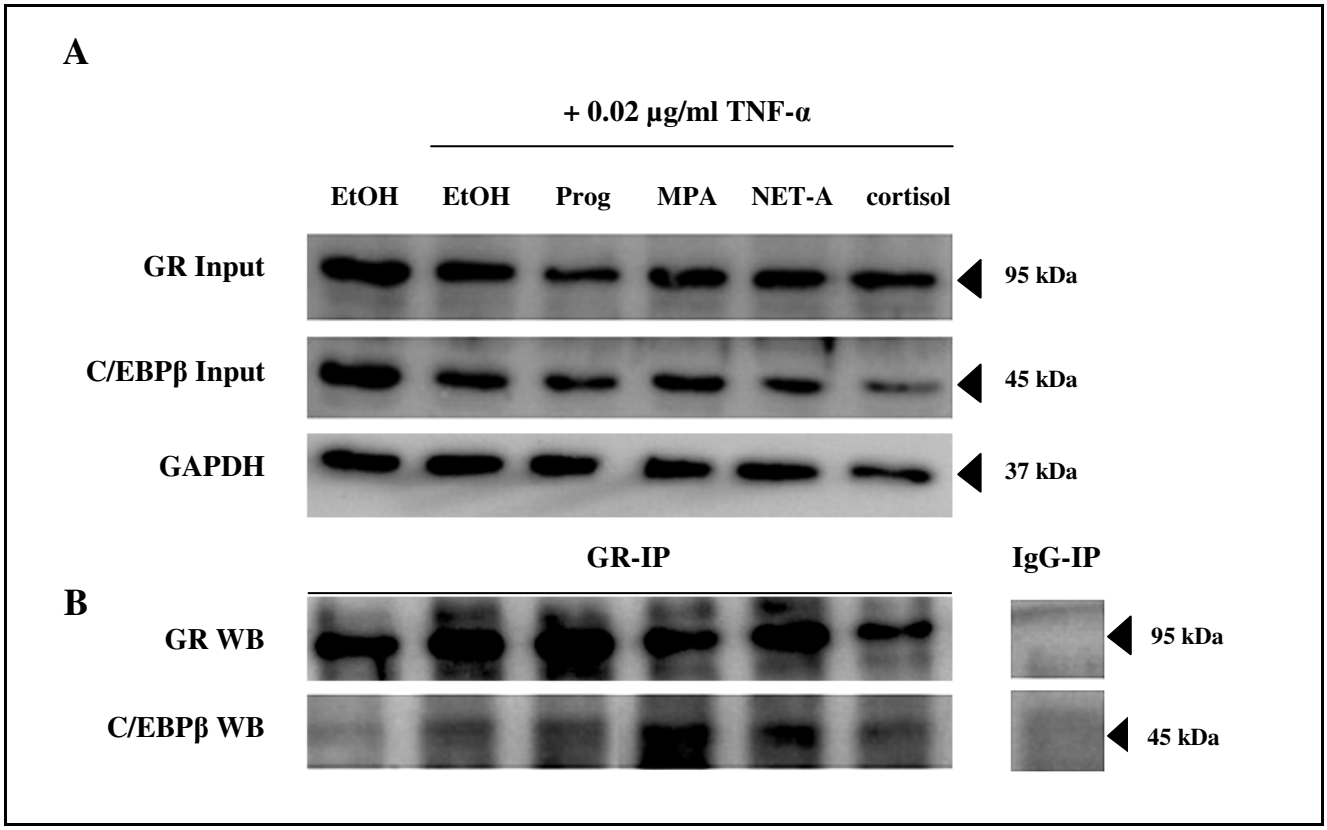

Figure 3.7. The liganded GR and C/EBPβ occurs in a complex in the Ect1/E6E7 cell line. Human Ect1/E6E7 cells were incubated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for an hour. Cellular extracts were immunoprecipitated with a GR-specific antibody, and Western blots were probed firstly GR-specific antibody and then stripped and reporbed with the C/EBPβ antibody. A representative blot of three independent experiments is shown. **(A)** Both the GR and C/EBPβ are present in the input samples. **(B)** C/EBPβ co-immunoprecipitated with the GR only in the presence of ligand, indicating that the liganded GR and C/EBPβ can occur in a complex in the Ect1/E6E7 cell line.

Having shown that C/EBPβ is required for the upregulation of IL-12p40 gene expression by Prog, MPA and NET-A via the GR, and that the GR and C/EBPβ can occur as a complex, the co-localisation of the GR-C/EBPβ protein complex on the endogenous IL-12p40 promoter was next investigated by using a re-ChIP assay. Intact Ect1/E6E7 cells were treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 2 hours. The immunoprecipitated GR-C/EBPβ proteins bound to the endogenous IL-12p40 promoter were detected using primers spanning 190 bp of the IL-12p40 promoter, which comprises the C/EBPβ regulatory element. The immunoprecipitated DNA fragments and input DNA were analysed by

realtime qPCR. Similar results were obtained for the re-ChIP assay (figure 3.8A) when compared to that of the ChIP assay (figure 3.4B). Figure 3.8A shows that only the Prog, MPA and cortisol treatment resulted in significant recruitment. Although NET-A shows a possible recruitment of the GR-C/EBPβ complex to the C/EBPβ binding site in the IL-12p40 promoter, significance could not be established. Significance for NET-A could possibly be obtained by performing more experiments (increasing the "n" value), thereby increasing the statistical power of the experiments. The IgG negative control confirmed the specificity of this GR-C/EBPβ co-localisation on the IL-12p40 promoter, as no recruitment of this complex was observed in the presence of an anti-IgG antibody (figure 3.8B). Taken together, our results show that, in response to Prog, MPA and possibly NET-A, the GR interacts with C/EBPβ, and that endogenous GR and C/EBPβ are in a common complex bound to the IL-12p40 promoter.

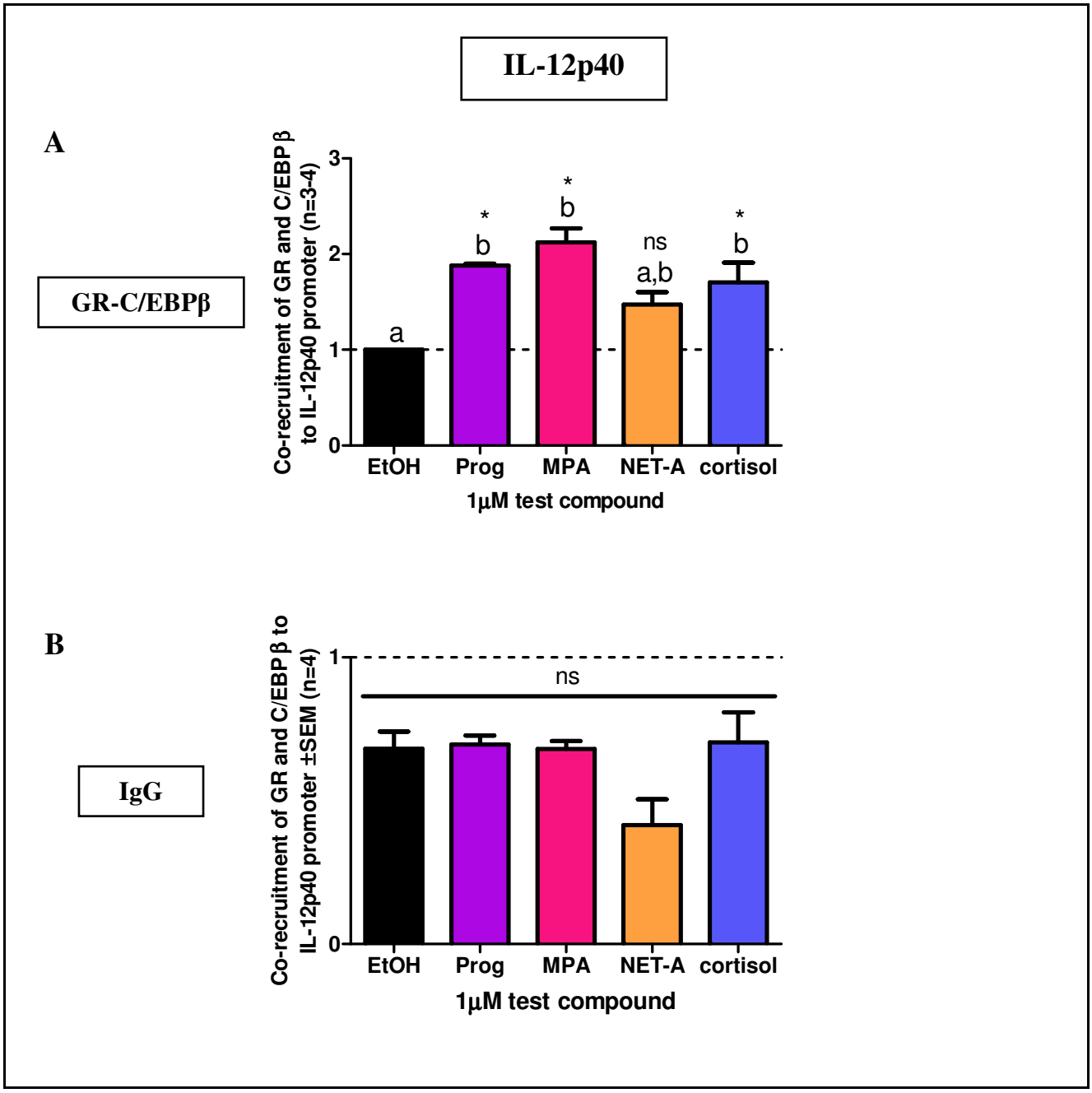

Figure 3.8. The endogenous GR and C/EBPβ are in a complex recruited to the endogenous IL-12p40 promoter in the Ect1/E6E7 cell line in response to Prog, MPA and NET-A. Human Ect1/E6E7 cells were incubated with 0.02 μg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 μM Prog, MPA, NET-A or cortisol for 2 hours. Cell lysates were subjected to immunoprecipitation first with **(A)** the GR-specific antibody (ChIP) and then with the C/EBPβ antibody (re-ChIP) or **(B)** IgG. The immunoprecipitation DNA fragments and input DNA were analysed by realtime qPCR. Data shown are normalised to input and expressed as the fold-response relative to EtOH (control) in A, which was set as one. Results shown are the average (± SEM) of at least three independent experiments. One-way ANOVA analysis of variance and both Bonferroni (compares all pairs of columns) and Dunnett (compares all columns vs. control (EtOH) column) post-tests were used for statistical analysis.

3.2.5. STAT-3 is required for the GR-mediated downregulation of IL-10 gene expression in response to MPA and NET-A

The results indicating that the GR is recruited to the Sp1/STAT-3 regulatory elements of the endogenous IL-10 promoter, but not to Sp1/GRE regulatory elements, suggest that the STAT-3 regulatory element is important for the transcriptional suppression of IL-10 gene expression. The involvement of the transcription factor STAT-3 in the ligand dependent GR-mediated downregulation of IL-10 mRNA expression was thus further investigated by siRNA. The Ect1/E6E7 cell line was transfected with 10 nM validated NSC siRNA or STAT-3-specific siRNA oligonucleotides, followed by treatment with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. Western blot analysis showed 55% reduction in the expression of endogenous STAT-3 protein levels as compared to the NSC (figure 3.9A and 3.9B). Reduced STAT-3 levels significantly reversed the cortisol-induced downregulation of IL-10 (figure 3.9C). Similarly, the Prog, MPA and NET-A induced downregulation of IL-10 gene expression was significantly attenuated by the decrease in STAT-3 levels, suggesting a role for STAT-3 in the GR-mediated downregulation of IL-10 gene in response to these ligands. Interestingly, an increase for Prog and MPA was observed when the STAT-3 levels were reduced.

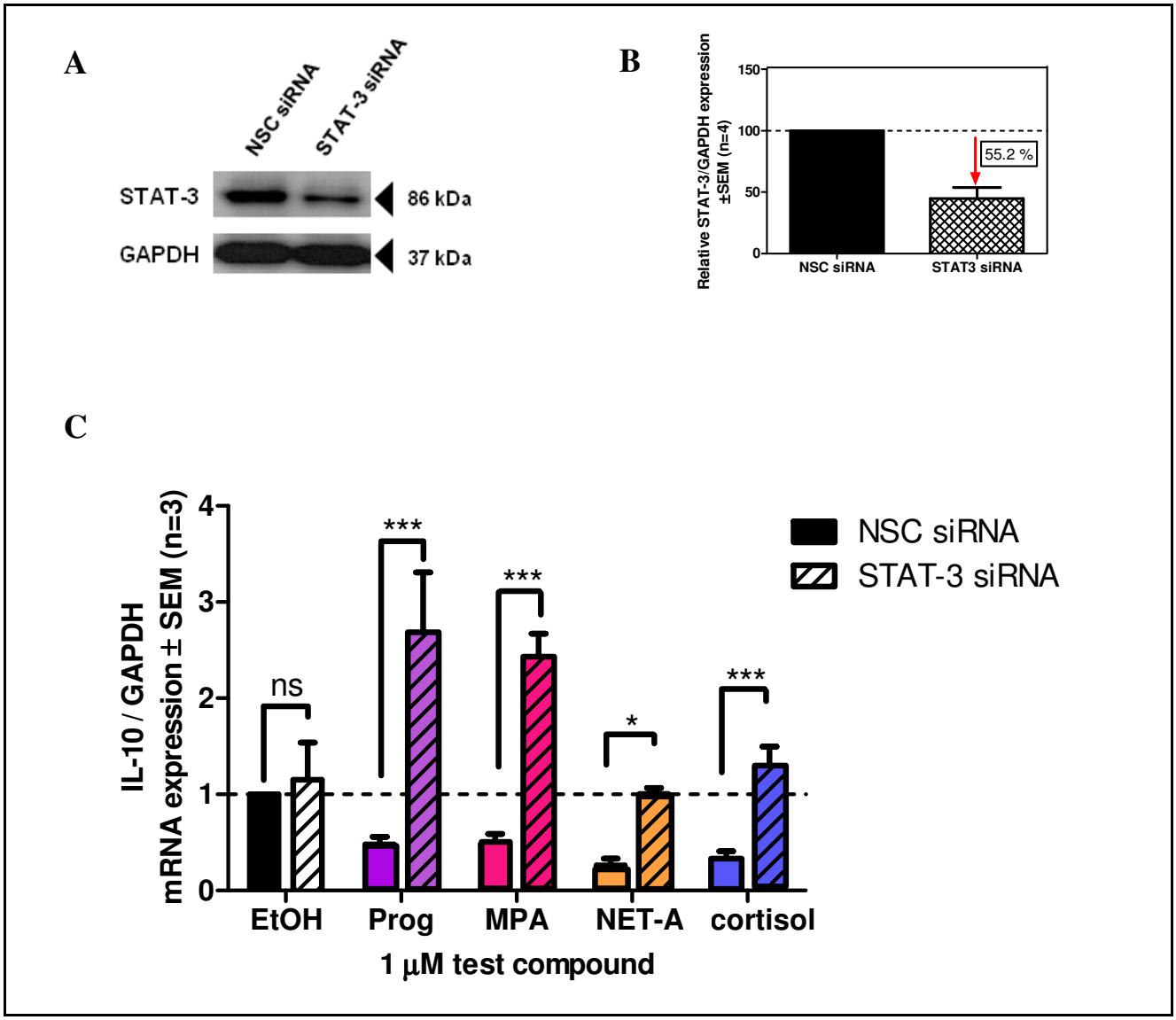

Figure 3.9. Reduced STAT-3 protein by siRNA indicates a role for STAT-3 in the GR mediated downregulation of IL-10 gene expression in the Ect1/E6E7 cell line. Human Ect1/E6E7 cells were transfected with 10 nM NSC or STAT-3 siRNA oligonucleotides and after 24 hours stimulated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. **(A)** For verification of STAT-3 knockdown, total protein from the untreated cells was harvested to perform Western blotting using antibodies specific for STAT-3 and GAPDH. The latter was used as a loading control. A representative blot is shown. **(B)** STAT-3 expression levels relative to GAPDH were quantified using UN-SCAN-IT. Western blots of at least four independent experiments were quantified to determine the percentage STAT-3 protein knockdown. **(C)** Total RNA was isolated and reversed transcribed to cDNA. Thereafter realtime qPCR was performed to determine the mRNA expression levels of IL-10, using GAPDH as the internal standard. Relative IL-10 gene expression of treated samples was calculated relative to vehicle control (EtOH) of the NSC siRNA, which was set as one. Results shown are the average (± SEM) of at least three independent experiments. Two-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis.

Taken together, these results suggest that STAT-3 is needed for the downregulation of IL-10 gene expression by Prog-, MPA- or NET-A- bound GR, suggesting that the GR and STAT-3 are co-recruited to the IL-10 promoter. Co-immunoprecipitation assays were thus used to investigate whether these two proteins interact with each other. The Ect1/E6E7 cell line was treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for an hour, and immunoprecipitated using a GR-specific antibody. Western Blot analysis confirmed the presence of the GR and STAT-3 in the input samples (figure 3.10A). Figure 3.10B shows that the GR is present in all the immunoprecipitated samples, independent of the presence of ligand. When the same membrane was stripped to remove the anti-GR antibody, and reprobed with a STAT-3-specific antibody, the results like those observed for C/EBPβ, show that STAT-3 co-immunoprecipitated with the GR, in the presence of TNF-α, and that the ligands do not disturb the interaction. These results suggest that the GR and STAT-3 can occur in a complex in the Ect1/E6E7 cell line. No interaction between GR and STAT-3 was observed when immunoprecipitated with IgG. As for the GR-C/EBPβ co-immunoprecipitation assay (figure 3.7B), high signal background was observed for the GR Western blot. Thus, the co-immunoprecipitation assay should be repeated by firstly probing with a STAT-3-specific antibody, and then reprobing with a GR-specific antibody.

Having shown that STAT-3 is required for the downregulation of IL-10 gene expression by Prog, MPA and NET-A via the GR, and that the GR and STAT-3 can occur as a complex, the co-localisation of the GR-STAT-3 protein complex on the endogenous IL-10 promoter was next investigated by using a re-ChIP assay. Intact Ect1/E6E7 cells were treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 2 hours. The immunoprecipitated GR-STAT-3 proteins bound to the endogenous IL-10 promoter were detected using primers spanning 84 bp of the IL-10 promoter, which comprises the STAT-3 regulatory

element. The immunoprecipitated DNA fragments and input DNA were analysed by realtime qPCR. Similar results were obtained for the re-ChIP assay (figure 3.11A) when compared to that of the ChIP assay (figure 3.5B). The level of recruitment of the GR-STAT-3 complex to the endogenous IL-10 promoter at the STAT-3 binding site by Prog, MPA, NET-A and cortisol treatment didn't significantly differ from each other (figure 3.11A). The IgG negative control confirmed the specificity of this GR-STAT-3 co-localisation on the IL-10 promoter, as no recruitment of this complex was observed in the presence of an anti-IgG antibody (figure 3.11B). Taken together, our results show that, in response to Prog, MPA and possibly NET-A, the GR interacts with STAT-3, and that endogenous GR and STAT-3 are in a common complex bound to the IL-10 promoter.

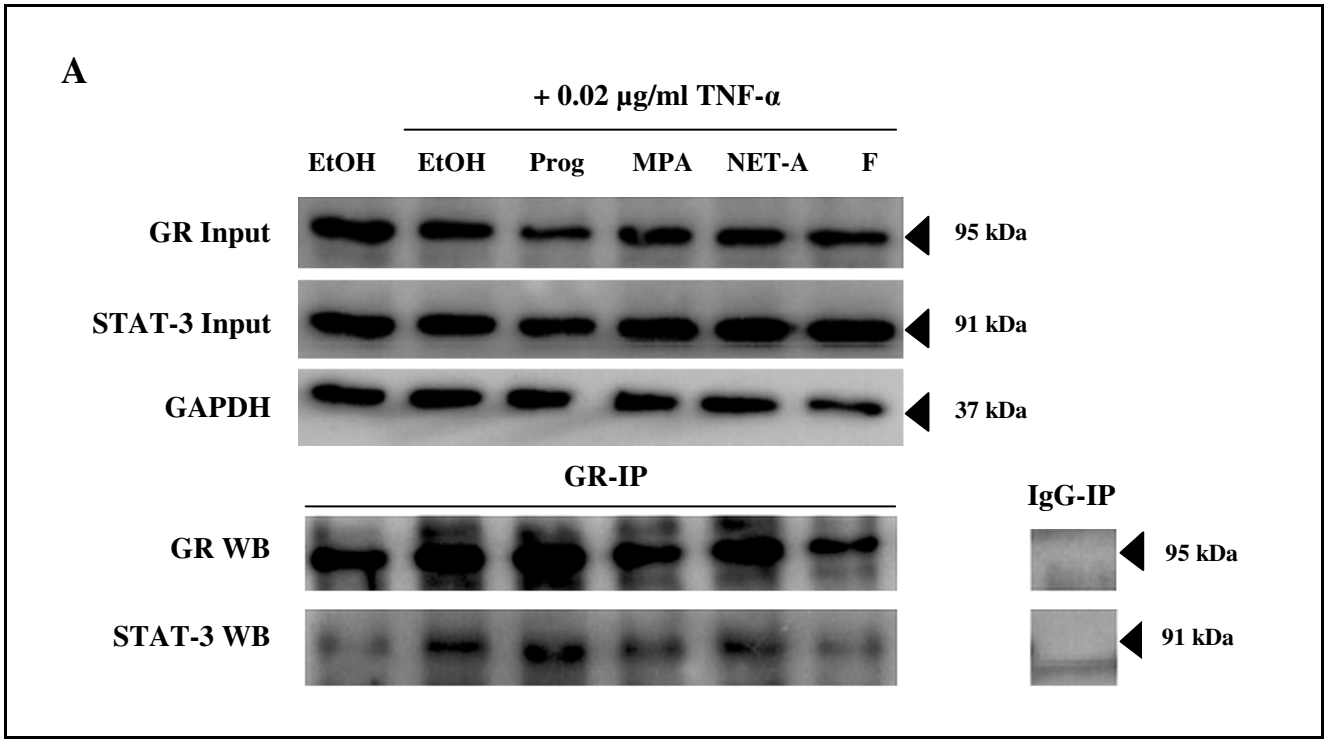

Figure 3.10. The liganded GR and STAT-3 occurs in a complex in the Ect1/E6E7 cell line. Human Ect1/E6E7 cells were incubated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for an hour. Cellular extracts were immunoprecipitated with a GR-specific antibody, and Western blots were probed firstly with the GR-specific antibody and then stripped and reporbed with the STAT-3 antibody. A representative blot of three independent experiments is shown. **(A)** Both the GR and STAT-3 are present in the input samples. **(B)** STAT-3 co-immunoprecipitated with the GR only in the presence of ligand, indicating that the liganded GR and STAT-3 can occur in a complex in the Ect1/E6E7 cell line.

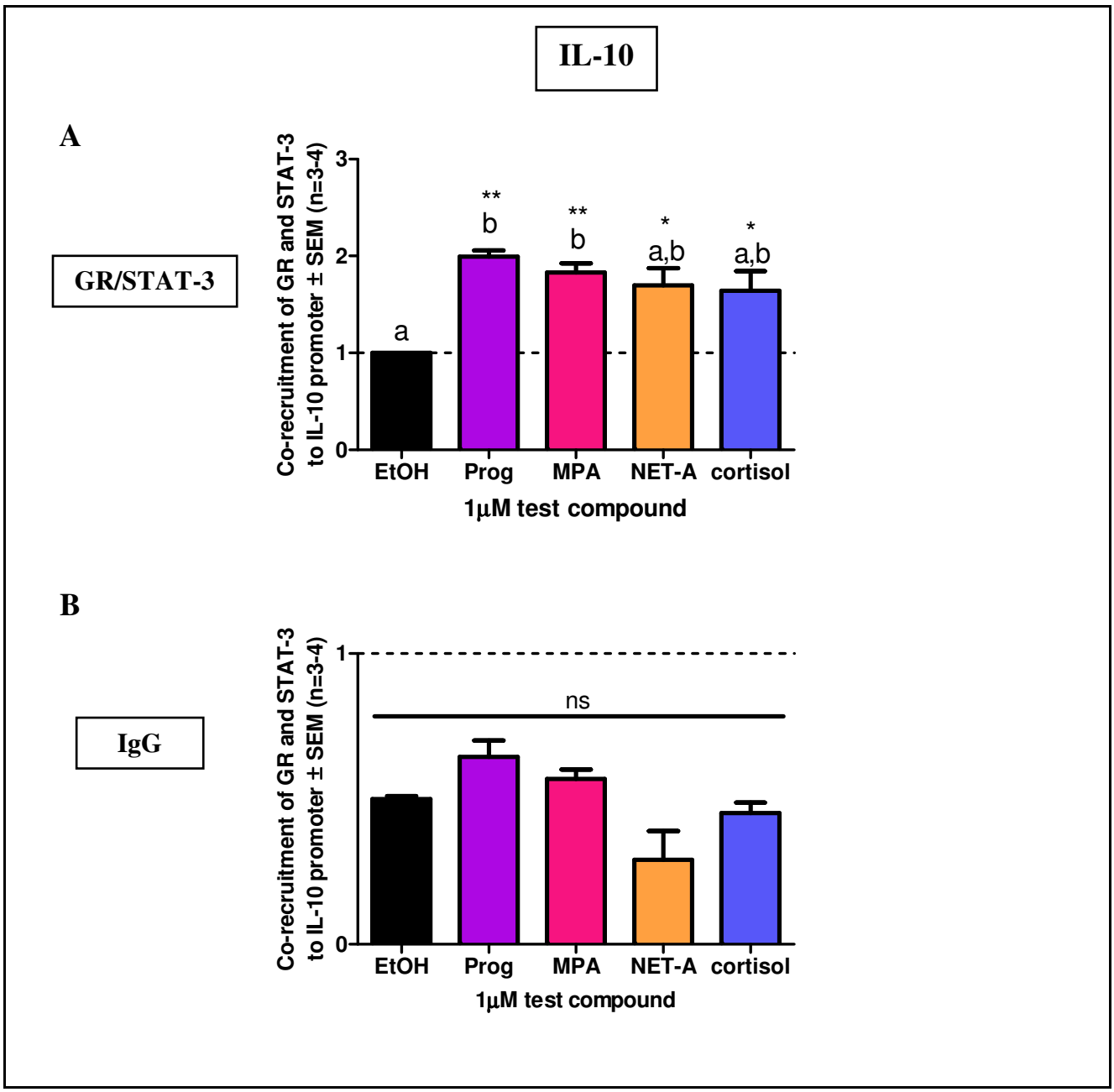

Figure 3.11. The endogenous GR and STAT-3 are in a complex recruited to the endogenous IL-10 promoter in the Ect1/E6E7 cell line in response to Prog, MPA and NET-A. Human Ect1/E6E7 cells were incubated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 2 hours. Cell lysates were subjected to immunoprecipitation first with **(A)** the GR-specific antibody (ChIP) and then with the STAT-3 antibody (re-ChIP) or **(B)** IgG (re-ChIP). The immunoprecipitation DNA fragments and input DNA were analysed by realtime qPCR. Data shown are normalised to input and expressed as the fold-response relative to EtOH (control) in A, which was set as one. Results shown are the average (± SEM) of at least three independent experiments. One-way ANOVA analysis of variance and both Bonferroni (compares all pairs of columns) and Dunnett (compares all columns vs. control (EtOH) column) post-tests were used for statistical analysis.

3.3. Discussion

Inflammation in the female genital tract is influenced by immune mediators produced by epithelial cells (Givan et al., 1997, Ochiel et al., 2008), and may modulate the mucosal immune function (Alfano and Poli, 2005). For example, pro-inflammatory cytokines attract and stimulate immune cells, thereby promoting inflammation (Dinarello, 2000, Wira et al., 2005b, Ochiel et al., 2008), while anti-inflammatory cytokines reduce inflammation by suppressing the activity of pro-inflammatory cytokines (Opal and DePalo, 2000). Correlation analysis suggest that an increase in inflammation of the human female genital tract may increase susceptibility to infections such as HIV-1 (reviewed in (Kaushic et al., 2010, Blish and Beaten, 2011, Roberts et al., 2012a), (Kreiss et al., 1994, Gumbi et al., 2008, Nkwanyana et al., 2009). Thus the cytokine milieu in the female genital tract, including the ectocervical mucosa, is thought to be a vital determinant of susceptibility to infections such as HIV-1 (reviewed in (Gravitt and Ghanem, 2010, Kaushic et al., 2010). The pro-inflammatory cytokine, IL-12, and anti-inflammatory cytokine, IL-10, have been reported to play a role in HIV-1 infection (Chehimi et al., 1992, Chehimi et al., 1994, Kelly et al., 1997, Cohen et al., 1999, Ma and Montaner, 2000, Little et al., 2006, Gee et al., 2007, Byrnes et al., 2008). A decrease in the secretion of IL-12 and an increase in IL-10 production may alter susceptibility of HIV-1. Several factors such as the female sex steroid hormones and hormonal contraceptives have been shown to modulate mucosal immunity in the female genital tract by modifying the cytokine milieu (reviewed in (Gravitt and Ghanem, 2010, Hel et al., 2010, Blish and Beaten, 2011, Wira et al., 2011), and thereby influence the balance between pro- and anti-inflammatory immune responses. Understanding factors such as endogenous hormones or hormonal contraception that may influence the local immune response in the cervicovaginal environment is essential, as it is the first portal of entry for HIV-infection. Little is known about the effects of the synthetic progestins MPA and NET-A widely used in contraception on local epithelial immune function. Our recent study, using human ectocervical and vaginal epithelial cell lines as *in vitro* models for mucosal

immunity, showed that Prog, MPA and NET-A differentially regulate TNF-α induced pro-inflammatory cytokine (IL-6) and chemokine (IL-8 and RANTES) gene expression, in a ligand-, promoter- and cell-specific manner (Africander et al., 2011a). Using the human ectocervical epithelial cell line (Ect1/E6E7) as an *in vitro* cell culture model system for mucosal immunity, the present study investigated the effects, and the underlying mechanisms, of Prog, MPA or NET-A on the mRNA expression of the cytokines, IL-12 and IL-10. The Ect1/E6E7 cell line expresses characteristics of multiple layers of stratified squamous non-keratinising epithelial cells (Fichorova et al., 1997, Fichorova and Anderson, 1999, Quayle, 2002, Wira et al., 2005a, Wira et al., 2005b), and expresses a number of immune mediators either constitutively or when stimulated with TNF-α (Fichorova and Anderson, 1999). For the purpose of this study, the ectocervical cell line was treated with TNF-α to mimic infection in the non-sterile ectocervical environment.

The general observation was that Prog, MPA and NET-A significantly upregulate the expression of the pro-inflammatory cytokine gene IL-12p40 (figure 3.1C), while significantly downregulating the expression of the anti-inflammatory cytokine gene IL-10 (figure 3.1D). Considering that these cytokine genes are known to have opposing roles in the inflammatory response, the results from this study showing opposing effects on IL-12p40 and IL-10 gene expression were not unexpected. Surprisingly however, was the fact that Prog upregulated IL-12p40 gene expression. Several studies have previously shown that Prog inhibits the production of IL-12 or IL-12p40 in other cell types (Par et al., 2003, Enomoto et al., 2007, Hughes et al., 2008, Jones et al., 2008). Similarly, the contraceptive use of MPA has previously been shown to downregulate the protein expression of IL-12p40 in human peripheral blood mononuclear cells (PBMCs) (Kleynhans et al., 2011). However, it has been reported that IL-12 levels were higher in the vaginal lavage fluid of adolescent female Depo-Provera users, than non-users (Barousse et al., 2007). In agreement with this, the protein concentration of IL-12 in cervical secretions has also been shown to be elevated by oral

contraceptives (Gravitt et al., 2003). The results showing downregulation of IL-10 by Prog and MPA are consistent with other studies. For example, IL-10 produced in PBMCs have been shown to be downregulated in MPA users (Kleynhans et al., 2011), while MPA decreased the lipopolysaccharide (LPS)-induced IL-10 mRNA and protein levels in the cervix of mice (Elovitz and Gonzalez, 2008). Nonetheless, a number of other studies have shown that Prog has no effect on the protein production of IL-10 (reviewed in Bouman et al., 2005; (Visser et al., 1998, Shrier et al., 2003, Jones et al., 2008, Hickey et al., 2012). To the best of our knowledge, our study is the first to report on the gene regulation of IL -12p40 and IL10 by NET-A. Attempts to determine the effects of these ligands on IL-12p40 and IL-10 protein levels (Addendum A, figure A3) were less successful. Although there are some changes in response to certain ligands, these results may not be significant as the absolute protein concentration values determined were at the lower end of the standard curve. Thus, further optimisation of these experiments need to be performed.

The physiological implications of our results are potentially important and should be considered in the light of the fact that a balance between pro- and anti-inflammatory cytokines should be maintained in order to mediate homeostasis in the cervicovaginal environment. Our results showing upregulation of the pro-inflammatory IL-12 gene and downregulation of the anti-inflammatory IL-10 gene by the progestins, suggest that the balance is disrupted, favouring a pro-inflammatory milieu, and thus promoting chronic inflammation. Initially, the upregulation of IL-12p40 gene expression in the ectocervix may be protective, as IL-12 plays an important role in the early events of an immune response and is essential in the defense against pathogens (Hasko and Szabo, 1999, Barousse et al., 2007, Hamza et al., 2010), for example *Chlamydia trachomatis* infection (Cummins and Doncel, 2009). It has also been shown that the addition of exogenous IL-12 protected thermally injured mice against local HSV-1 infection (Matsuo et al., 1996). On the other hand, it has been shown that chronic inflammation may result due to prolonged production of pro-inflammatory

mediators. Thus long term use of MPA or NET-A, as in the case of contraception, may lead to chronic inflammation of the ectocervical environment, which may increase a women's risk of susceptibility to viral infections such as HIV-1 and HSV. Consistent with a pro-inflammatory environment increasing susceptibility to HIV-1, Naicker and co-workers proposed that a decrease in the production of IL-10 in blood may increase susceptibility to HIV-1 and enhance disease progression (Naicker et al., 2009). However, a limitation of this study is that only a single cell line was used as an *in vitro* model for mucosal immunity. Based on the results from a recent study conducted by our group showing ligand-, promoter- and cell-specific effects of MPA and NET-A, it may be that the effects of the progestogens on IL-12p40 and IL-10 gene expression is cell-specific. For this reason, it would be ideal to investigate the regulation of the IL-12p40 and IL-10 genes by the progestogens using other cell lines.

Considering that our previous study showed that the GR is the only endogenous steroid receptor transcriptionally competent in both transactivation and transrepression in the Ect1/E6E7 cell line (Africander et al., 2011a), the role of the GR in the regulation of the mRNA expression of IL-12 and IL-10 by the progestogens was further investigated. Firstly using competitive whole cell binding and Western blotting, we showed that Prog, MPA and NET-A bind to the endogenous GR in the Ect1/E6E7 cell line (figure 3.2A) and that none of these ligands modulate GR protein levels (figure 3.2B and 3.2C). Secondly, using siRNA to reduce the GR protein levels, the progestin-mediated upregulation of IL-12p40 (figure 3.3C) and downregulation of IL-10 (figure 3.3D) was significantly attenuated. As this study showed that the effects of the progestogens on IL-12p40 and IL-10 mRNA expression was completely lost when the GR protein levels were reduced, the role of other steroid receptors endogenously expressed in this cell line was not investigated. The finding that MPA upregulates IL-12p40 gene expression and downregulates IL-10 gene expression via the GR was unexpected as the glucocorticoid properties of MPA have previously been suggested to be

responsible for its anti-inflammatory effects (Bamberger et al., 1999, Kurebayashi et al., 2003, Koubovec et al., 2004, Koubovec et al., 2005). Similarly, the lack of anti-inflammatory activity of the natural glucocorticoid cortisol was also somewhat unexpected (John et al., 1998, Richards et al., 2000, Mozo et al., 2003, Peek et al., 2005). Generally GR ligands, including Dex, cortisol and MPA, downregulate pro-inflammatory immune mediators and upregulate anti-inflammatory immune mediators (reviewed in De Bosscher and Haegeman, 2009). However, our study shows that this is not always the case. Consistent with our results (figure 3.3C and 3.3D), GR ligands such as Dex have been shown to elicit pro-inflammatory immune responses in other cell types (Visser et al., 1998, Lannan et al., 2012). In addition, a role for the GR in the Prog-induced downregulation of IL-12p40 in macrophages (Jones et al., 2008) has been shown.

Considering that Prog, MPA and NET-A have previously been shown to have different relative binding affinities (RBAs) and transcriptional activities via the GR (reviewed in Africander et al., 2011b; (Kontula et al., 1983, Koubovec et al., 2005, Ronacher et al., 2009), it was surprising that, in these cells, these progestogens displayed similar effects on IL-12 and IL-10 gene regulation via the GR. As it has recently been shown that MPA and NET-A differentially regulate cytokine gene expression, in a ligand-, cell- and promoter-specific manner (Africander et al., 2011a), discrepancies between the results from this study, showing similar activities of MPA and NET-A on IL12p40 and IL-10 gene expression via the GR in the ectocervical epithelial cell line, and those showing differing activities on synthetic GRE-containing promoters in COS-1 and human embryonic kidney cells (HEK293), may be due to either cell- or promoter-specific effects. The hypothesis of a promoter-specific effect is further strengthened by the result in this study showing that the GR is recruited to the endogenous GRE-containing GILZ promoter (Chen et al., 2006) in response to MPA, but not NET-A (figure 3.5D). However, considering that we do not see

recruitment of the GR to this promoter in response to NET-A, it would be of interest to investigate whether MPA and NET-A differentially regulate GILZ gene expression in the Ect1/E6E7 cell line.

The classically accepted mechanism of GR function, is transactivation via direct binding of the ligand-bound GR to glucocorticoid response elements (GREs) within promoters of target genes, while transrepression would occur via direct protein-protein interactions between the liganded GR and other transcription factors such as NFκB, AP-1 or C/EBP (Edwards et al., 1995, Cato and Wade, 1996, McKay and Cidlowski, 1999, Webster and Cidlowski, 1999, De Bosscher et al., 2000, Newton, 2000, Osborne et al., 2000, Almawi and Melemedjian, 2002, De Bosscher et al., 2003, Leonhardt et al., 2003, Zhou and Cidlowski, 2005, Liberman et al., 2007, Kramarova et al., 2009, Africander et al., 2011b). The latter protein-protein interaction is referred to as a tethering mechanism. However, human IL-12 and IL-10 gene expression can be regulated via several inducible transcription factors that bind to specific binding sites, such as NFκB, activator protein (AP)-1, Sp1, STAT and C/EBP and GREs (Jones and Tjian, 1985, Grimm and Baeuerle, 1993, Kube et al., 1995, Murphy et al., 1995, Plevy et al., 1997, Gri et al., 1998, Benkhart et al., 2000, Becker et al., 2001, Ma et al., 2001, Cao et al., 2002, Ziegler-Heitbrock et al., 2003, Chambers et al., 2004, Ma et al., 2004, Kremer et al., 2007, Mosser and Zhang, 2008, Szalmas et al., 2008, Unterberger et al., 2008), within the promoters of these cytokine genes. Thus, considering that IL-12p40 gene expression is upregulated and IL-10 gene expression is downregulated, it is expected that the liganded GR binds to a GRE in the IL-12p40 promoter and one of the above-mentioned transcription factors in the IL-10 promoter, respectively. Using a combination of siRNA, co-immunoprecipitation, ChIP and re-ChIP assays, we show that the regulation of IL-12p40 and IL-10 by these ligands activate distinct signalling pathways, leading to binding of the GR to specific regulatory elements. It is important to note, that tethering of the GR to other DNA-bound transcription factors may also enhance target gene expression (Johansson-Haque et al., 2008). For

example, glucocorticoids (Dex) have been shown to positively regulate transcription of the MAPK-phosphatase gene in human epithelial lung carcinoma cells, by recruiting the transcription factor C/EBPβ to the promoter, with C/EBPβ tethered to glucocorticoid-bound GR (Johansson-Haque et al., 2008). Similarly, the GR has been shown to interact with C/EBPβ to transactivate haptoglobin (Hp) gene expression in rats during acute phase responses (Arambasic et al., 2010). The results in this study suggest the formation of a cellular complex of progestin-bound GR and C/EBPβ with the endogenous IL-12p40 promoter, likely via binding to C/EBPβ regulatory elements to positively regulate transcription of the human IL-12p40 gene. C/EBPβ has previously been reported to play a crucial role in the transcriptional activation of the mouse IL-12p40 gene (Plevy et al., 1997). In terms of the GR-mediated downregulation of the IL-10 gene in response to Prog, MPA and NET-A (figure 3.9C), this study shows a requirement for STAT-3. Using Co-IP assays, a protein-protein interaction between the GR and STAT-3 has previously been shown (Zhang et al., 1997, Arambasic et al., 2010). The results in this study suggest the formation of a cellular complex of progestin-bound GR and STAT-3 with the endogenous IL-10 promoter, likely via binding to STAT-3 regulatory elements to negatively regulate transcription of the human IL-10 gene.

To summarise, we propose a model for the molecular mechanism of progestin-induced regulation of IL-12p40 (figure 5.1) and progestin-induced regulation of IL-10 (figure 5.2) in the human ectocervical epithelial cell line. Upon progestogen binding, the GR undergoes a conformation change and translocates to the nucleus. To activate transcription of the human IL-12p40 gene the liganded GR tethers to the transcription factor C/EBPβ and forms a cellular complex, with the endogenous IL-12p40 promoter, likely via binding to the C/EBPβ *cis*-regulatory element. To repress transcription of the human IL-10 gene the liganded GR tethers to the transcription factor STAT-3 to form a protein complex, with the endogenous IL-10 promoter, likely via binding to the STAT-3 *cis*-regulatory element. Our results indicate that Prog, MPA and NET-A used in hormonal

therapy may have important implications for the ectocervical immune function, as pro-inflammatory cytokines have been associated with increased levels of HIV-1 shedding in the female genital tract (Gumbi et al., 2008). The proposed mechanism may be particularly relevant to infections such as HIV-1 in the cervix, as both IL-12 and IL-10 have been shown to be key role players in HIV-1 pathogenesis. Considering the results from a recent clinical study indicating an association between the usage of MPA and increased risk of HIV-1 acquisition (Heffron et al., 2012), our results may provide a potential mechanism whereby the injectable contraceptive Depo-Provera, and possibly Nuristerate, increase women's risk of acquiring HIV-1 and other genital tract infections.

CHAPTER 4

BRIEF INTRODUCTION, RESULTS AND DISCUSSION

MODULATION OF CYTOKINE GENE EXPRESSION IN A HUMAN ECTOCERVICAL EPITHELIAL CELL LINE BY THE HIV-1 ACCESSORY PROTEIN Vpr

4.1. Brief Introduction

HIV-1 is considered a global pandemic with the majority of infections occurring in the female population (Heikinheimo and La, 2008, Kaushic et al., 2010, Le Douce et al., 2010). The majority of these women acquire HIV infection via the female genital tract during male to female transmission (reviewed in (Cummins and Dezzutti, 2000, Pope and Haase, 2003, Hladik and Hope, 2009, Kaushic et al., 2010). The ectocervix is thought to be the first target site to become infected with sexually transmitted HIV-1 (Howell et al., 2005). Although there are studies indicating that epithelial cells of the female genital tract can be infected by HIV-1 and may serve as potential targets for the initial infection by HIV-1 and its systemic spread (Howell et al., 1997, Maher et al., 2005, Bobardt et al., 2007)(reviewed in (Hladik and McElrath, 2008), there has been no conclusive evidence of HIV-1 infection of the genital epithelium *in vivo* (reviewed in Shacklett, 2009 and Haase, 2010) (Chapter 1, literature review). In addition, evidence suggest that direct exposure of the genital epithelial cells to HIV-1 may disrupt the mucosal barrier of the female genital tract and increase the permeability of the mucosal epithelium, possibly due to genital tract inflammation (Nazli et al., 2010).

In the light of the fact that it has been suggested that steroid receptors such as the GR may facilitate successful HIV-1 pathogenesis (Chapter 1, literature review), our strategy was thus to investigate whether HIV-1 Vpr protein modulates TNFα-induced IL-12p35, as well as IL-12p40 and IL-10 gene expression by Prog, MPA and NET-A, and to determine whether the GR plays a role in mediating these effects. IL-12p35 was included in this part of the study as Vpr has previously been reported to modulate transcriptional regulation of IL-12p35, but not that of IL-12p40 (Mirani et al., 2002).

4.2. Results

4.2.1. As observed for IL-12p40, Prog, MPA and NET-A also upregulate IL-12p35 mRNA expression in the Ect1/E6E7 cell line

The previous chapter showed that Prog, MPA and NET-A regulated the mNA expression of IL-12p40 and IL-10. As IL-12 is a heterodimeric glycoprotein consisting of IL-12p40 and IL-12p35 (Kobayashi et al., 1989, Stern et al., 1990, Aragane et al., 1994, Trinchieri and Scott, 1995b, Chizzonite et al., 1998, Trinchieri, 2003, Byrnes et al., 2008), the regulation of IL-12p35 gene expression by Prog, MPA and NET-A was also investigated in this chapter. The human Ect1/E6E7 cell line was treated with 0.02 µg/ml TNF-α and 0.1% EtOH (control) or 1 µM Prog, MPA or NET-A for 6 hours, followed by qPCR analysis for the mRNA expression of IL-12p40 and IL-12p35, respectively. Figure 3.1A and 4.1B shows the effects of the ligands on IL-12p40 and IL-12p35 mRNA expression in response to Prog, MPA and NET-A. No significant differences were detected between any of the ligands in the regulation of IL-12p40 ($p>0.05$), while IL-12p35 gene expression was upregulated to a significantly higher extent by NET-A, than Prog or MPA ($p<0.01$). These results indicate that both IL-12p40 and IL-12p35 gene expression are upregulated by the synthetic progestins, MPA and NET-A, and the endogenous ovarian hormone, Prog.

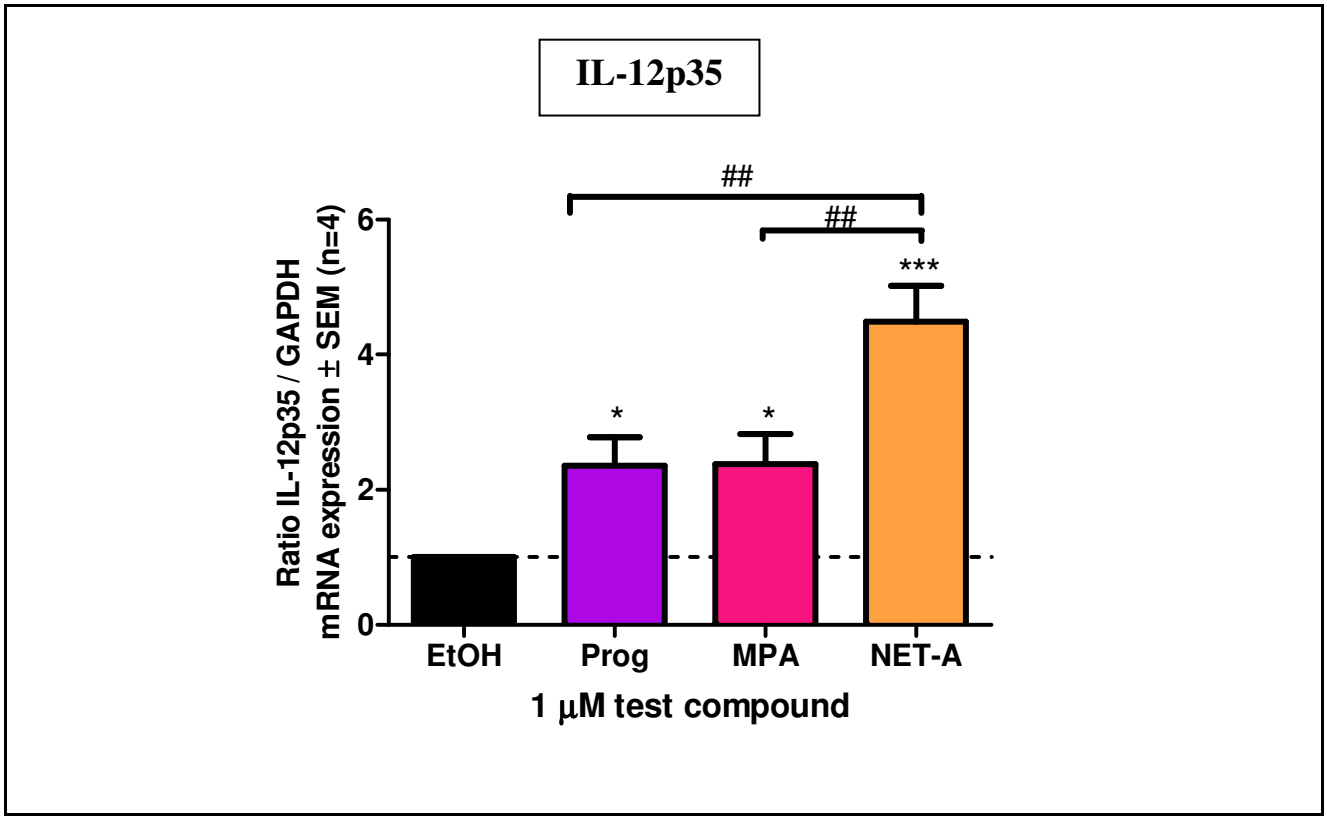

Figure 4.1. Prog, MPA and NET-A upregulate the TNF-α induced expression of IL-12p35 to different extents. The human Ect1/E6E7 cell line was incubated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA or NET-A for 6 hours. Total RNA was isolated and reversed transcribed to cDNA. Realtime qPCR was performed to determine the mRNA expression levels of IL-12p35, using GAPDH as the internal standard. Results shown are the averages (± SEM) of at least four independent experiments. One-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis.

4.2.2. A role for the GR in the regulation of IL-12p35 by Prog and NET-A

As we have shown a role for the GR in mediating the effects of Prog, MPA and NET-A on IL-12p40 (figure 3.3C), we hypothesised that the effects of these ligands on IL-12p35 are also mediated by the GR. To further investigate the role of the GR in mediating the regulation of Prog, MPA and NET-A on IL-12p35 gene expression, the GR was silenced using siRNA transfections. The Ect1/E6E7 cell line was transiently transfected with validated GR specific siRNA oligonucleotides or NSC siRNA oligonucleotides (control) for 48 hours, followed by treatment with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. To confirm GR knockdown, Western blot analysis confirmed reduction of GR protein

levels of approximately 83% (figure 3.3A and 3.3B). Consistent with the GR knockdown result for IL-12p40 (Chapter 3), reduction of GR protein levels significantly abrogates the upregulation of IL-12p35 in response to Prog and partially NET-A, indicating a role for the GR. Although not statistically significant, the GR also seemed to mediate the effect of MPA on IL-12p35. As expected, although not statistically significant, the natural glucocorticoid cortisol decreased IL-12p35 mRNA expression (figure 4.2), and reducing the GR protein levels significantly abrogated this downregulation. Taken together, we show that the GR mediates the effects of Prog, while the effects of NET-A and possibly MPA are partially mediated by the GR.

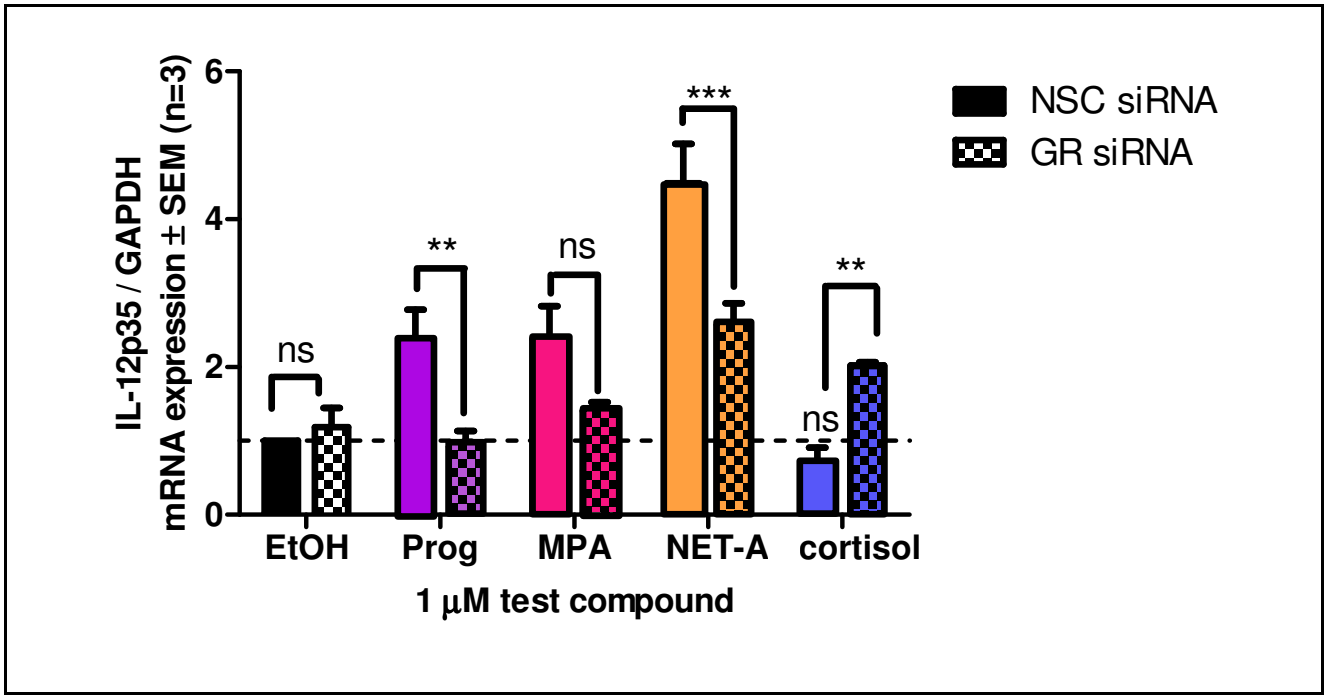

Figure 4.2. Decreasing GR protein levels by siRNA indicates a role for the GR in mediating the effects of Prog, and a partial role for mediating the effects of NET-A, and possibly MPA, on IL-12p35 mRNA expression in the Ect1/E6E7 cell line. The human Ect1/E6E7 cell line transfected with 10 nM GR or NSC siRNA oligonucleotides, was treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. Total RNA was isolated and reversed transcribed to cDNA. Thereafter realtime qPCR was performed to determine the mRNA expression levels of IL-12p35, using GAPDH as the internal standard. Relative IL-12p35 gene expression of treated samples was calculated relative to vehicle control (EtOH) of the NSC siRNA, which was set as one. Results shown are the average (± SEM) of at least three independent experiments. Two-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis.

4.2.3. The HIV-1 accessory protein, Vpr, modulates the effects of the progestogens on IL-12p40, IL-12p35 and IL-10 mRNA expression

Having shown that the progestogens regulate TNF-α-induced IL-12p40 (figure 3.1C), IL-12p35 (figure 4.1) and IL-10 (figure 3.1D) gene expression in the Ect1/E6E7 cell line, thereby possibly providing an environment prone to HIV infections, and that *in vitro* evidence suggest that the GR interacts with the HIV-1 accessory Vpr protein (Refaeli et al., 1995, Kino et al., 1999, Muthumani et al., 2006); reviewed in Hapgood and Tomasicchio, 2010), we investigated whether HIV-1 Vpr modulates the regulation of these cytokine genes to Prog, MPA and NET-A. The HA-tagged Vpr was overexpressed in the human Ect1/E6E7 cell line and treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA or NET-A for 6 hours. Realtime qPCR results show that Vpr significantly reversed the effects of Prog, MPA and NET-A on the mRNA expression of both IL-12p40 (figure 4.3A) and IL-10 (figure 4.3C), but only the NET-A effect on the IL-12p35 gene (figure 4.3B). Although not statistically significant, Vpr inhibited the increase of IL-12p35 mRNA expression in response to Prog and MPA.

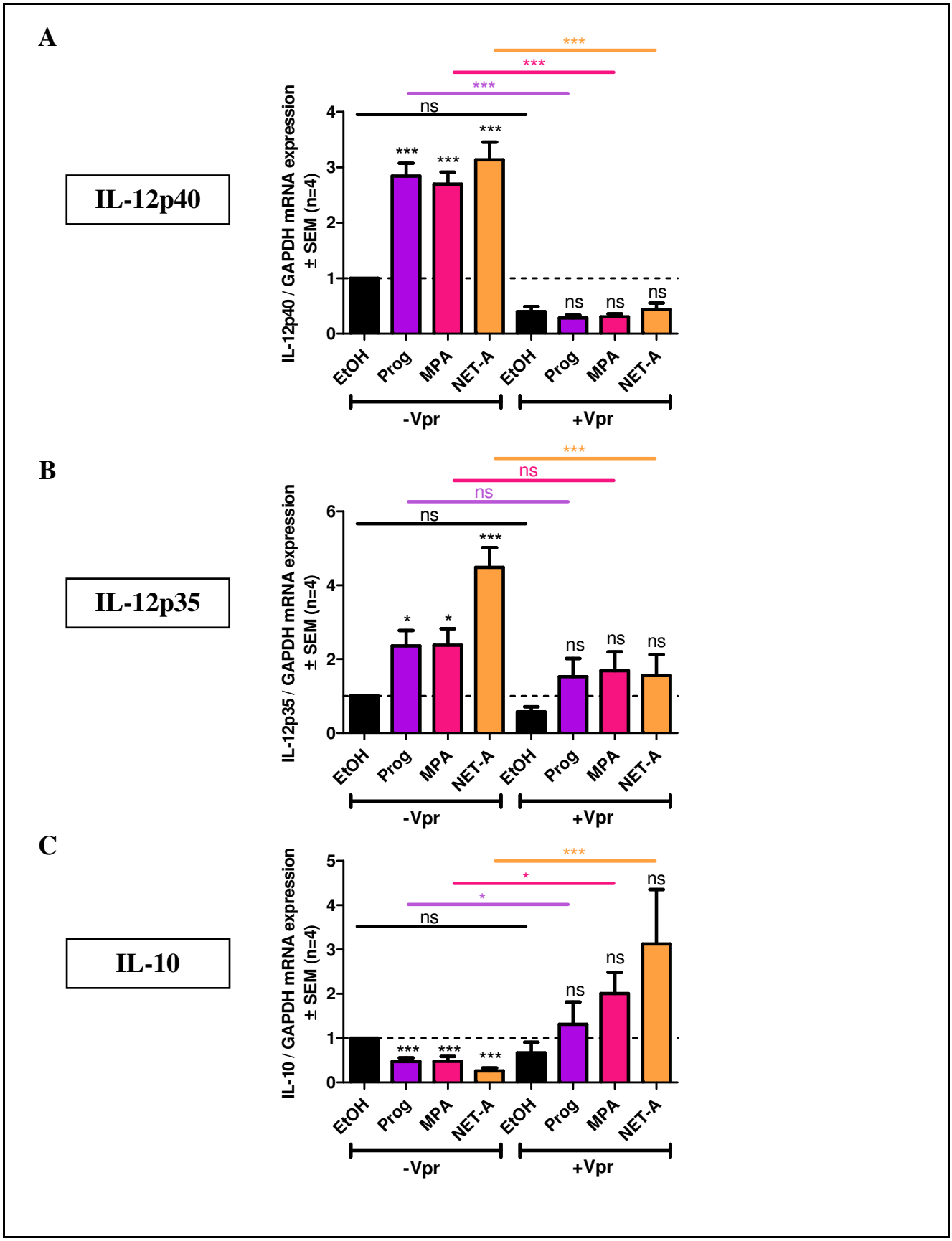

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Figure 4.3. Vpr modulates the effects of Prog, MPA and NET-A on IL-12 and IL-10 gene expression in the Ect1/E6E7 cell line. Human Ect1/E6E7 cells, in the absence (-Vpr) and presence of transiently transfected with 1 µg pCMV4-3HA.Vpr (+Vpr), were treated with 0.02 µg/ml TNF-α and 0.1% EtOH (control) or 1 µM Prog, MPA or NET-A for 6 hours. Total RNA was isolated and reversed transcribed to cDNA. Realtime qPCR was performed to determine the mRNA expression levels of **(A)** IL-12p40, **(B)** IL-12p35 and**(C)** IL-10, using GAPDH as the internal standard. Relative IL-12p40, IL-12p35 and IL-10 gene expression of treated samples was calculated relative to vehicle control lacking Vpr (-Vpr) set as one. One-way ANOVA analysis of variance and Dunnett post-test was peformed to compare each of the treated bars to their corresponding condition's control (i.e. EtOH –Vpr or EtOH +Vpr) bar. This is indicated by *, **, *** or ns above bars. Two-way ANOVA and Bonferroni post-test was used for statistical analysis to compare between the group lacking Vpr (-Vpr) and the group expressing Vpr (+Vpr). This is indicated by the lines above the bars. Results shown are the averages (±SEM) of at least four independent experiments.

4.2.4. HIV-1 Vpr requires the GR for the modulation of cytokine gene regulation in the Ect1/E6E7 cell line

Having shown that HIV-1 Vpr modulates the regulation of the cytokine genes in response to some of the progestogens, we investigated whether the GR was involved in mediating these effects of Vpr. The human Ect1/E6E7 cell line was transfected with 10 nM validated GR-specific siRNA oligonucleotides or NSC siRNA oligonucleotides (control), as well as HA-tagged Vpr, followed by treatment with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. To confirm GR knockdown, Western blot analysis showed reduction of GR protein levels of approximately 70% (figure 4.4A and 4.4B). As indicated in figure 4.5A, the GR is needed for modulating the effects of Vpr on progestogen-induced IL-12p40 gene expression. Surprisingly, when GR protein levels are reduced in the presence of Vpr, cortisol treatment significantly increases IL-12p40 mRNA expression compared to when Vpr is absent. Interestingly, the GR does not mediate the effect of Vpr on the mRNA expression of IL-12p35 in response to NET-A (figure 4.5B). Furthermore, the effect of Vpr on Prog and MPA induced upregulation of IL-10 mRNA expression was not reversed by the reduction of GR protein levels. Surprisingly, although not statistically significant, MPA increased IL-10 gene expression when the GR levels

were reduced. This study shows that Vpr partially requires the GR to modulate the mRNA expression of IL-10 in response to NET-A.

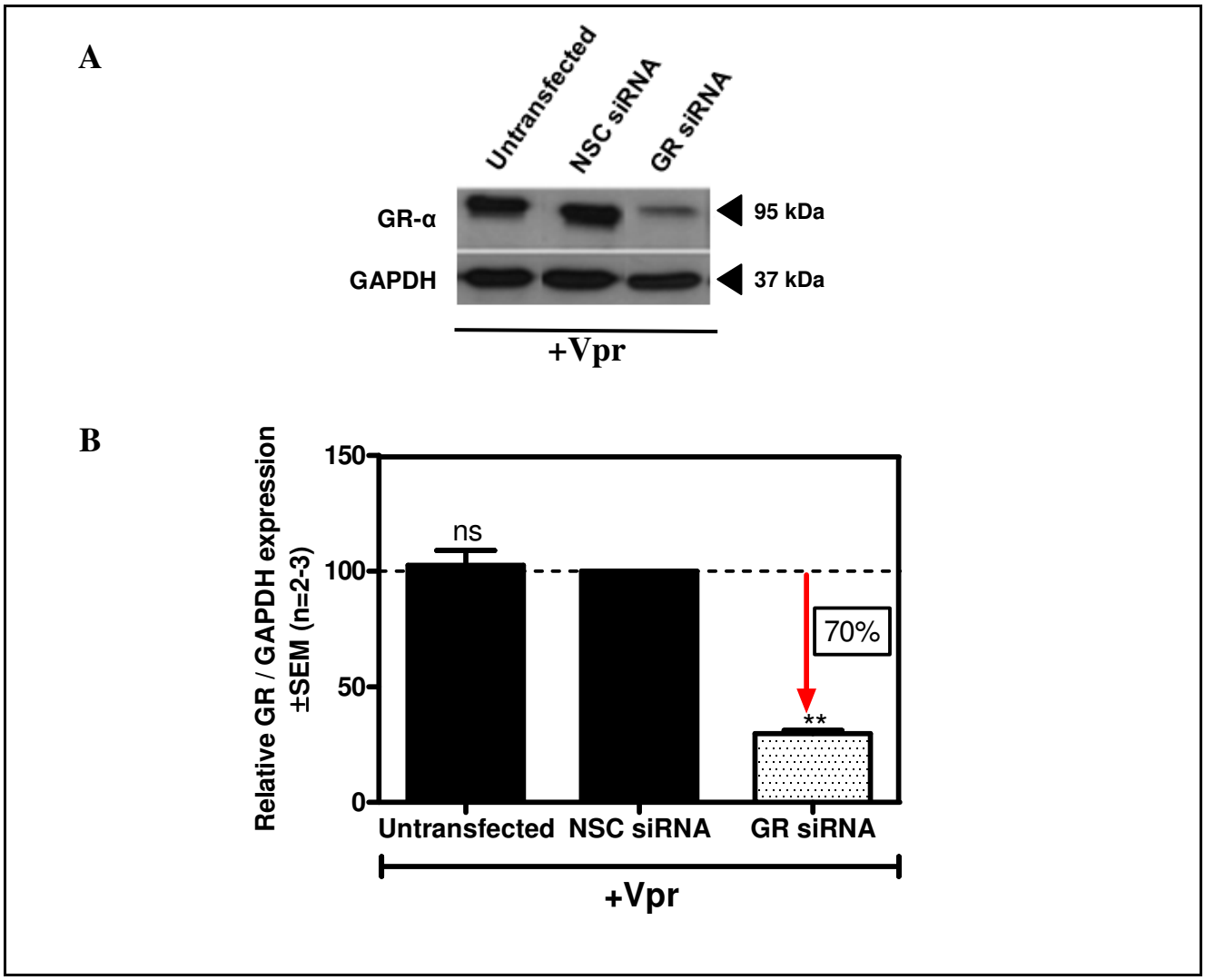
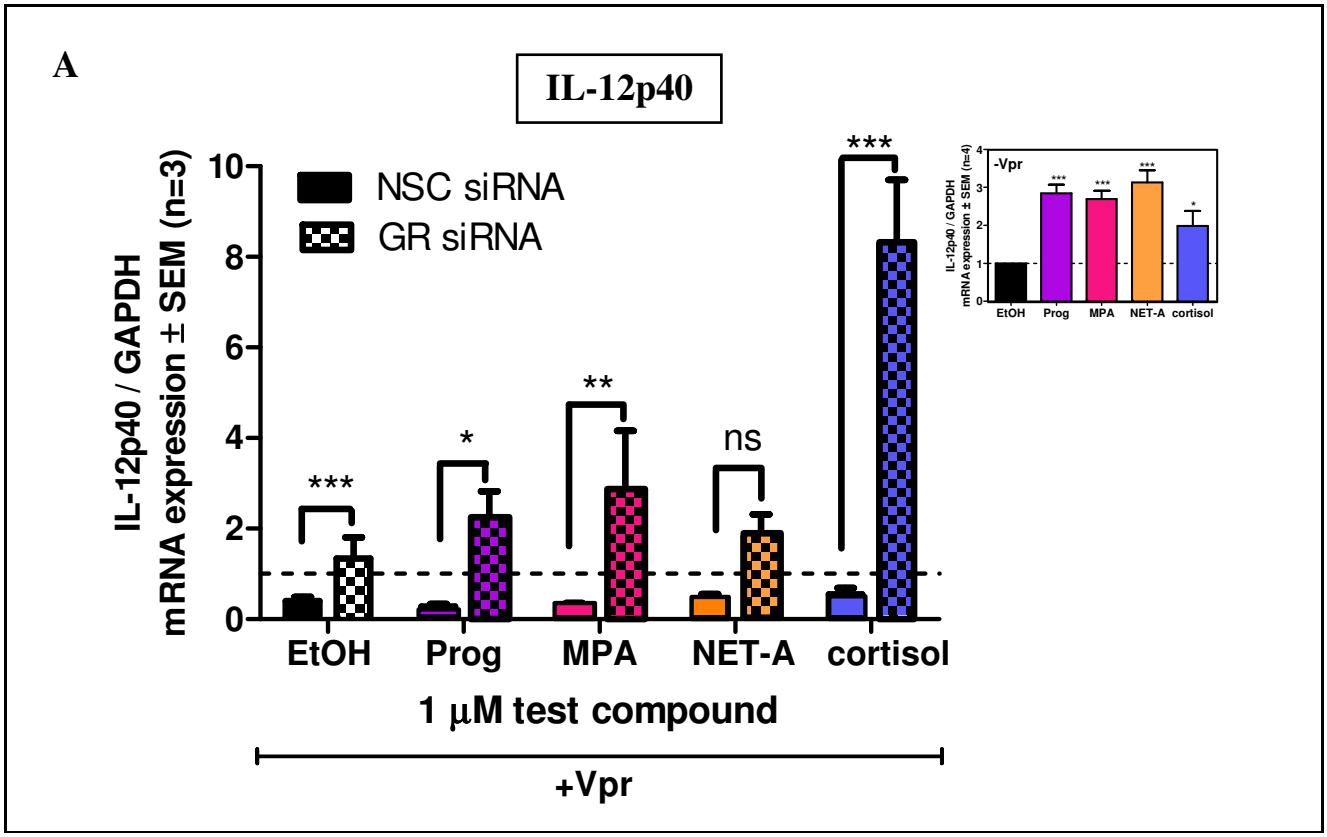
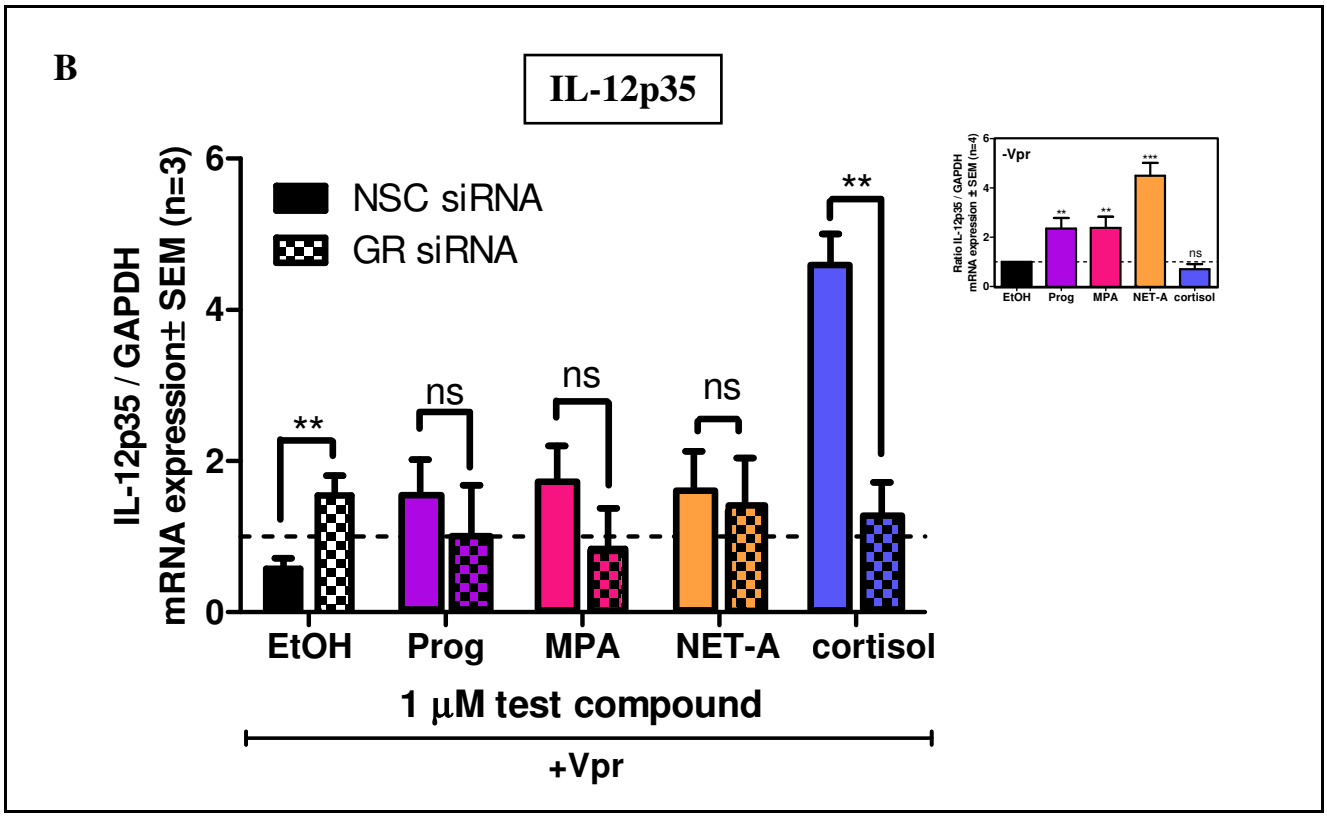


Figure 4.4. Verification of GR knockdown. Total protein from the untreated human Ect1/E6E7 cell line, transiently transfected with 1 µg pCMV4-3HA.Vpr, as well as 10 nM GR or NSC siRNA oligonucleotides, were harvested to perform Western blotting, using antibodies specific for the GR and GAPDH. The latter was used as a loading control. **(A)** A representative blot is shown. **(B)** GR expression levels relative to GAPDH were quantified using UN-SCAN-IT. Western blots of at least two independent experiments were quantified to determine the percentage GR protein knockdown. One-way ANOVA analysis of variance and Dunnett (compares all columns vs. control (NSC siRNA) column) post-test was used for statistical analysis.





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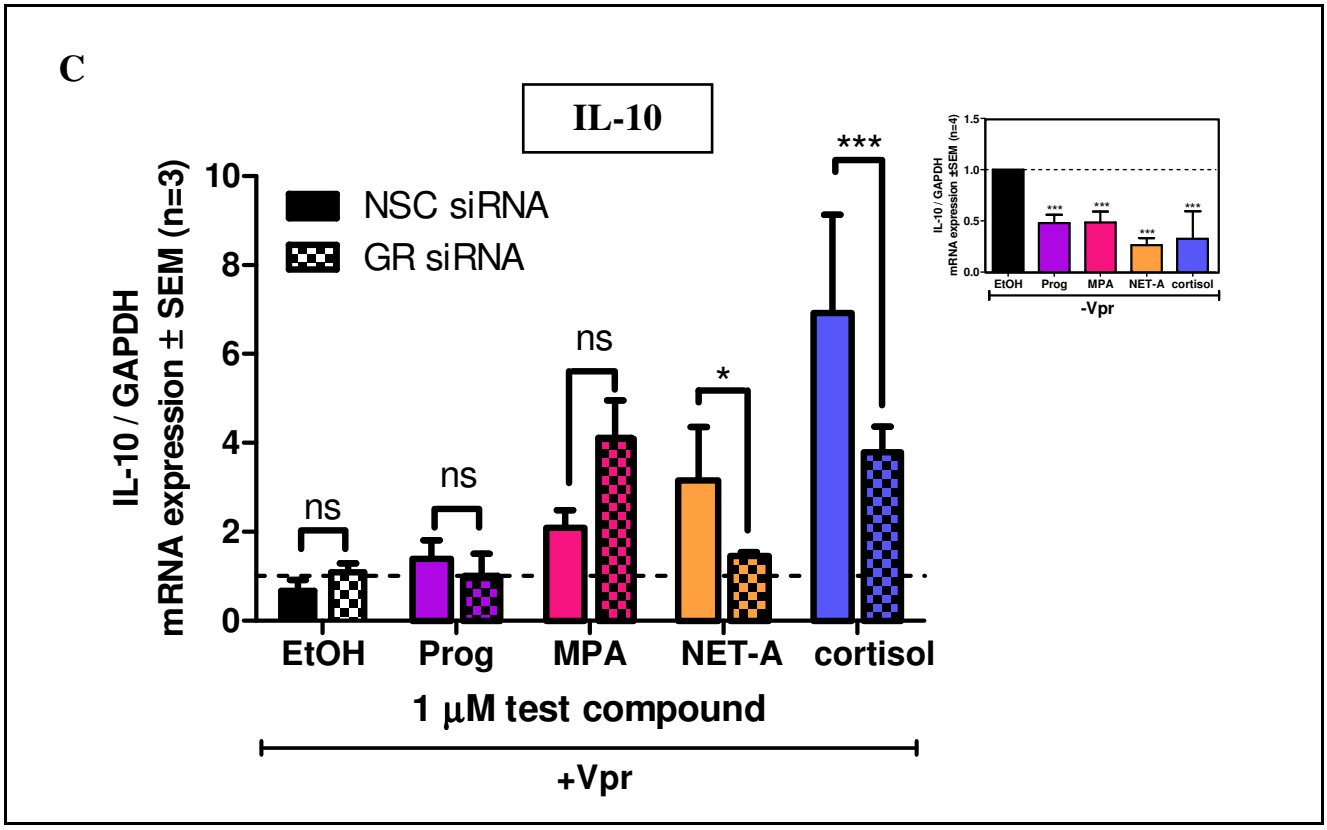
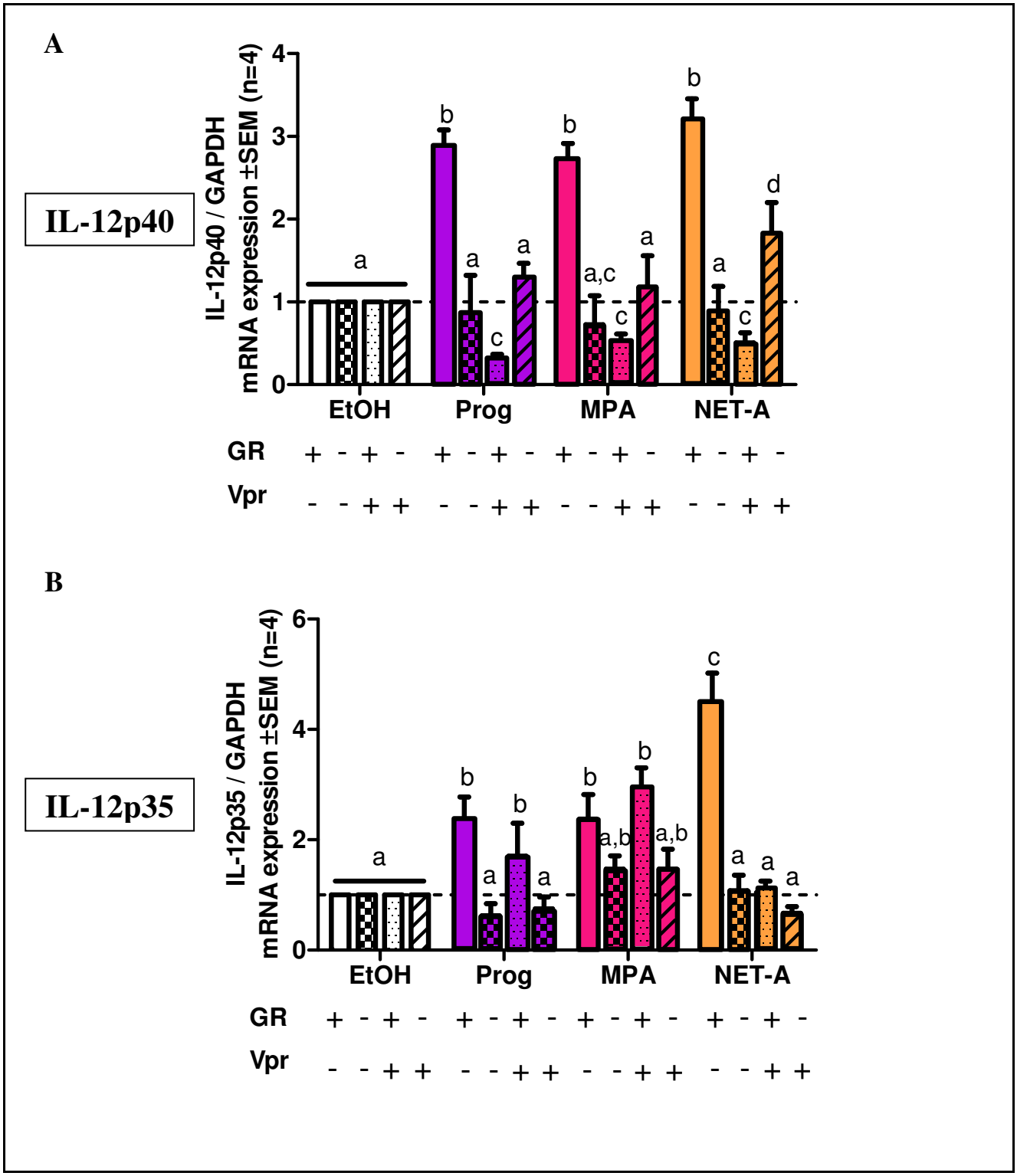


Figure 4.5. Influence of Vpr on the mRNA expression of cytokine genes in response to progestins in a GR-dependent manner in the Ect1/E6E7 cell line. The human Ect1/E6E7 cell line was transfected with 10 nM GR or NSC siRNA oligonucleotides, and after 24 hours transiently transfected with 1 µg pCMV4-3HA.Vpr (+Vpr). The cells were treated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. Total RNA was isolated and reversed transcribed to cDNA. Realtime qPCR was performed to determine the mRNA expression levels of IL-12p40 **(A)**, IL-12p35 **(B)** and IL-10 **(C)**, using GAPDH as the internal standard. Relative IL-12p40, IL-12p35 and IL-10 gene expression of treated samples was calculated relative to the control (NSC siRNA EtOH) lacking Vpr, which was set as one. Two-way ANOVA and Bonferroni post-test was used for statistical analysis. Results shown are the averages (±SEM) of at least four independent experiments. The insert shows the effect of the ligands on the cytokine gene expression in the absence of Vpr.

Figure 4.6 shows the replotting of the results from figures 3.3, 4.2 and 4.5, where each different transfection condition was normalised to its own EtOH+TNF-α which was set as one. An interpretation of these results is proposed in a model in Chapter 5 (figure 5.3).



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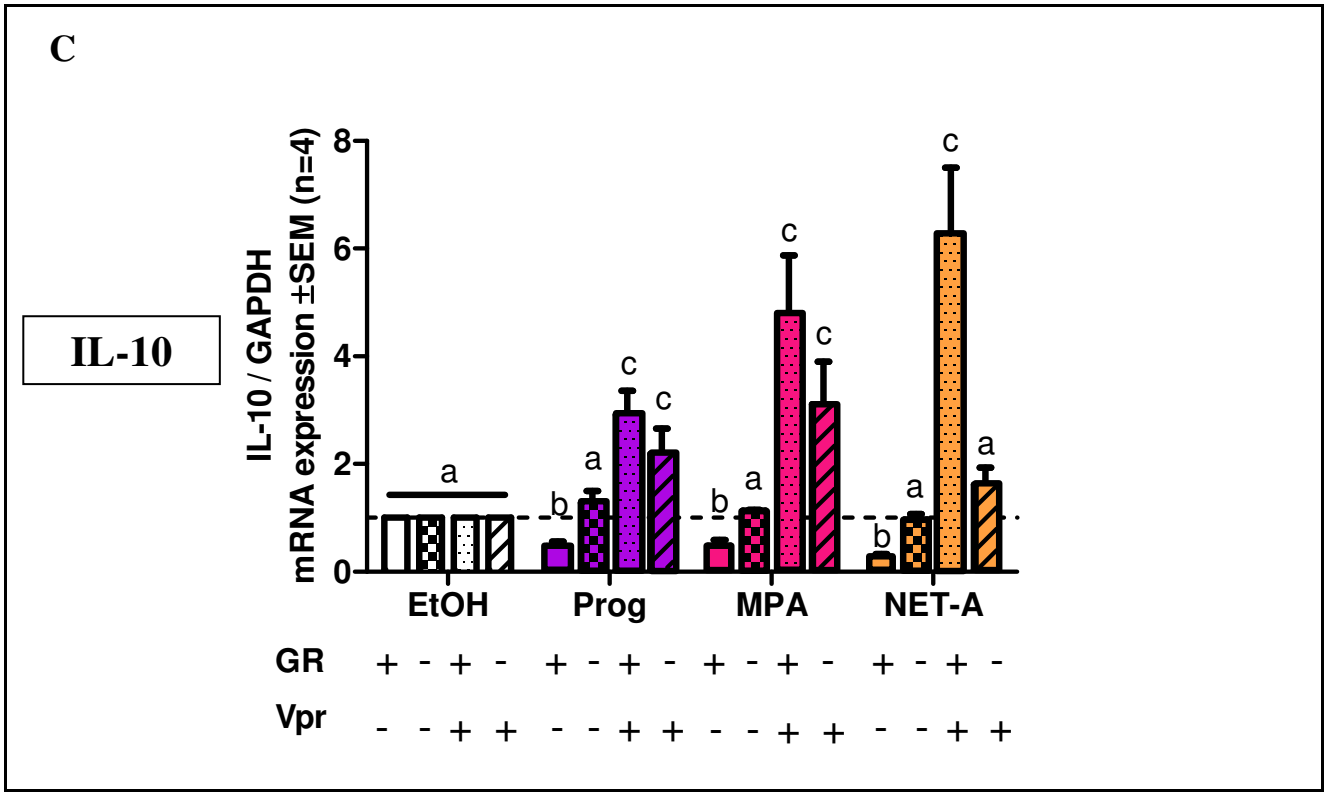


Figure 4.6. A summary of the effects of Prog, MPA and NET-A, in the absence and presence of Vpr, as well as in the absence and presence of GR, on (A) IL-12p40, (B) IL-12p35 and (C) IL-10 mRNA expression in the Ect1/E6E7 cell line. The figure is replotted from figures 3.4, 4.2 and 4.6. The effects of these progestogens on **(A)** IL-12p40, **(B)** IL-12p35 and **(C)** IL-10 mRNA expression is plotted relative to the EtOH+TNF-α of each different transfection condition, which was set as one. Two-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis.

Interestingly, the qPCR results revealed that, in the presence of Vpr, reduced GR protein levels resulted in an increase in TNFα-induced IL-12p40, IL-12p35 and IL-10 gene expression in the absence of GR ligand (figure 4.5A-C). Figure 4.7 shows the replotting of the EtOH+TNF-α results from figures 4.3 and 4.5 and suggests that Vpr requires the unliganded GR for its effect on these cytokine genes. Taken together, these results suggest that upon treatment of the Ect1/E6E7 cells with TNF-α, Vpr reduces inflammation by decreasing the gene expression of pro-inflammatory IL-12p40 and IL-12p35 via the GR.

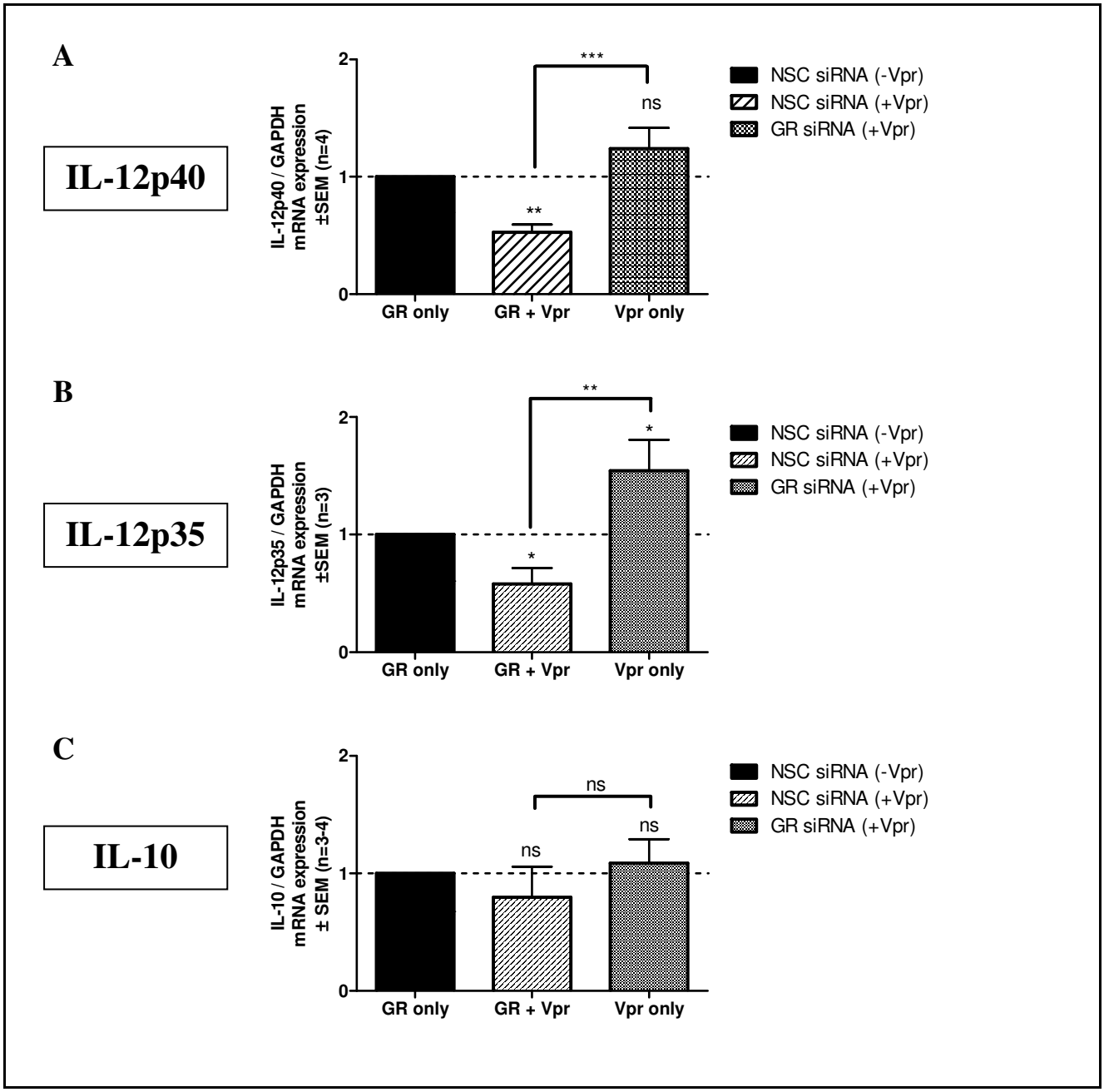
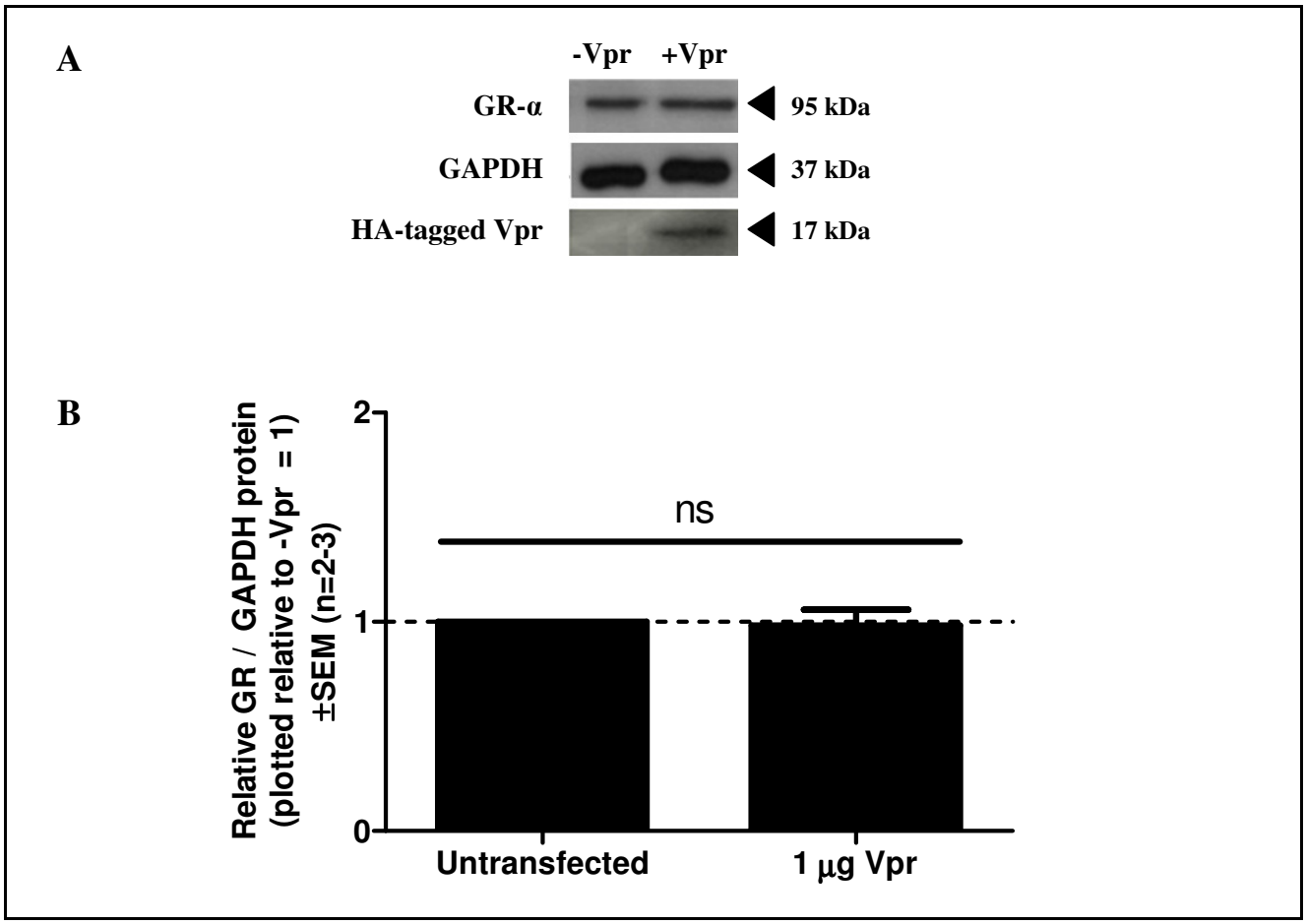


Figure 4.7. The GR is needed for Vpr mediated suppression of TNF-α induced mRNA expression of IL-12p40 and IL-12p35 in the human ectocervical epithelial cell line. qPCR data for EtOH+TNF-α from figures 4.3 and 4.5 were replotted. The Ect1/E6E7 cell line lacking Vpr was set as 1, and all other conditions were plotted relevant to that. One-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis.

4.2.5. GR protein levels are not significantly changed by HIV-1 Vpr in the human ectocervical cell line

Next we determined whether the overexpression of Vpr affected GR protein levels. The human Ect/E6E7 cell line was transiently transfected with 1 µg pCMV4-3HA.Vpr and treated with 0.02 µg/ml TNF-α and 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. Western blot analysis in figure 4.8A and 4.8B show that Vpr is indeed overexpressed in the Ect1/E6E7 cell line and it does not affect the unliganded GR protein levels. Although not statistically significant, in the presence of all the ligands (figure 4.8C and 4.8D), Vpr appeared to decrease the GR protein levels.



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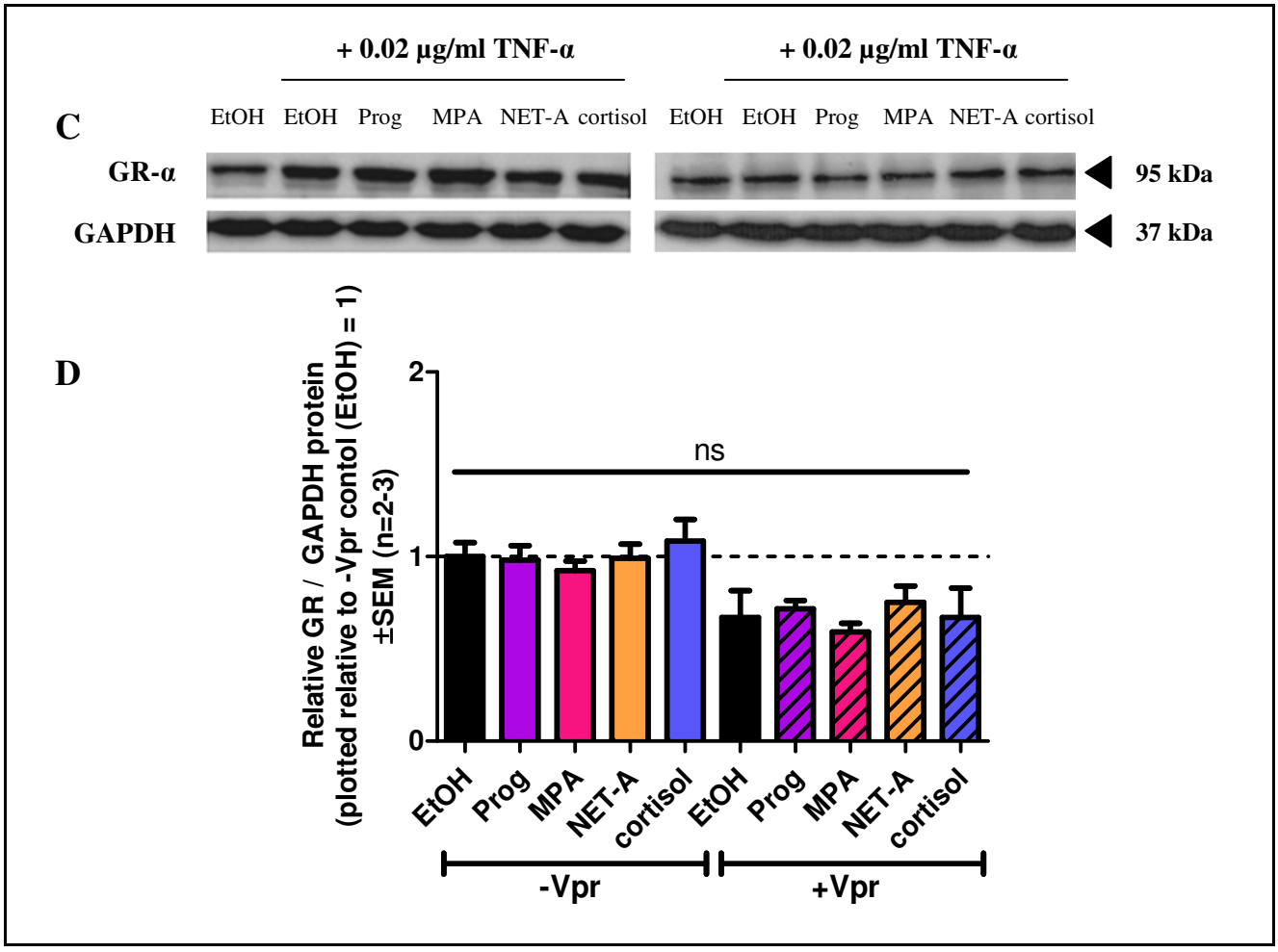

Figure 4.8. Vpr does not modulate endogenous GR protein levels in the Ect1/E6E7 cell line. Whole cell extracts were prepared from the human Ect1/E6E7 cell line, lacking Vpr expression (-Vpr) and cells transiently transfected with 1 µg pCMV4-3HA.Vpr (+Vpr). Protein lysates were subjected to electrophoresis on a 10% SDS polyacrylamide gel. The GR and Vpr protein levels were analysed using GR- and HA-specific antibodies. GAPDH was used as a loading control. **(A-B)** A single representative gel of at least two independent experiments is shown. **(C-D)** The cells were treated with 0.02 µg/ml TNF-α and 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 6 hours. **(B and D)** GR protein levels were quantified using UN-SCAN-IT and normalised to the quantified GAPDH levels. The effect of the test compounds on the endogenous GR protein levels is shown as fold induction relative to EtOH set as 1. Two-way ANOVA analysis of variance and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis.

4.3. Discussion

Numerous epidemiological studies have investigated the association between the use of hormonal contraceptives and HIV-1 acquisition and cervical shedding of the virus (Clemetson et al., 1993, Mostad et al., 1997, Martin et al., 1998, Wang, 2004, Beaten et al., 2007, Kleinschmidt et al., 2007,

Morrison et al., 2007, Myer et al., 2007, Kumwenda et al., 2008, Morrison et al., 2010, Heffron et al., 2012, Morrison et al., 2012). However, whether hormonal contraceptives are indeed a risk factor for HIV-1 acquisition remains controversial. Results from Chapter 3 suggest that the synthetic progestins used in contraception, MPA and NET-A, modulate the inflammatory process in the female genital tract by affecting the gene expression of IL-12p40 and IL-10. Similar effects were seen for IL-12p35 (figure 4.1). Taken together, these results suggest that MPA and NET-A used in hormonal therapy may lead to chronic inflammation in the ectocervical environment, thereby providing a potential environment conducive to infections such as HIV-1.

In Chapter 3 we also showed that the GR mediates the effects of the progestins on IL-12p40 (figure 3.3C) and IL-10 (figure 3.3D), while in this chapter the same is shown for IL-12p35 (figure 4.2). Moreover, there is evidence to suggest that the GR may play a role in HIV-1 pathogenesis and that its activity and function becomes enhanced during HIV-1 infection (Kino et al., 2003)(reviewed in (Hapgood and Tomasicchio, 2010). It has been suggested that the HIV-1 accessory Vpr is in part responsible for the observed increase in the GR transcriptional activity, as Vpr has been shown to act as a co-activator for the GR (Kino et al., 1999, Sherman et al., 2000, Kino et al., 2002, Mirani et al., 2002, Muthumani et al., 2004). The main aim of the study in this chapter, was thus to investigate whether Vpr could modulate the effects of the progestins on the mRNA expression of these cytokine genes. The Ect1/E6E7 cell line was transiently transfected with 1 µg of the pCMV4-3HA.Vpr plasmid. This concentration was suggested by the laboratory of Prof Janet Hapgood at the University of Cape Town (personal communication).

The general trend observed was that Vpr significantly modulated the effects of Prog, MPA and NET-A on the mRNA expression of IL-12p40 (figure 4.3A) and IL-10 (figure 4.3C), respectively. In contrast, Vpr modulated only the NET-A effect on the mRNA expression of IL-12p35 (figure

4.3B). Moreover, this study shows that Vpr downregulates IL-12p40 and IL-12p35 gene expression in the absence of ligands (figure 4.7). Collectively, these results suggest that Vpr has anti-inflammatory activities and immunsuppressive properties, i.e. Vpr downregulates the pro-inflammatory cytokine IL-12 and upregulates the anti-inflammatory cytokine IL-10. Consistent with this results, Vpr has been reported to downregulate the production of pro-inflammatory cytokines and chemokines (reviewed in (Muthumani et al., 2004). Results suggest that in the presence of Vpr, these progestins may increase susceptibility to other genital infections due to an increase in the IL-10/IL-12 ratio, which is thought to weaken the hosts defense mechanisms against infections (Kelly et al., 1997). It should be noted that the effect of the progestogens on these cytokine genes in the absence of Vpr, was investigated without transfecting the cells with an empty vector. A more appropriate control for these experiments may be transfection with a pCMV-driven empty plasmid.

Having shown that Vpr can modulate the effects of the GR ligands Prog, MPA and NET-A (Koubovec et al., 2005, Ronacher et al., 2009, Avenant et al., 2010a) on IL-12 and IL-10 gene expression, we next investigated whether the GR was involved in mediating these effects using GR knockdown experiments. Reduction of the GR protein levels showed that Vpr significantly alters the cortisol induced regulation of IL-12p40 (figure 4.5A), IL-12p35 (figure 4.5B) and IL-10 (figure 4.5C) mRNA expression in a GR-dependent manner. Unexpectedly, when the GR protein levels were reduced in the presence of Vpr, cortisol treatment significantly increased IL-12p40 mRNA expression compared to when Vpr is absent (figure 4.5A). The reason for this observation is not clear, but we speculate that it may be due to non-genomic effects of cortisol. Furthermore, although Vpr modulated the NET-A induced mRNA expression of IL-12p35 (figure 4.5B or figure 4.6B), this response did not require the GR. One can postulate that in the presence of Vpr, NET-A elicits its response via a different signalling pathway, which could entail binding to another steroid

receptor, as Vpr have previously been shown to also interact with the PR and ER (Kino et al., 1999). A similar explanation could account for why the GR is not needed for the Vpr modulation of Prog- and MPA-induced IL-10 expression (figure 4.5C or figure 4.6C).

Furthermore, our results in the human ectocervical epithelial cell line showed that Vpr, independent of ligand, significantly suppresses the TNF-α induced mRNA expression of both IL-12p40 and IL-12p35 (figure 4.7A and figure 4.7B), but not that of IL-10 (figure 4.7C), in a GR-dependent manner. This result is inconsistent with the study of Mirani and co-workers who found that Vpr suppressed the mRNA expression of IL-12p35, but not that of IL-12p40 in human peripheral monocytes stimulated with LPS (Mirani et al., 2002). This thesis suggests that Vpr may reduce inflammation in the ectocervical environment by decreasing the expression of pro-inflammatory cytokines via the GR.

As ligand binding has been shown to destabilise the GR, by degradation via the ubiquitination-proteasome pathway (Webster *et al.* 1997, Wallace & Cidlowski 2001, Ismaili & Garabedian 2004, Kinyamu et al 2005), it could be argued that these progestogens could have altered the endogenous GR levels, or that Vpr could have (i) stabilised the GR against degradation and thereby increased the GR levels prior to stimulation or (ii) acted like the GR ligands and reduced GR levels. However, using Western blotting (figure 4.8C and 4.8D), we showed that Vpr did not significantly alter ligand bound GR levels. After the initial Western blot showing that Vpr could be overexpressed in the Ect1/E6E7 cell line, we subsequently were unable to detect Vpr using the same commercial antibody for detection of HA as before. Thus, it could not be determined whether ligand treatment affected Vpr expression levels. These experiments should thus be repeated as soon as the conditions for the currently available antibody are optimised or by using a different HA-tagged antibody or one specific for Vpr. Once an optimimal antibody for the detection of Vpr has been identified, it would

be crucial to show that silencing of the GR protein levels do not affect the expression of Vpr. Furthermore, once the experimental conditions for the Multiplex bead array and/or the ELISA (Chapter 3; Addendum A, figure A3) has been finalised, the effects of Vpr should be investigated at the protein level.

In conclusion, although the results from this study suggest that Vpr influences the regulation of IL-12 and IL-10 mRNA expression by the progestogens Prog, MPA and NET-A, and in most part via the GR, these results should be interpreted with caution considering the trend of decreasing GR protein levels in the presence of Vpr. Nonetheless, the results may suggest that in the presence of Vpr (HIV-1 infection) inflammation in the ectocervical environment is reduced, which may be beneficial in terms of HIV-1, but may increase women's susceptibility to other genital infections due to weaker defense mechanisms against infections (Kelly et al., 1997, Stringer and Antonsen, 2008).

CHAPTER 5

CONCLUDING DISCUSSION

5. Introduction

Although MPA, and also NET, are widely used in female reproductive therapy, little is known about their effects on local immune function, and the implications on susceptibility to infections such as HIV-1. In the first part of this thesis (Chapter 3), using the Ect1/E6E7 immortalised ectocervical epithelial cell line, the regulation of two cytokine genes by MPA and NET-A, as compared to the endogenous hormone Prog, and the underlying molecular mechanism was investigated. Although immortalised, the Ect/E6E7 cell line has been reported to have similar morphological and immunocytochemical features to that of their tissue of origin as well as primary cultures (Fichorova et al., 1997, Fichorova and Anderson, 1999). In the second part of the thesis (Chapter 4), the influence of the HIV-1 Vpr on the regulation of cytokine genes by the progestogens, was examined.

5.1. Comparing the regulation of a pro- and anti-inflammatory cytokine gene in the human Ect1/E6E7 cell line

Inflammation is characterised by an increase in the production of pro-inflammatory cytokines which attracts and stimulates immune cells. In Chapter 3, the regulation of IL-12 and IL-10 by Prog, MPA and NET-A was investigated. These specific cytokines were selected based on their immuneregulatory role during infections (Trinchieri and Scott, 1995b, Trinchieri and Gerosa, 1996, Trinchieri, 1998, Schottelius et al., 1999, Leifeld et al., 2002, Pestka et al., 2004, Nemeth et al., 2005, Couper et al., 2008), as well as having distinct roles in HIV-1 pathogenesis (Chehimi et al., 1992, Chehimi et al., 1994, Kelly et al., 1997, Cohen et al., 1999, Ma and Montaner, 2000, Little et al., 2006, Gee et al., 2007, Byrnes et al., 2008).

The results presented in Chapter 3 showed that Prog, MPA and NET-A significantly upregulated the TNF-α stimulated IL-12p40 (figure 3.1C) gene expression to similar extents, while the mRNA expression of IL-10 was significantly downregulated (figure 3.1D). The results from this study showing upregulation of the pro-inflammatory cytokine IL-12p40 by Prog is inconsistent with previous studies showing inhibition of protein levels in mouse bone-marrow derived macrophages (Jones et al., 2008) and mouse spleen dendritic cells (Hughes et al., 2008). Consistent with the results from this study, MPA has been shown to increase the concentration of IL-12 in vaginal lavage fluid of DMPA adolescent female users (Barousse et al., 2007). Furthermore, as seen for IL-12p40, the results for this study showing downregulation of the anti-inflammatory cytokine IL-10 by Prog is inconsistent with previous reports, while that of MPA correlates to the literature. Previous studies reported that Prog has no effect on the production of IL-10 (Visser et al., 1998, Shrier et al., 2003, Bouman et al., 2005), while a decrease in the LPS-induced IL-10 mRNA and protein levels in the cervix of mice was observed for MPA (Elovitz and Gonzalez, 2008). Addressing the discrepancies between results of this study and that of previous studies is not easy. However, it is likely that the differences could be ascribed to factors such as cell-specific effects or differences at the transcript (mRNA) vs protein level. Indeed, the immune responses of cervical lymphocytes have been reported to differ from that observed in PBMCs (Vats et al., 2007). In addition, it is well-known that protein levels do not always correlate to the transcript levels as the production of proteins is regulated not only at the transcriptional level, but also other processes such as mRNA degradation, translation, post-transcriptional modifications and protein degradation (Vogel and Marcotte, 2012). Results from the present study indicate that progestins are pro-inflammatory in the ectocervical epithelial cells of the female genital tract. Interestingly clinical (Barousse et al., 2007) and animal (Elovitz and Gonzalez, 2008) evidence, for IL-12 and IL-10, respectively, suggest that MPA is indeed anti-inflammatory in the female genital tract.

Chapter 3 also investigated the effects of these ligands on IL-12p40 and IL-10 (Addendum A, figure A3) protein levels using both a Multiplex bead array and ELISA. Firstly, the IL-12p40 and IL-10 protein levels were measured in the cell culture supernatant after 24 hours using the Multiplex bead array. Although, the results obtained for the IL-12p40 and IL-10 protein levels were below the detectable (linear) range, 0.24 – 1.79 pg/ml and ~ 0.90 pg/ml, respectively, we were able to quantify the protein levels of other immune mediators such as IL-6, IL-8 and RANTES (Addendum A, figure A4). Interestingly, the general trends observed for the effects of progestogens on the protein expression of these pro-inflammatory cytokines/chemokines correlates with the recently reported mRNA expression (Africander et al., 2011a). Subsequently, we found a study showing that IL-12 and IL-10 are time-dependently increased in the vaginal epithelial cell line, Vk2/E6E7, from 24 to 96 hours (Steele and Fidel, 2002). Our next strategy was thus to measure IL-12p40 and IL-10 protein levels in response to Prog, MPA and NET-A after 48 and 96 hours, using ELISA due to the expense of the Multiplex bead array. The protein levels of IL-12p40 and IL-10 were still below the detectable range. Thus, the optimal time for detecting IL-12 and IL-10 protein in the ectocervical epithelial cell line should be determined by performing a more in-depth time course experiment. In addition, the media could be concentrated to improve detection of these cytokines. However, the possibility that the protein levels of these cytokines are too low to be detected in this cell line cannot be excluded. It may also be useful to use fluorescence activated cell-sorting (FACS) as an alternative method.

Taken together, the results suggest that Prog, MPA and NET-A promote inflammation in the ectocervical environment. This may increase susceptibility to viral infections such as HIV, HSV and HPV since several lines of evidence suggest that an increase in inflammation of the human female genital tract may increase susceptibility to infections. The physiological implication of this result is not straightforward. In terms of Prog, upregulation of IL-12p40 and downregulation of IL-

10 mRNA expression may be beneficial. The concentrations of Prog fluctuates due to reproductive processes such as pregnancy and menstruation (Bouman et al., 2005, Wira et al., 2005a, Gravitt and Ghanem, 2010), thus Prog will not induce chronic inflammation, but rather acute inflammation during times of high Prog concentrations such as during the luteal phase of the menstrual cycle, thereby providing protection for the development of a potential fetus (Fleischman and Fessler, 2010). In contrast, the long term use of MPA or NET-A as contraceptives, may lead to chronic inflammation of the ectocervical environment. This chronic inflammation may lead to an increase in the recruitment of HIV-1 target cells into the site of infection, and potentially increase susceptibility to HIV-1 (Bebell et al., 2008, Gumbi et al., 2008, Nkwanyana et al., 2009), reviewed in (Roberts et al., 2012a). However, the effects on the expression levels of IL-12 and IL-10 in the ectocervical epithelial cells cannot be considered in isolation as there is a constant release of various pro- and anti-inflammatory mediators during an immune response. Furthermore, the defense mechanisms in the female genital tract are dependent on a number of different factors in different anatomical sites. It would thus be critical to investigate the pro- and anti-inflammatory cytokine profiles *ex vivo,* using explants from the ectocervix, and possibly endocervix and vagina, from healthy versus HIV infected women using MPA and NET-A as injectable contraceptives, to those not using these contraceptives. Although the concentrations of MPA and NET-A used in this study were much higher than the serum concentrations reported in the literature (Chapter 1, literature review), the possibility that higher concentrations are present in ectocervical tissues *in vivo* compared with the concentrations usually measured in the blood, should not be excluded. There is evidence that MPA at low and high concentrations elicit similar effects on AR responsive genes (Ghatge, 2005). However, future studies should include a dose response analysis for MPA and NET-A on IL-12p40 and IL-10 gene expression in the Ect1/E6E7 cell line.

Progestogens mediate their biological effects by binding to steroid receptors, including the PR, AR, GR and possibly the ER. Although all these steroid receptors are endogenously expressed in the Ect1/E6E7 cell line, only the GR is transcriptionally active for both transactivation and transrepression in this cell line (Africander et al., 2011a). In the current study is was hypothesised that the effects of the progestogens on the cytokine genes are due to those ligands acting via the GR. Using a combination of whole cell binding assays (figure 3.2A) and Western blotting (figure 3.2B and 3.2C), the present study shows that these ligands bind to the endogenous GR and do not affect the levels of the protein. This is consistent with previous studies in human lung carcinoma cells (A549) endogenously expressing the GR or COS-1 cells with overexpressed human GR (Koubovec et al., 2005, Ronacher et al., 2009). Furthermore, as it has previously been shown that the GR bound to Prog, MPA and NET-A has a half-life of 28, 17 and 37 hours, respectively (Avenant et al., 2010b), we did not expect these progestogens to modulate the endogenous GR protein levels in the Ect1/E6E7 cell line at 6 hours. We next determined whether the GR is involved in the response of these progestogens on the IL-12p40 and IL-10 genes by reducing the GR protein levels using siRNA. The results suggest that the induction of IL-12p40 (figure 3.3C) and inhibition of IL-10 (figure 3.3D) by Prog, MPA and NET-A is via the GR, since complete abrogation of the effects on these cytokine genes were seen when the GR protein levels were reduced. Interestingly, IL-10 mRNA expression (figure 3.3D) was upregulated by NET-A in the presence of GR siRNA. This suggests that when GR levels are reduced, NET-A, or one of its metabolites, act via another steroid receptor, as it is known that the PR, AR and ER are endogenously expressed in this cell line (Africander et al., 2011a). Considering that similar effects are not seen for MPA, it is speculated that this effect is likely due to the activity of metabolites of NET, rather than NET itself, via the ER. Future studies should thus investigate the role of the ER in the NET-A induced regulation of IL-10, using either ER-specific antagonists such as ICI 182,780 or ER siRNA. The effects of NET-A

metabolites such 3α-hydroxy-5α-hydrogen-NET (Schoonen et al., 2000) on IL-10 gene expression could also be investigated.

It was rather surprising that MPA and the natural glucocorticoid cortisol, previously shown to elicit anti-inflammatory activity via the GR (Bamberger et al., 1999, Kurebayashi et al., 2003, Koubovec et al., 2004, Koubovec et al., 2005)(reviewed in (Griekspoor et al., 2007), exhibited pro-inflammatory activity in the ectocervical epithelial cell line. However, both anti-inflammatory (De Bosscher and Haegeman, 2009) and pro-inflammatory activities (Lannan et al., 2012) have previously been reported for the potent synthetic GR ligand, Dex.

The fact that MPA and NET-A displayed similar effects on IL-12p40 and IL-10 gene regulation via the GR was also unexpected, as these progestogens are known to have different binding affinities for the GR, as well as potencies and efficacies for transactivation and transrepression via the GR (Koubovec et al., 2005, Ronacher et al., 2009). Thus, the results from this study showing similar activities of 1 μM MPA and NET-A via the GR in the Ect1/E6E7 cell line are difficult to explain. It may be that the amount of activated GR is sufficient for mediating similar effects of these progestins on these genes, as demonstrated by the ChIP results in Chapter 3, even though the fractional occupancy of the GR is different for MPA and NET-A. Alternatively, the relative affinities of these ligands for the GR may be different in this cell line compared to other cell lines, as it has previously been shown that the concentration of GR determines the binding affinity of a ligand for the receptor (Robertson, 2011). To address this possibility, the relative binding affinities of these progestins for the endogenous GR in the Ect1/E6E7 cell line could be determined.

Having shown that Prog, MPA and NET-A regulate the gene expression of both IL-12p40 and IL-10 via the GR, a detailed investigation was followed into the molecular mechanism involved. Whether the progestogen-bound GR could be recruited to the endogenous IL-12p40 and IL-10 promoters were investigated by ChIP analysis (figure 3.4 and figure 3.5). The results for IL-12p40

showed that the GR was recruited to the endogenous IL-12p40 promoter region encompassing the NFkB and C/EBPβ *cis*-elements (figure 3.4B) in the presence of TNF-α and Prog, MPA, NET-A or cortisol. TNF-α is known to activate both the transcription factors NFkB (Smoak and Cidlowski, 2004) and C/EBPβ (Cardinaux et al., 2000, Kim et al., 2009). Tethering of the GR to NFkB is mostly seen when pro-inflammatory genes are repressed (Smoak and Cidlowski, 2004, Kassel and Herrlich, 2007). A recent study proposed that tethering of the GR to C/EBPβ can either prevent DNA-binding to responsive elements and inhibit transcription of pro-inflammatory cytokines, for example IL-1β, or induce transcription of glucocorticoid-responsive genes lacking GREs, by binding to C/EBPβ binding sites (Roos and Nord, 2012). Further analysis by siRNA showed that C/EBPβ is needed for the progestogen-bound GR to mediate the upregulation of IL-12p40 gene expression (figure 3.6C). These results are consistent with a previous study showing that C/EBPβ plays a crucial role in the transcriptional activation of the mouse IL-12p40 gene (Plevy et al., 1997). Furthermore, using the co-immunoprecipitation (figure 3.7) and re-ChIP (figure 3.8) analyses this study also provides evidence that the endogenous GR and C/EBPβ occurs as a cellular complex in Ect1/E6E7 cell line. Moreover, the re-ChIP analysis revealed significant recruitment of the GR-C/EBPβ complex to the endogenous IL-12p40 promoter, most likely via the C/EBPβ regulatory element, by Prog, MPA and NET-A.

In summary, a model is proposed in figure 5.1 for the mechanism whereby IL-12p40 mRNA expression is upregulated by the progestogens in the human ectocervical epithelial cell line. It is proposed that the transcription factor C/EBPβ is recruited to the C/EBPβ regulatory element of the Il-12p40 promoter in response to TNF-α. However, the recruitment of C/EBPβ to the IL-12p40 promoter will need to be confirmed by ChIP analysis. When Prog, MPA or NET-A binds to the endogenous GR, the liganded-GR then tethers to the DNA-bound C/EBPβ, thereby increasing the transcription of the IL-12p40 gene. The mechanism proposed here may not be unique to IL-12p40,

but may also be relevant to other pro-inflammatory cytokine or chemokine genes containing C/EBPβ *cis*-regulatory elements within their promoters, such as MIP-1α. Indeed, the upregulation of MIP-1α by Prog and MPA in human vaginal epithelial cells has previously been shown to be mediated by the GR (Noeth, 2012).

The reason for the observed recruitment of the progestogen-bound GR to the NFκB-C/EBPβ regulatory element, rather than to AP-1 or Sp1 elements of the endogenous IL-12p40 promoter in the ectocervical cell line is not clear. Considering that gene regulation is dependent on the nature of the chromatin, in terms of whether it is open or closed, certain *cis*-regulatory elements may or may not be blocked by nucleosomes, thereby affecting the accessibility of these elements to transcription factors (Bell et al., 2011). Thus, it may be that the AP-1 or Sp1 binding sites of the IL-12p40 promoter are blocked by nucleosomes in these cells, and are thus not accessible to the progestogen-bound GR. It should be noted that role of the NFκB binding element was not investigated as the results showed complete abrogation of the progestogen-induced upregulation of IL-12p40 gene expression when C/EBPβ protein levels were reduced. Although unlikely, the possibility that the GR may be recruited to the NFκB binding element can thus not be definitively excluded. Future studies can address this by performing similar experiments as for C/EBPβ, using siRNA and ChIP assays.

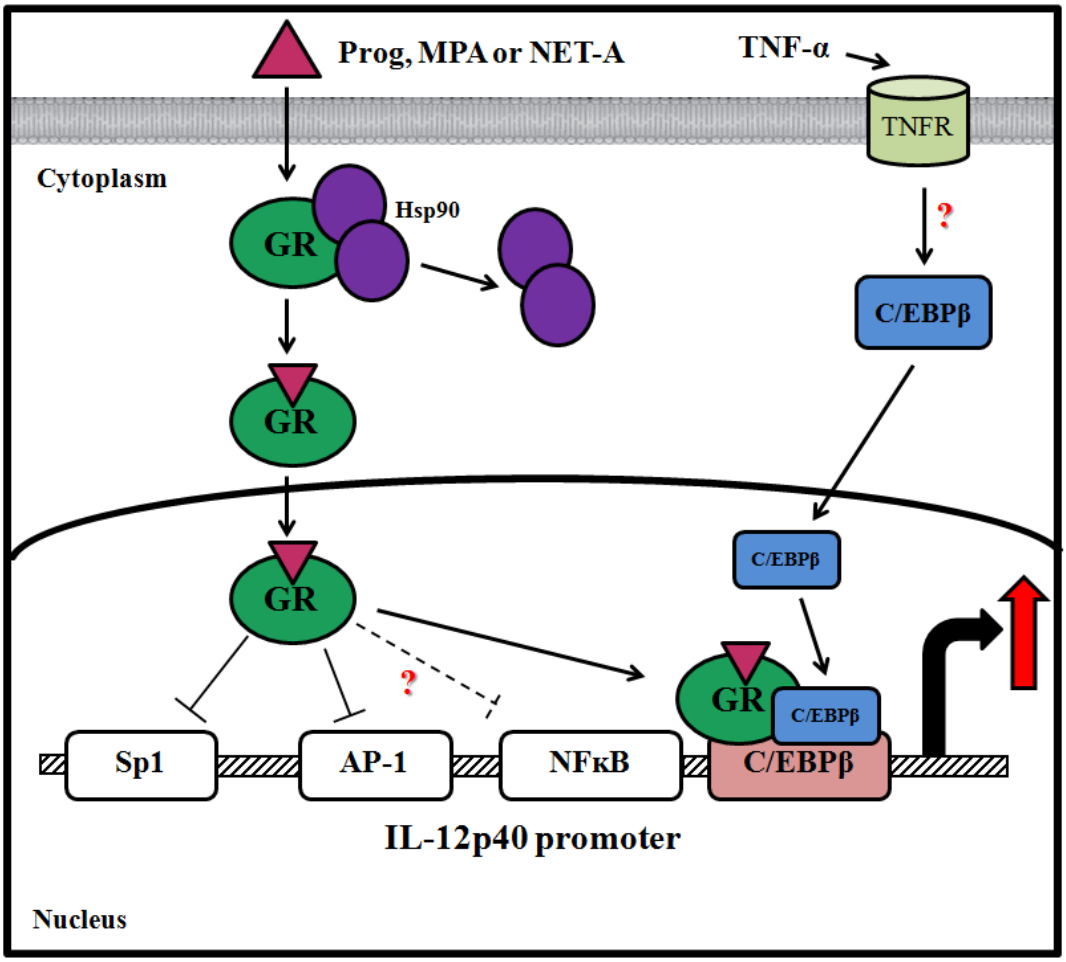

Figure 5.1. Schematic model for the progestogen-induced upregulation of IL-12p40 gene expression via the GR in the ectocervical epithelial cell line. Upon Prog, MPA or NET-A binding to the GR, the GR undergoes a conformational change and the progestogen-bound GR translocates to the nucleus, where it co-localise to the IL-12p40 promoter with the transcription factor C/EBPβ via the C/EBPβ binding elements to upregulate transcription of the IL-12p40 gene. Abbreviations: AP-1: activator protein-1; C/EBPβ: CCAAT enhancer binding protein-β; GR: glucocorticoid receptor; Hsp90: heat shock protein-90; MPA: medroxyprogesterone acetate; NET-A: norethisterone acetate; NFκB: nuclear factor kappa-B; Prog: progesterone; Sp1: specific protein-1; TNF-α: tumor necrosis factor-α; TNFR: tumor necrosis factor receptor;

ChIP, siRNA, co-immunoprecipitation and re-ChIP analyses were also used for an in-depth investigation into the GR-mediated downregulation of IL-10 gene expression by the progestins (figure 3.9 - figure 3.11). Figure 5.2 summarises the results of these experiments, and illustrates that once Prog, MPA and NET-A bind to the GR, the liganded-GR co-localises with the transcription

factor STAT-3, on the endogenous IL-10 promoter, in the Ect1/E6E7 cell line, likely via a STAT-3 regulatory element in the IL-10 promoter. It is proposed that the transcription factor STAT-3 is recruited to the STAT-3 regulatory element of the Il-10 promoter in response to TNF-α, as other studies have shown that TNF-α can activate STAT-3 (Miscia et al., 2002, Robinson et al., 2006). Using co-immunoprecipitation assays, previous studies identified the formation of a complex between the GR and STAT-3 in rat liver extracts, to upregulate the mRNA expression of the haemoglobin binding protein haptoglobin (Arambasic et al., 2010), and a rat hepatoma cell line, indicating a novel mechanism for the interaction between the glucocorticoid and IL-6 signaling pathways (Zhang et al., 1997). Interestingly, IL-10 gene expression was upregulated by Prog and MPA when STAT-3 was silenced. The latter may suggest that when the levels of STAT-3 are reduced, the GR recruits to other transcription factors at other *cis*-regulatory elements within the endogenous IL-10 promoter, for example a GRE. Future studies should investigate the role of other transcription factors and recruitment of the GR to the endogenous IL-10 promoter using ChIP assays after STAT-3 levels are reduced. As mentioned for IL-12p40, the mechanism proposed here may not be unique for IL-10, but may also be relevant to other anti-inflammatory cytokine genes containing STAT-3 *cis*-regulatory elements within their promoters.

As discussed above the nature of the chromatin could affect the accessibility of the GRE or Sp1 elements of endogenous IL-10 promoter, and therefore explain why the progestogen-bound GR recruits to STAT-3 regulatory elements, rather than to GRE or Sp1 elements. Thus, it may be that the GRE and Sp1 binding sites of the IL-10 promoter are blocked by nucleosomes in these cells, and are thus not accessible to the progestogen-bound GR. Considering that the role for the AP-1 binding element was not investigated, the possibility that the GR may be recruited to the AP-1 binding element cannot be definitively excluded. Future studies can address this by performing similar experiments as for STAT-3, using siRNA and ChIP assays.

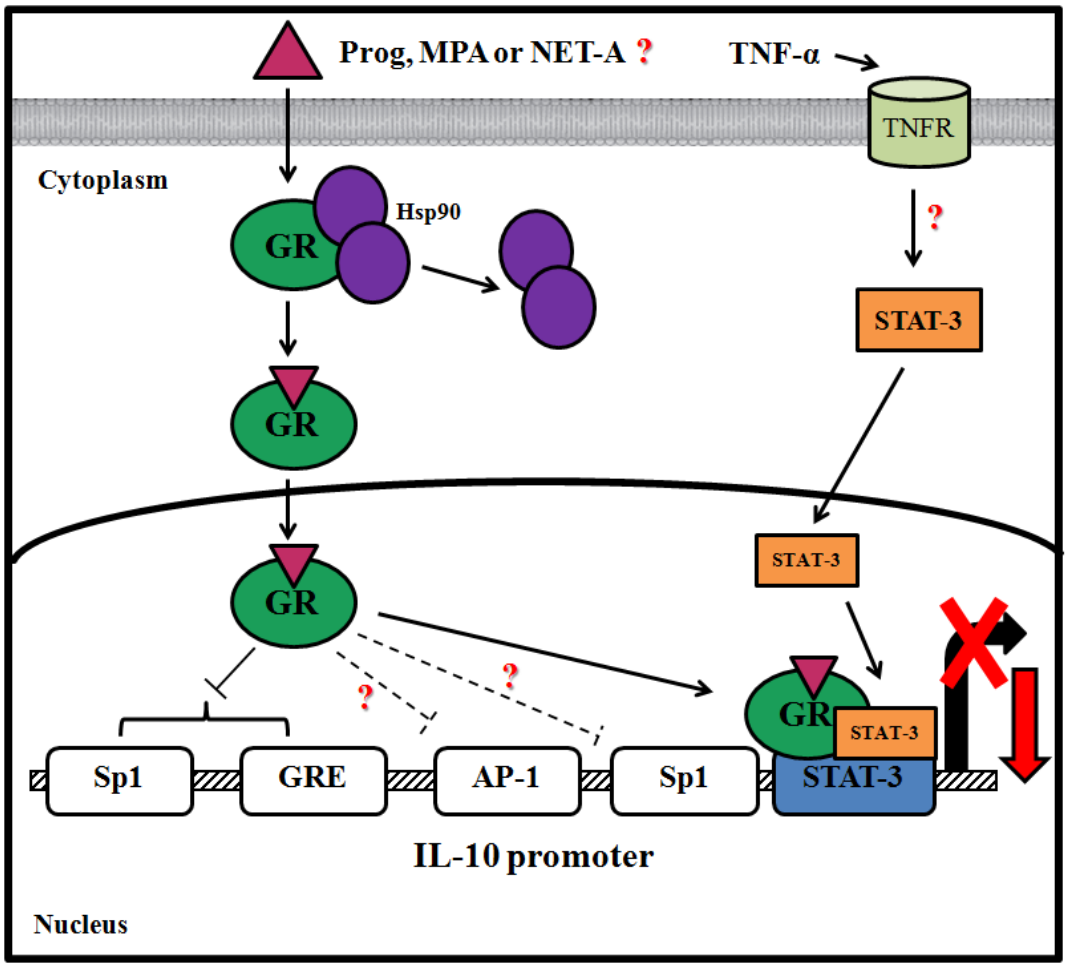

Figure 5.2. . Schematic model for the progestogen-induced downregulation of IL-10 gene expression via the GR in the ectocervical epithelial cell line. Upon Prog, MPA or NET-A binding to the GR, the GR undergoes a conformational change and the progestogen-bound GR translocates to the nucleus, where it co-localise to the IL-10 promoter with the transcription factor STAT-3 via the STAT-3 binding elements to decrease transcription of the IL-10 gene. Abbreviations: AP-1: activator protein-1; GR: glucocorticoid receptor; GRE: glucocorticoid response element; Hsp90: heat shock protein-90; MPA: medroxyprogesterone acetate; NET-A: norethisterone acetate; Prog: progesterone; Sp1: specific protein-1; STAT-3:signal transducer and activator of transcription-3; TNF-α: tumor necrosis factor-α; TNFR: tumor necrosis factor receptor;

5.2. Vpr modulates the effects of the progestogens on the expression of the endogenous cytokine genes

Previous studies have described HIV-1 Vpr as a co-activator of the GR (Kino et al., 1999, Kino et al., 2002), that downregulates the expression of pro-inflammatory cytokine genes and upregulates the expression of anti-inflammatory cytokine genes (reviewed in Muthumani et al., 2004). The original hypothesis at the onset of this thesis was thus that Vpr would modulate the mRNA

expression of IL-12 and IL-10, by decreasing the expression of the former and increasing the expression of the latter via the GR. Chapter 4 thus focussed on the influence of Vpr on cytokine gene expression, specifically on progestogen-induced regulation of IL-12p40, IL-10 and IL-12p35. The latter was included as it has previously been shown that Vpr suppresses the mRNA expression of IL-12p35, but not IL-12p40 and IL-10, in human peripheral monocytes (Mirani et al., 2002). Figure 5.3 summarises the results of this chapter in the form of a model showing the role of the GR in the modulation of progestogen-induced cytokine gene expression by Vpr.

Results from this study show that Vpr, independent of ligand, significantly suppresses the TNF-α induced mRNA expression of both IL-12p40 and IL-12p35 (figure 4.7A and figure 4.7B), but not that of IL-10 (figure 4.7C). The Vpr results on IL-12p35 and IL-10, but not IL-12p40, are in agreement with the study in PBMCs (Mirani et al., 2002). These authors showed that Vpr had no effect on IL-12p40 gene expression. However, the results presented in this study, indicating a decrease in IL-12p40 gene expression in the presence of Vpr, correlate to the literature showing decreased or impaired IL-12 levels in PBMCs of HIV-infected individuals (Chehimi and Trinchieri, 1994, Ma and Montaner, 2000, Byrnes et al., 2008). Vpr significantly modulated the effects of Prog, MPA and NET-A on the mRNA expression of IL-12p40 (figure 4.3A) and IL-10 (figure 4.3C), while only the effect of NET-A on IL-12p35 was modulated (figure 4.3B). As the effects of NET-A on IL-12p35 gene expression is only partly mediated by the GR, suggesting that other steroid receptors such as the PR, AR or ER may be involved, it may be that Vpr needs one of these steroid receptors rather than the GR.

Reducing the GR protein levels suggested that Vpr requires the GR to alter the Prog, MPA and NET-A induced regulation of IL-12p40 (figure 4.5A or figure 4.6A) mRNA expression. Furthermore, although Vpr modulated the NET-A induced mRNA expression of IL-12p35 (figure

4.5B or figure 4.6B), GR siRNA indicated that the GR is not needed for this response. This result supports the above suggestion that Vpr needs a receptor other than the GR, for its effects on NET-A-induced upregulation of IL-12p35 gene expression (figure 4.3B). As Vpr have previously been shown to also interact with the PR and ER (Kino et al., 1999), one can postulate that in the presence of Vpr, NET-A or one of its metabolites, elicits its response by binding to either one of these receptors, but most likely the ER. A similar speculation could explain why the Prog- and MPA-induced IL-10 gene expression in the presence of Vpr does not change when the GR protein levels are reduced (figure 4.5C or figure 4.6C). Surprisingly, Vpr requires the GR to modulate the NET-A, but not the MPA, effect on IL-10 mRNA expression. As it could be argued that the differences observed with Vpr and the progestogens could be due to Vpr altering the endogenous GR levels, Western blotting was performed. This study showed that Vpr did not significantly alter the progestogen-bound GR levels (figure 4.8C and 4.8D). Although Vpr has previously been shown to be a co-activator of the GR (Kino et al., 1999, Kino et al., 2002), the results in this chapter do not agree with this. Vpr did not enhance the GR-mediated activation of the IL-12 gene, or the GR-mediated repression of the IL-10 gene by Prog, MPA and NET-A. Furthermore, the results in Chapter 4 showed that Vpr needs the unliganded GR to significantly suppress the TNF-α induced mRNA expression of both IL-12p40 and IL-12p35 (figure 4.7A and figure 4.7B). The results suggest that HIV-1 Vpr may reduce inflammation in the ectocervical environment by decreasing the expression of pro-inflammatory cytokines. Considering that pro-inflammatory cytokines/chemokines and mucosal inflammation in the genital tract are associated with lower systemic $CD4^+$ cell counts during acute HIV-1 infection, reducing genital tract inflammation may slow disease progression (Bebell et al., 2008, Roberts et al., 2012b). On the other hand, reducing inflammation in the cervix in the presence of HIV may facilitate other opportunistic infections (Ma and Montaner, 2000, Guha and Chatterjee, 2009).

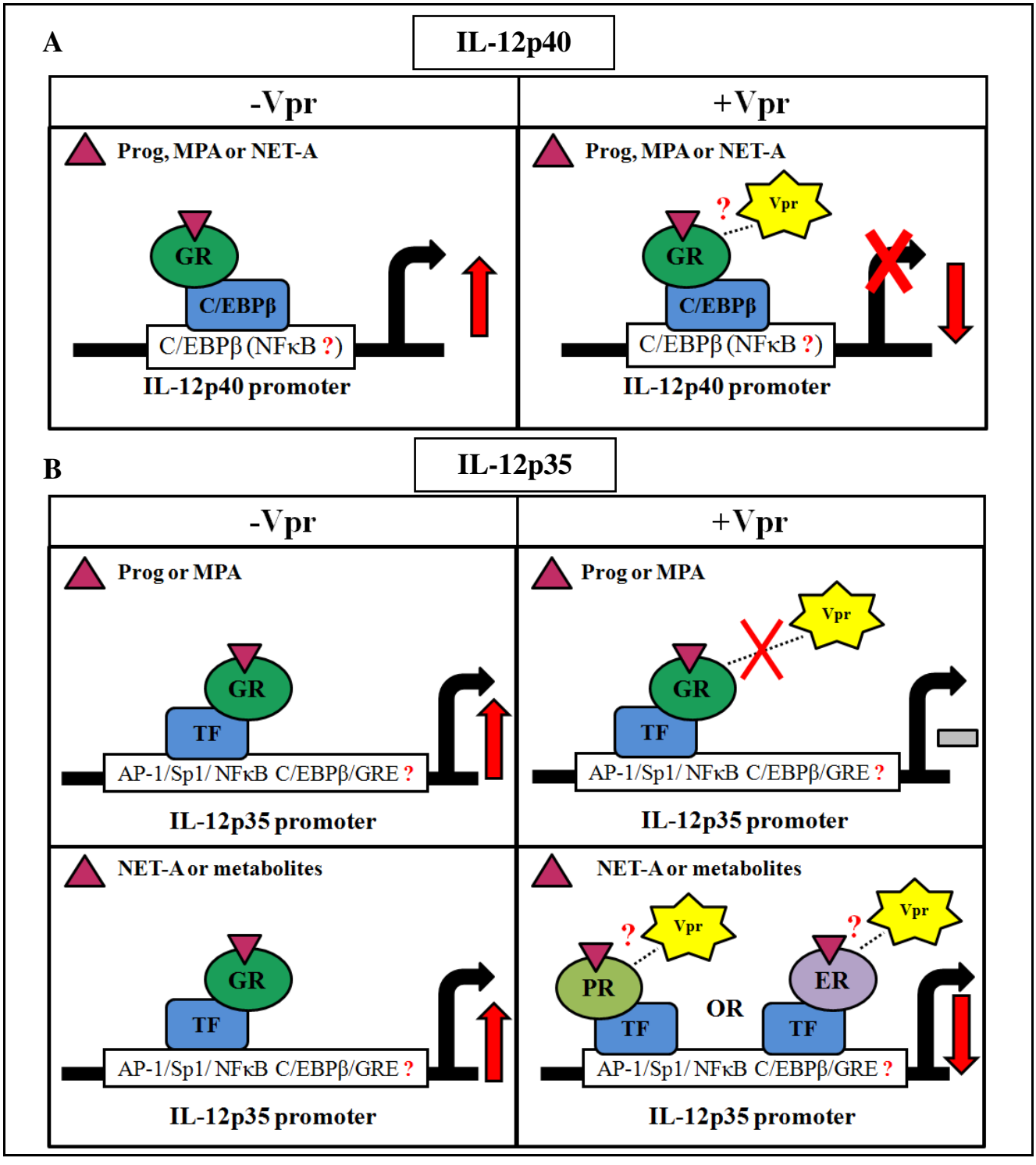

Figure 5.3 continues on next page.

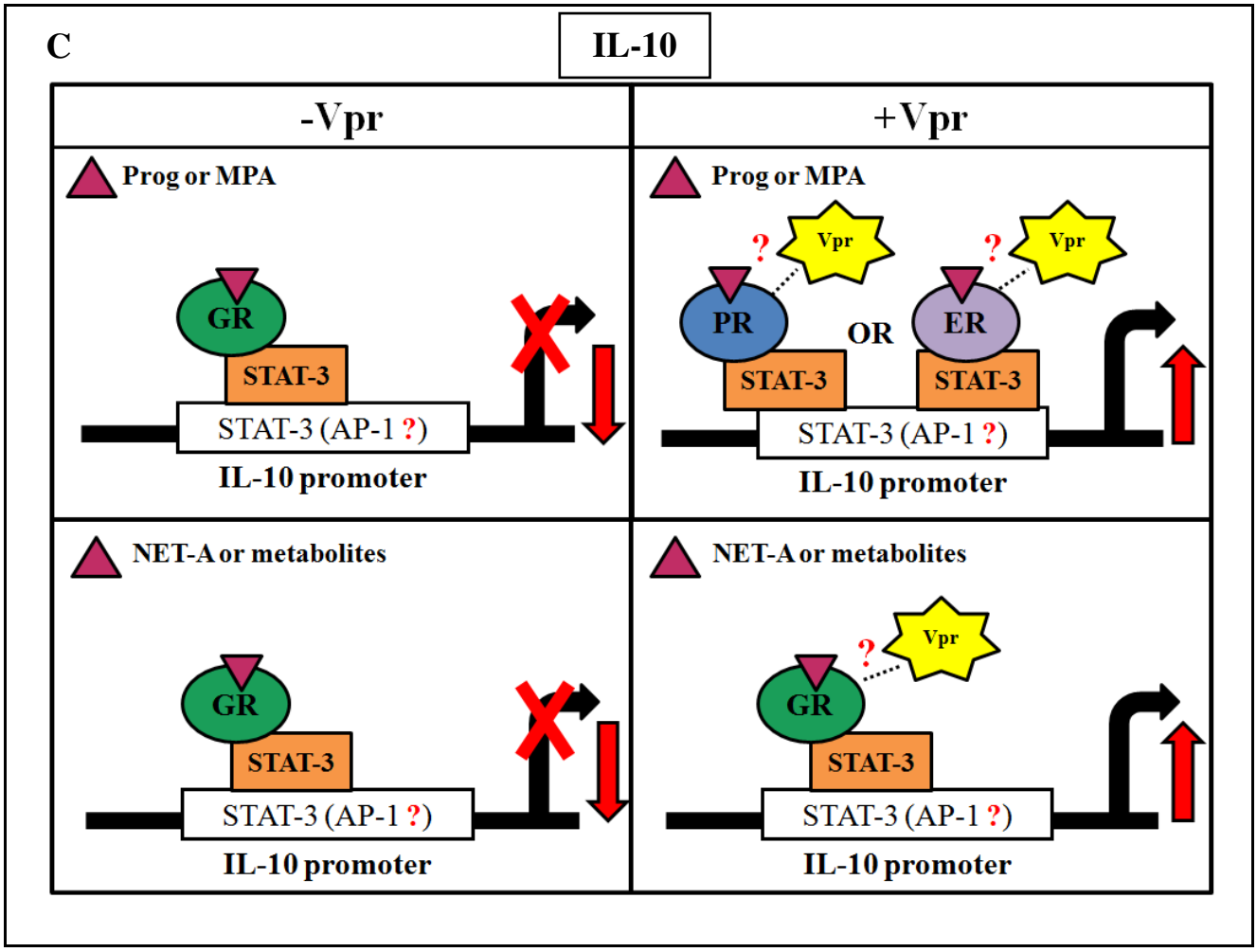

Figure 5.3. Proposed schematic model summarising the possible role of the GR in the modulation of the progestogen-induced cytokine gene expression by Vpr in the ectocervical epithelial cell line. (A) IL-12p40, **(B)** IL-12p35, **(C)** IL-10. The proposed model was constructed from the replotted data in figure 4.6 of Chapter 4. Abbreviations: AP-1: activator protein-1; C/EBPβ: CCAAT enhancer binding protein-β; ER: estrogen receptor; GR: glucocorticoid receptor; GRE: glucocorticoid response element; MPA: medroxyprogesterone acetate; NET-A: norethisterone acetate; NFκB: nuclear factor kappa-B; Prog: progesterone; PR: progesterone receptor; Sp1: specific protein-1; SR, STAT-3: signal transducer and activator of transcription-3; TF: transcription factor.

Taken together, the results presented in Chapter 4 shows a shift from pro-inflammatory (IL-12) to anti-inflammatory (IL-10) immune responses in the presence of Vpr, which are consistent with the evidence in the literature suggesting a switch from the Th1 (e.g. IL-12, IFNγ and TNF-α) to Th2 (e.g. IL-4 and IL-10) cytokine profiles in PBMCs (Clerici and Shearer, 1994, Hasko and Szabo, 1999) during HIV-1 disease progression. As this study showed that Vpr altered the regulation of IL-12p40 and IL-12p35, in the absence and presence of ligand, and IL-10, in the presence of ligand, in

most part requiring the GR, this study supports the hypothesis that the GR is used by HIV-1 Vpr to ensure successful pathogenesis (reviewed in Hapgood and Tomasicchio, 2010). However, although *in vitro* cell culture models have shown that Vpr interacts with the GR (Kino et al., 1999, Sherman et al., 2000, Kino et al., 2002, Muthumani et al., 2006), evidence for a direct *in vivo* association between Vpr and the GR is lacking. To determine whether Vpr associates directly with the GR *in vivo*, a fluorescence resonance energy transfer (FRET) assay could be performed. This assay uses the principle of photo-excitation to excite an electron on a fluorophore tagged protein, which transfer its energy to a second fluorophore tagged protein when these proteins are in close association (Karpova and McNally, 2006). As an alternative, the putative association between Vpr and the GR could also be investigated by using a mammalian two-hybrid assay, a technique used to study protein-protein interactions (Luo et al., 1997).

Considering the inhibition of IL-12p40 gene activation when Vpr is present (figure 4.3.A), Vpr may also be a co-repressor of the GR. It may be better to refer to Vpr as a co-regulator, as some proteins can have either a role of a co-activator or co-repressor depending on the cell context. For example, GRIP-1 has been shown to act as both a GR co-activor and a GR co-repressor in different cell types (Rogatsky et al., 2002, Avenant et al., 2010a, Verhoog et al., 2011). Furthermore, it would be interesting to investigate the localisation of the GR in the presence of Vpr on the IL-12p40 and IL-10 promoters, using ChIP assays. In addition, Vpr appears to prevent Prog and MPA from binding to the GR and thereby induce IL-10 mRNA expression. It would be interesting to investigate whether Vpr can affect the dissociation constant (K_d) values of Prog and MPA for the GR, or even other steroid receptors present in the ectocervical epithelial cell line, by means of whole cell binding assays. Furthermore, Western blotting can also be performed to determine whether Vpr affects the expression levels of other steroid receptors in the ectocervical epithelial cell line.

Although not statistically significant, the results in Chapter 4 show a general trend for Vpr decreasing the GR protein levels, in the absence and presence of ligands, in the ectocervical epithelial cell line. However, a shortcoming of this study was that we were unable to verify the overexpression of Vpr in these experiments as Western blotting using the commercially available antibody for the detection of HA (HA tagged Vpr) was not reproducible. Thus, these experiments should be repeated by either optimising the conditions of the currently available antibody or by acquiring another HA-tagged antibody with more specificity. In fact, it would be ideal to use a Vpr specific antibody to exclude the possibility that Vpr is endogenously expressed in this cell line. Moreover, overexpressing the HIV-1 Vpr in the Ect1/E6E7 cell line may not be the best way to investigate the effects of Vpr on the regulation of IL-12 and IL-10 in these cells. It may be useful to investigate alternate methods for the delivery of Vpr into the ectocervical epithelial cell line, such as delivering Vpr with a virus and/or adding Vpr as a whole protein.

Finally, the current study could be expanded by investigating concentration-dependent effects of MPA and NET-A, on a wide variety of immune mediators in cell lines and primary tissues of different anatomical sites of the female genital tract from healthy and HIV infected women using PCR arrays, multiplex protein arrays and ChIP-sequencing.

5.3. Conclusion

In summary, the results presented in this thesis provide evidence that Prog, MPA and NET-A may promote the pro-inflammatory milieu in the ectocervical environment. The expression of both the pro-inflammatory cytokine genes, IL-12p40 and IL-12p35, by Prog, MPA and NET-A, was upregulated, while the expression of the anti-inflammatory cytokine IL-10, was downregulated. These results imply that the use of MPA or NET *in vivo* may have adverse local immunosuppressive effects by causing chronic inflammation of the ectocervical environment,

which may increase the risk of susceptibility to infections such as HIV-1 (Bebell et al., 2008, Gumbi et al., 2008, Nkwanyana et al., 2009), reviewed in (Roberts et al., 2012a). The proposed mechanisms (figure 5.1. and 5.2.) may be particularly relevant to infections such as HIV-1 in the cervix, as both IL-12 and IL-10 have been shown to be key role players in HIV-1 pathogenesis. The results from the first part of the study showing increased inflammation (Chapter 3), may provide a potential mechanism whereby the injectable contraceptive Depo-Provera (Heffron et al., 2012), and possibly Nuristerate, increase the risk of women using these contraceptives of acquiring HIV-1.

In the second part of the study (Chapter 4), we showed that the progestogen-induced cytokine milieu in the ectocervical environment changes from pro- to anti-inflammatory when HIV-1 Vpr is present. In the absence of the progestogens, Vpr also suppressed IL-12p40 and IL-12p35, but not IL-10 gene expression. These results for IL-12p40 and IL-12p35 in the ectocervical epithelial cells are in agreement with the evidence in the literature showing decreased or impaired IL-12 levels in PBMCs of HIV-infected individuals, but are inconsistent with the reports of increased IL-10 levels in PBMCs of HIV-infected individuals (Chehimi and Trinchieri, 1994, Trinchieri and Scott, 1995a, Ma and Montaner, 2000, Li and Lau, 2007, Byrnes et al., 2008). The results from this study may suggest that in the presence of Vpr (HIV-1 infection), inflammation in the ectocervical environment is reduced, and may render women more susceptibility to other opportunistic genital infections due to a weaker defense mechanism (Kelly et al., 1997, Stringer and Antonsen, 2008). Moreover, the result of this study is in agreement with others showing that the GR is needed for the effects elicited by Vpr. This implies that the GR could be a key protein exploited by HIV-1 to ensure successful pathogenesis. To conclude, the combined results from this thesis suggest that the contraceptive use of MPA and NET-A may affect local immune function in the human ectocervical epithelial cells.

References

AAGAARD, M. M., SIERSBAEK, R. & MANDRUP, S. (2011) Molecular basis for gene-specific transactivation by nuclear receptors. *Biochim Biophys Acta,* 1812**,** 824-35.

AFRICANDER, D., LOUW, R., VERHOOG, N., NOETH, D. & HAPGOOD, J. P. (2011a) Differential regulation of endogenous pro-inflammatory cytokine genes by medroxyprogesterone acetate and norethisterone acetate in cell lines of the female genital tract. *Contraception,* 84**,** 423-435.

AFRICANDER, D., VERHOOG, N. & HAPGOOD, J. P. (2011b) Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception. *Steroids,* 76**,** 636-652.

AFRICANDER, D. J. (2010) Comparative study of the molecular mechanism of action of the synthetic progestins, Medroxyprogesterone acetate and Norethisterone acetate. *Biochemistry.* Stellenbosch, University of Stellensbosch, South Africa.

AHMAD, N. & KUMAR, R. (2011) Steroid hormone receptors in cancer development: a target for cancer therapeutics. *Cancer Lett,* 300**,** 1-9.

ALFANO, M. & POLI, G. (2005) Role of cytokines and chemokines in the regulation of innate immunity and HIV infection. *Molecular immunology,* 42**,** 161-82.

ALMAWI, W. Y. & MELEMEDJIAN, O. K. (2002) Negative regulation of nuclear factor-kappaB activation and function by glucocorticoids. *Journal of molecular endocrinology,* 28**,** 69-78.

ALTMAN, G. B., GOWN, A. M., LUCHTEL, D. L. & BAKER, C. (1999) RANTES production by cultured primate endometrial epithelial cells. *Am J Reprod Immunol,* 42**,** 168-74.

ANDERSEN, J. L. & PLANELLES, V. (2005) The Role of Vpr in HIV-1 Pathogenesis. *Current***,** 43-51.

ANDERSON, G. L., LIMACHER, M., ASSAF, A. R., BASSFORD, T., BERESFORD, S. A., BLACK, H., BONDS, D., BRUNNER, R., BRZYSKI, R., CAAN, B., CHLEBOWSKI, R., CURB, D., GASS, M., HAYS, J., HEISS, G., HENDRIX, S., HOWARD, B. V., HSIA, J., HUBBELL, A., JACKSON, R., JOHNSON, K. C., JUDD, H., KOTCHEN, J. M., KULLER, L., LACROIX, A. Z., LANE, D., LANGER, R. D., LASSER, N., LEWIS, C. E., MANSON, J., MARGOLIS, K., OCKENE, J., O'SULLIVAN, M. J., PHILLIPS, L., PRENTICE, R. L., RITENBAUGH, C., ROBBINS, J., ROSSOUW, J. E., SARTO, G., STEFANICK, M. L., VAN HORN, L., WACTAWSKI-WENDE, J., WALLACE, R. & WASSERTHEIL-SMOLLER, S. (2004) Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA,* 291**,** 1701-12.

ANSAR AHMED, S. & TALAL, N. (1990) Sex hormones and the immune system - part 2. Animal data. *Bailliere's Clinical Rheumatology,* 4**,** 13-30.

ARAGANE, Y., RIEMANN, H., BHARDWAJ, R. S., SCHWARZ, A., SAWADA, Y., YAMADA, H., LUGER, T. A., KUBIN, M., TRINCHIERI, C. & SCHWARZ, T. (1994) IL-12 is expressed and released by human keratinocytes and epidermoid carcinoma cell lines. *The Journal of Immunology,* 153**,** 5366-72.

ARAMBASIC, J., POZNANOVIC, G., IVANOVIC-MATIC, S., BOGOJEVIC, D., MIHAILOVIC, M., USKOKOVIC, A. & GRIGOROV, I. (2010) Assocation of the

glucocorticoid receptor with STAT3, C/EBPβ, and the hormone-responsive element within the rat haptoglobin gene promoter during the acute phase response. *IUBMB Life,* 62**,** 227-236.

ARCHER, M., MADDEN, C. & ODERDA, G. (2011) Drug class review: Hormonal contraceptives. Salt Lake City, University of Utah College of Pharmacy.

ASADULLAH, K., STERRY, W. & VOLK, H. D. (2003) Interleukin-10 Therapy — Review of a New Approach. *Pharmacological Reviews,* 55**,** 241-269.

ATTARDI, B. J., KODURI, S. & HILD, S. A. (2010) Relative progestational and androgenic activity of four progestins used for male hormonal contraception assessed in vitro in relation to their ability to suppress LH secretion in the castrate male rat. *Mol Cell Biol,* 328**,** 16-21.

AVENANT, C., KOTITSCHKE, A. & HAPGOOD, J. P. (2010a) Glucocorticoid receptor phosphorylation modulates transcription efficacy through GRIP-1 recruitment. *Biochemistry,* 49**,** 972-985.

AVENANT, C., RONACHER, K., STUBSRUD, E., LOUW, A. & HAPGOOD, J. P. (2010b) Role of ligand-dependent GR phosphorylation and half-life in determination of ligand-specific transcriptional activity. *Mol Cell Endocrinol*.

AYYAVOO, V., MAHALINGAM, S., RAFAELI, Y., KUDCHODKAR, S., CHANG, D., NAGASHUNMUGAM, T., WILLIAMS, W. V. & WEINER, D. B. (1997) HIV-1 viral protein R (Vpr) regulates viral replication and cellular proliferation in T cells and monocytoid cells in vitro. *Journal of leukocyte biology,* 62**,** 93-99.

AYYAVOO, V., MUTHUMANI, K., KUDCHODKAR, S., ZHANG, D., RAMANATHAN, P., DAYES, N. S., KIM, J. J., SIN, J. I., MONTANER, L. J. & WEINER, D. B. (2002) HIV-1 viral protein R compromises cellular immune function in vivo. *Int Immunol,* 14**,** 13-22.

BAGAMASBAD, P. & DENVER, R. J. (2011) Mechanisms and significance of nuclear receptor auto- and cross-regulation. *Gen Comp Endocrinol,* 170**,** 3-17.

BAI, Z. & GUST, R. (2009) Breast cancer, estrogen receptor and ligands. *Arch Pharm (Weinheim),* 342**,** 133-49.

BAKRY, S., MERHI, Z. O., SCALISE, T. J., MAHMOUD, M. S. & FADIEL, A. (2008) Depot-medroxyprogesterone acetate : an update. *North***,** 1-12.

BALOTTA, C., LUSSO, P., CROWLEY, R., GALLO, R. C. & FRANCHINI, G. (1993) Antisense phosphorothioate oligodeoxynucleotides targeted to the vpr gene inhibit human immunodeficiency virus type 1 replication in primary human macrophages. *J Virol,* 67**,** 4409-14.

BAMBERGER, C., ELSE, T., BAMBERGER, A.-M., BEIL, F. & SCHULTE, H. (1999) Dissociative glucocorticoid activity of medroxyprogesterone acetate in normal human lymphocytes. *Journal of Clinical Endocrinology & Metabolism,* 84**,** 4055-4061.

BAROUSSE, M. M., THEALL, K. P., VAN DER POL, B., FORTENBERRY, D., ORR, D. P. & FIDEL JR, P. L. (2007) Susceptibility of middle adolescent females to sexually transmitted infections: impact of hormone contraception and sexual behaviors on vaginal immunity. *American Journal of Reproductive Immunology,* 58**,** 159-168.

BEATEN, J. M., LAVREYS, L. & OVERBAUGH, J. (2007) The influence of hormonal contraceptive use on HIV-1 transmission and disease progression. *Clin Infect Dis,* 45**,** 360-369.

BEATO, M. (1989) Gene regulation by steroid hormones. *Cell,* 56**,** 335-344.

BEATO, M. & KLUG, J. (2000) Steroid hormone receptors: an update. *Hum Reprod Update,* 6**,** 225-236.

BEATTIE, C. W., HANSEN, N. W. & THOMAS, P. A. (1985) Steroid receptors in human lung cancer. *Cancer Res,* 45**,** 4206-14.

BEBELL, L. M., PASSMORE, J.-A. S., WILLIAMSON, C., MLISANA, K., IRIOGBE, I., VAN LOGGERENBERG, F., ABDOOL KARIM, Q. & ABDOOL KARIM, S. (2008) Relationship between levels of inflammatory cytokines in the genital tract and CD4+ cell counts in women with acute HIV-1 infection. *The Journal of infectious diseases,* 198**,** 710-714.

BECKER, C., WIRTZ, S., MA, X., BLESSING, M., GALLE, P. R. & NEURATH, M. F. (2001) Regulation of IL-12p40 promoter activity in primary human monocytes: Roles of NF-kB, CCAAT/enhancer-binding protein beta, and PU.1 and indentification of a novel repressor element (GA-12) that responds to IL-4 and Prostaglandin E2. *The Journal of Immunology,* 167**,** 2608-2618.

BEIJERINK, N. J., BHATTI, S. F. M., OKKENS, A. C. & DIELEMAN, S. J. (2008) Pulsatile plasma profiles of FSH and LH before and during medroxyprogesterone acetate treatment in the bitch. *Obstetrics & Gynecology,* 70**,** 179-185.

BELL, O., TIWARI, V. K., THOMÄ, N. H. & SCHÜBELER, D. (2011) Determinants and dynamics of genome accessibility. *Nature reviews Genetics,* 12**,** 554-564.

BENKHART, E. M., SIEDLAR, M., WEDEL, A., WERNER, T. & ZIEGLER-HEITBROCK, H. W. L. (2000) Role of Stat 3 in lipopolysaccharide-induced IL-10 gene expression. *The Journal of Immunology,* 165**,** 1612-1617.

BENTEL, J. M., BIRRELL, S. N., PICKERING, M. A., HOLDS, D. J., HORSFALL, D. J. & TILLEY, W. D. (1999) Androgen receptor agonist activity of the synthetic progestin, medroxyprogesterone acetate, in human breast cancer cells. *Mol Cell Endocrinol,* 154**,** 11-20.

BERAL, V., BANKS, E., BULL, D. & REEVES, G. (2003) Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet,* 362**,** 419-427.

BERGINK, E. W., VAN MEEL, F., TURPIJN, E. W. & VAN DER VIES, J. (1983) Binding of progestagens to receptor proteins in MCF-7 cells. *J Steroid Biochem,* 19**,** 1563-70.

BLISH, C. A. & BAETEN, J. M. (2011) Hormonal Contraception and HIV-1 Transmission. *Am J Reprod Immunol,* 65**,** 302-7.

BLISH, C. A. & BEATEN, J. M. (2011) Hormonal contraception and HIV-1 transmission. *Am J Reprod Immunol,* 65**,** 302-307.

BOBARDT, M. D., CHATTERJI, U., SELVARAJAH, S., SCHUEREN, B. V. D., DAVID, G., KAHN, B. & GALLAY, P. A. (2007) Cell-Free Human Immunodeficiency Virus Type 1 Transcytosis through Primary Genital Epithelial Cells ▪. *Society,* 81**,** 395-405.

BOST, K. L., RAMP, W. K., NICHOLSON, N. C., BENTO, J. L., MARRIOTT, I. & HUDSON, M. C. (1999) Staphylococcus aureus infection of mouse or human osteoblasts induces high levels of interleukin-6 and interleukin-12 production. *J Infect Dis,* 180**,** 1912-20.

BOUMAN, A., HEINEMAN, M. J. & FAAS, M. M. (2005) Sex hormones and the immune response in humans. *Human reproduction update,* 11**,** 411-423.

BRABIN, L. (2002) Interactions of the female hormonal environment, susceptibility to viral infections, and disease progression. *AIDS Patient Care STDS,* 16**,** 211-21.

BRUNDA, J. (1994) Interleukin-12. *Journal of Leukocyte Biology,* 55**,** 280-8.

BUSER, A. C., GASS-HANDEL, E. K., WYSZOMIERSKI, S. L., DOPPLER, W., LEONHARDT, S. A., SCHAACK, J., ROSEN, J. M., ANDERSON, S. M. & EDWARDS, D. P. (2007) Progesterone receptor repression of prolactin/signal transducer and activator of transcription 5-mediated transcription of the beta-casein gene in mammary epithelial cells. *Molecular Endocrinology,* 21**,** 106-125.

BYRNES, A. A., HARRIS, D. M., ATABANI, S. F., SABUNDAYO, B. P., LANGAN, S. J., MARGOLICK, J. B. & KARP, C. L. (2008) Immune activation and IL-12 production during acute/early HIV infection in the absence and presence of highly active, antiretroviral therapy. *Journal of Leukocyte Biology,* 84**,** 1447-53.

CAO, S., LIU, J., CHESI, M., BERGSAGEL, P. L., HO, J. L., DONELLY, R. P. & MA, X. (2002) Differential regulation of IL-12 and IL-10 gene expression in macrophages by the basic leucine zipper transcrtiption factor c-Maf fibrosarcoma. *The Journal of Immunology,* 169**,** 5715-2725.

CARDINAUX, J. R., ALLAMAN, I. & MAGISTRETTI, P. J. (2000) Pro-inflammatory cytokines induce the transcription factors C/EBPbeta and C/EBPdelta in astrocytes. *Glia,* 29**,** 91-7.

CATO, A. C. B. & WADE, E. (1996) Molecular mechanisms of anti-inflammatory action of glucocorticoids. *BioEssays,* 18**,** 371-378.

CEJTIN, H. E., JACOBSON, L., SPRINGER, G., WATTS, D. H., LEVINE, A., GREENBLATT, R., ANASTOS, K., MINKOFF, H. L. & MASSAD, L. S. (2003) Effect of hormonal contraceptive use on plasma HIV-1-RNA levels among HIV-infected women. *AIDS,* 17**,** 1702-1704.

CHAMBERS, K. A., PARKS, R. J. & ANGEL, J. B. (2004) Disruption of MAP kinase activation and nuclear factor binding to the IL-12 p40 promoter in HIV-infected myeloid cells. *Clin Exp Immunol,* 137**,** 329-40.

CHEHIMI, J., STARR, S. E., FRANK, I., RENGARAJU, M., JACKSON, S. J., YOUNG, D., NICKBARG, E., WOLF, S. F. & TRINCHIERI, G. (1992) Natural killer (NK) cell stimulatory factor increases the cytotoxic activity of NK cells from both healthy donors and human immunodeficiency virus-infected patients. *Journal of Experimental Medicine,* 175**,** 789-796.

CHEHIMI, J., START, S. E., FRANK, I., ANDREA, A. D., MARL, S. X., ROY, R., SENNELIER, J. & TRINCHIERIS, G. (1994) Impaired Interleukin 12 production in Human Immunodeficiency Virus-infected patients. *Journal of Experimental Medicine,* 179**,** 1361-1366.

CHEHIMI, J. & TRINCHIERI, G. (1994) Interleukin-12: a bridge between innate resistance and adaptive immunity with a role in infection and acquired immunodeficiency. *Journal of Clinical Immunology,* 14**,** 149-61.

CHEN, W., ROGATSKY, I. & GARABEDIAN, M. J. (2006) MED14 and MED1 Differentially Regulate Target-Specific Gene Activation by the Glucocorticoid Receptor. *Mol Endocrinol,* 20**,** 560-572.

CHIZZONITE, R., GUBLER, U., MAGRAM, J. & STERN, A. S. (1998) Interleukin-12. *Analysis*, 183-198.

CHRIGUER, R. S., ELIAS, L. L., DA SILVA, I. M., JR., VIEIRA, J. G., MOREIRA, A. C. & DE CASTRO, M. (2005) Glucocorticoid sensitivity in young healthy individuals: in vitro and in vivo studies. *J Clin Endocrinol Metab,* 90**,** 5978-84.

CLEMETSON, D. B. A., MOSS, G. B., WILLERFORD, D. M., HENSEL, M., EMONYI, W., HOLMES, K. K., PLUMMER, F., NDINYA-ACHOLA, J., ROBERTS, P. L., HILLIER, S. & KREISS, J. K. (1993) Detection of HIV DNA in Cervical and Vaginal Secretions. Prevalance and correlates among women in Niarobi, Kenya. *JAMA: The Jounal of the American Medical Association,* 269**,** 2860-2864.

CLERICI, M. & SHEARER, G. M. (1994) Viewpoint: The Thl-Th2 hypothesis of HIV infection : new insights. *Immunology today,* 15**,** 575-581.

COHEN, E. A., SUBBRAMANIAN, R. A. & GOTTLINGER, H. G. (1996) Role of auxiliary proteins in retroviral morphogenesis. *Curr Top Microbiol Immunol,* 214.

COHEN, G. R., PLUMMER, F. A., MUGO, N., MACLEAN, I., SHEN, C., BUKUSI, E. A., IRUNGU, E., SINEI, S., BWAYO, J. J. & BRUNHAM, R. C. (1999) Increased interleukin-10 in the endocerivcal secretions of women with non-ulcerative sexually transmitted diseases: a mechanism for enhanced HIV-1 transmission? *AIDS,* 13**,** 327-332.

CONNEELY, O. M., MULAC-JERICEVIC, B., DEMAYO, F., LYDON, J. P. & O'MALLEY, B. W. (2002) Reproductive functions of progesterone receptors. *The Endocrine Society*, 339-355.

COUPER, K. N., BLOUNT, D. G. & RILEY, E. M. (2008) IL-10: the master regulator of immunity to infection. *Journal of immunology* 180**,** 5771-5777.

COURTIN, A., COMMUNAL, L., VILASCO, M., CIMINO, D., MOURRA, N., DE BORTOLI, M., TAVERNA, D., FAUSSAT, A., CHAOUAT, M., FORGEX, P. & GOMPEL, A. (2011) Glucocorticoid receptor activity discriminates between progesterone and medroxyprogesterone acetate effects in breast cells. *Breast Cancer Res Treat*.

CUMMINS, J. E. & DEZZUTTI, C. S. (2000) Sexual HIV-1 Transmission and Mucosal Defense Mechanisms. *Prevention*.

CUMMINS, J. E. & DONCEL, G. F. (2009) Biomarkers of cervicovaginal inflammation for the assessment of microbicide safety. *Sexually transmitted diseases,* 36**,** S84-91.

D'ANDREA, A., ASTE-AMEZAGA, M., VALIANTE, N. M., MA, X., KUBIN, M. & TRINCHIERI, G. (1993) Interleukin 10 inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *Journal of Experimental Medicine,* 178**,** 1041-8.

D'ANDREA, A., RENGARAJU, M., VALIANTE, N. M., CHEHIMI, J., KUBIN, M., ASTE, M., CHAN, S. H., KOBAYASHI, M., YOUNG, D., NICKBARG, E. & ET AL. (1992) Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J Exp Med,* 176**,** 1387-98.

DAHLMAN-WRIGHT, K., SILTALA-ROOS, H., CARLSTEDT-DUKE, J. & GUSTAFSSON, J. A. (1990) Protein-protein interactions facilitate DNA binding by the glucocorticoid receptor DNA-binding domain. *J Biol Chem,* 265**,** 14030-5.

DARNEY, P. D. (1995) The androgenicity of progestins. *Am J Med,* 98**,** 104S-110S.

DE BOSSCHER, K., BERGHE, W. V. & HAEGEMAN, G. (2000) Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. *Journal of Neuroimmunology,* 109**,** 16-22.

DE BOSSCHER, K. & HAEGEMAN, G. (2009) Minireview: latest perspectives on antiinflammatory actions of glucocorticoids. *Mol Endocrinol,* 23**,** 281-91.

DE BOSSCHER, K., VANDEN BERGHE, W. & HAEGEMAN, G. (2003) The interplay between the glucocorticoid receptor and nuclear factor-kappa B or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev,* 24**,** 488-522.

DE KLOET, E. R., VAN ACKER, S. A., SIBUG, R. M., OITZL, M. S., MEIJER, O. C., RAHMOUNI, K. & DE JONG, W. (2000) Brain mineralocorticoid receptors and centrally regulated functions. *Kidney Int,* 57**,** 1329-36.

DE WAAL MALEFYT, R., YSSEL, H., RONCAROLO, M., SPITS, H. & DE VRIES, J. E. (1992) Interleukin-10. *Current opinion in Immunology,* 4**,** 314-320.

DECKERS, G. H., SCHOONEN, W. G. & KLOOSTERBOER, H. J. (2000) Influence of the substitution of 11-methylene, delta(15), and/or 18-methyl groups in norethisterone on receptor binding, transactivation assays and biological activities in animals. *J Steroid Biochem Mol Biol,* 74**,** 83-92.

DEL VECCHIO, M., BAJETTA, E., CANOVA, S., LOTZE, M. T., WESA, A., PARMIANI, G. & ANICHINI, A. (2007) Interleukin-12: biological properties and clinical application. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 13**,** 4677-85.

DI CARLO, F., GALLO, E., CONTI, G. & RACCA, S. (1983) Changes in the binding of oestradiol to uterine oestrogen receptors induced by some progesterone and 19-nor-testosterone derivatives. *J Endocrinol,* 98**,** 385-9.

DINARELLO, C. A. (2000) Proinflammatory cytokines. *Chest,* 118**,** 503-8.

DUBICKE, A., FRANSSON, E., CENTINI, G., ANDERSSON, E., BYSTROM, B., MALMSTROM, A., PETRAGLIA, F., SVERREMARK-EKSTROM, E. & EKMAN-ORDEBERG, G. (2010) Pro-inflammatory and anti-inflammatory cytokines in human preterm and term cervical ripening. *Journal of Reproductive Immunology,* 84**,** 176-185.

EDWARDS, D. P., ALTMANN, M., DEMARZO, A., ZHANG, Y., WEIGEL, N. L. & BECK, C. A. (1995) Progesterone receptor and the mechanism of action of progesterone antagonists. *Journal of Steroid Biochemistry and Molecular Biology,* 53**,** 449-58.

ELOVITZ, M. A. & GONZALEZ, J. (2008) Medroxyprogesterone acetate modulates the immune response in the uterus, cervix and placenta in a mouse model of preterm birth. *The Journal of Maternal-fetal and Neonatal Medicine,* 21**,** 223-230.

EMERMAN, M. (1996a) HIV-1, Vpr and the cell cycle. *Curr. Biol.,* 6.

EMERMAN, M. (1996b) HIV-1, Vpr and the cell cycle. *Current Biology,* 6**,** 1096-1103.

ENOMOTO, L. M., KLOBERDANZ, K. J., MACK, D. G., ELIZABETH, D. & WIENBERG, A. (2007) Ex vivo of estrogen and progesterone compared with dexamethasone on cell-mediated immunity of HIV-infected and uninfected subjects. *Journal of acquired immune deficiency syndromes,* 45**,** 137-143.

ETIENNE, M. C., MILANO, G., FRENAY, M., RENEE, N., FRANCOIS, E., THYSS, A., SCHNEIDER, M. & NAMER, M. (1992) Pharmacokinetics and pharmacodynamics of medroxyprogesterone acetate in advanced breast cancer patients. *J Clin Oncol,* 10**,** 1176-1182.

EVANS, R. M. (1988) The steroid and thyroid hormone receptor superfamily. *Science,* 240**,** 889-895.

FAHEY, J. V., SCHAEFER, T. M., CHANNON, J. Y. & WIRA, C. R. (2005) Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract. *Human Reproduction,* 20**,** 1439-1446.

FAHEY, J. V., SCHAEFER, T. M. & WIRA, C. R. (2006) Sex hormone modulation of human uterine epithelial cell immune responses. *Access,* 46**,** 1082-1087.

FAIVRE, E. J., DANIEL, A. R., HILLARD, C. J. & LANGE, C. A. (2008) Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. *Mol Endocrinol,* 22**,** 823-37.

FAN, X., XU, H., WARNER, M. & GUSTAFSSON, J. A. (2010) ERbeta in CNS: new roles in development and function. *Prog Brain Res,* 181**,** 233-50.

FERNANDEZ, E. J. & LOLIS, E. (2002) Structure, function and inhibition of chemokines. *Annu. Rev. Pharmacol. Toxicol.,* 42**,** 469-499.

FICHOROVA, R. N. (2004) Guiding the vaginal microbicide trials with biomarkers of inflammation. *Journal of acquired immune deficiency syndromes* 37 Suppl 3**,** S184-93.

FICHOROVA, R. N. & ANDERSON, D. J. (1999) Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells. *Biology of reproduction,* 60**,** 508-14.

FICHOROVA, R. N., CRONIN, A. O., ANDERSON, D. J. & INGALLS, R. R. (2002) Response to Neisseria gonorrhoeae by cervicovaginal epithelial cells occurs in the absence of toll-like receptor 4-mediated signalling. *Journal of Immunology,* 168**,** 2424-2432.

FICHOROVA, R. N., RHEINWALD, J. G. & ANDERSON, D. J. (1997) Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. *Biology of reproduction,* 57**,** 847-55.

FIORENTINO, D. F., BOND, M. W. & MOSMANN, T. R. (1989) Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med,* 170**,** 2081-95.

FLEISCHMAN, D. S. & FESSLER, D. M. T. (2010) Progesterone's effects on the psychology of disease avoidance: Support for the compensatory behavioral prophylaxis hypothesis. *Hormones and Behavior,* xxx**,** xxx-xxx.

FOTHERBY, K., HOWARD, G., SHRIMANKER, K., ELDER, M. & BYE, P. G. T. (1978) Plasma levels of norethisterone after single and multiple injections of norethisterone oenanthate. *Contraception,* 18**,** 1-6.

FOTHERBY, K., TOWOBOLA, O., MUGGERIDE, J. & ELDER, M. G. (1983) Norethisterone levels in maternal serum and milk after intramuscular injection of norethisterone oenanthate as a contraceptive. *Contraception,* 28**,** 405-411.

FRAGA, D., MEULIA, T. & FENSTER, S. (2008) Real-Time PCR. *Cycle***,** 1-34.

FUHRMANN, U., KRATTENMACHER, R., SLATER, E. P. & RFRITZEMEIER, K.-H. (1996) The novel progestin Drospirenone and its natural counterpart Progesterone: Biochemical profile and antiandrogenic potential. *Contraception,* 54**,** 243-251.

FUHRMANN, U., SLATER, E. P. & FRITZEMEIER, K. H. (1995) Characterization of the novel progestin gestodene by receptor binding studies and transactivation assays. *Contraception,* 51**,** 45-52.

FUNDER, J. W. (2004) Aldosterone, mineralocorticoid receptors and vascular inflammation. *Mol Cell Endocrinol,* 217**,** 263-9.

GALI, Y., ARIEN, K. K., PRAET, M., VAN DEN BERGH, R., TEMMERMAN, M., DELEZAY, O. & VANHAM, G. (2010) Development of an in vitro dual-chamber model of the female genital tract as a screening tool for epithelial toxicity. *J Virol Methods,* 165**,** 186-97.

GAMBRELL, R. D., JR., MASSEY, F. M., CASTANEDA, T. A., UGENAS, A. J., RICCI, C. A. & WRIGHT, J. M. (1980) Use of the progestogen challenge test to reduce the risk of endometrial cancer. *Obstet Gynecol,* 55**,** 732-8.

GARCIA-BECERRA, R., COONEY, A. J., BORJA-CACHO, E., LEMUS, A. E., PEREZ-PALACIOS, G. & LARREA, F. (2004) Comparative evaluation of androgen and progesterone receptor transcription selectivity indices of 19-nortestesterone-derived progestins. *Journal of Steroid Biochemistry and Molecular Biology,* 91**,** 21-27.

GEE, K., ANGEL, J. B., MISHRA, S., BLAHOIANU, M. A. & KUMAR, A. (2007) IL-10 regulation by HIV-Tat in primary human monocytic cells: Involvement of calmodulin/calmodulin-dependent protein kinase-activated p38 MAPK and Sp-1 and CREB-1 transcription factors. *The Journal of Immunology,* 178**,** 798-807.

GEIGER, A. M. & FOXMAN, B. (1996) Risk factors for vulvovaginal candidiasis: a case-control study among university students. *Epidemiology,* 7**,** 182-7.

GELLERSEN, B., FERNANDES, M. S. & BROSENS, J. J. (2009) Non-genomic progesterone actions in female reproduction. *Hum Reprod Update,* 15**,** 119-38.

GHATGE, R. P., JACOBSEN, B. M., SCHITTONE, S. A. & HORWITZ, K. B. (2005) Research article The progestational and androgenic properties of medroxyprogesterone acetate : gene regulatory overlap with dihydrotestosterone in breast cancer cells. *Breast Cancer Research,* 7.

GIULIETTI, A., OVERBERGH, L., VALCKX, D., DECALLONNE, B., BOUILLON, R. & MATHIEU, C. (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods (San Diego, Calif.),* 25**,** 386-401.

GIVAN, A. L., WHITE, H. D., STERN, J. E., COLBY, E., GOSSELIN, E. J., GUYRE, P. M. & WIRA, C. R. (1997) Flow cytometric analysis of leukocytes in the human female reproductive tract: comparison of fallopian tube, uterus, cervix, and vagina. *Am J Reprod Immunol,* 38**,** 350-9.

GOEBELSMANN, U., STANCZYK, F. Z., BRENNER, P. F., GOEBELSMANN, A. E., GENTZSCHEIN, E. K. & MISHELL, D. R., JR. (1979) Serum norethindrone (NET) concentrations following intramuscular NET enanthate injection. Effect upon serum LH, FSH, estradiol and progesterone. *Contraception,* 19**,** 283-313.

GOLDHAR, A. S., DUAN, R., GINSBURG, E. & VONDERHAAR, B. K. (2011) Progesterone induces expression of the prolactin receptor gene through cooperative action of Sp1 and C/EBP. *Mol Cell Endocrinol,* 335**,** 148-57.

GOMEZ, C. R., NOMELLINI, V. & KOVACS, E. J. (2009) Sex Hormones and Immunosenescence. *Hormones***,** 799-831.

GORIELY, S., DEMONTE, D., NIZET, S., WIT, D. D., WILLEMS, F., GOLDMAN, M. & LINT, C. V. (2003) Human IL-12(p35) gene activation involves selective remodeling of a single nucleosome within a region of the promoter containing critical Sp1-binding sites. *Blood,* 101**,** 4894-4902.

GRAHAM, J. D. & CLARKE, C. L. (1997) Physiological Action of Progesterone in Target Tissues *. *Endocrine Reviews,* 18**,** 502-519.

GRAHAM, J. D., YEATES, C., BALLEINE, R. L., HARVEY, S. S., MILLIKEN, J. S., BILOUS, A. M. & CLARKE, C. L. (1996) Progesterone receptor A and B protein expression in human breast cancer. *J Steroid Biochem Mol Biol,* 56**,** 93-98.

GRAVITT, P. & GHANEM, K. (2010) *Sex steroids and risk of female genital tract infection,* Heidelberg; New York, Springer.

GRAVITT, P. E., HILDESHEIM, A., HERRERO, R., SCHIFFMAN, M., SHERMAN, M. E., BRATTI, M. C., RODRIGUEZ, A. C., MORERA, L. A., CARDENAS, F., BOWMAN, F. P., SHAH, K. V. & CROWLEY-NOWICK, P. A. (2003) Correlates of IL-10 and IL-12 concentrations in cervical secretions. *Journal of Clinical Immunology,* 23**,** 175-83.

GREENDALE, G. A., LEE, N. P. & ARRIOLA, E. R. (1999) The menopause. *Lancet,* 353**,** 571-80.

GRESE, T. A. & DODGE, J. A. (1998) Selective estrogen receptor modulators (SERMs). *Curr Pharm Des,* 4**,** 71-92.

GREYDANUS, D. E., PATEL, D. R. & RIMSZA, M. E. (2001) Contraception in the adolescent: an update. *Pediatrics,* 107**,** 562-73.

GRI, G., SAVIO, D., TRINCHIERI, G. & MA, X. (1998) Synergistic regulation of the human IL-12p40 promoter by NFkB and Ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. *The Journal of biological chemistry,* 273**,** 6431-6438.

GRIEKSPOOR, A., ZWART, W., NEEFJES, J. & MICHALIDES, R. (2007) Visualizing the action of steroid hormone receptors in living cells. *Nucl Recept Signal,* 5**,** e003.

GRIMM, S. & BAEUERLE, P. A. (1993) The inducible transcription factor NF-kappa B: structure-function relationship of its protein subunits. *Biochem J,* 290 (Pt 2)**,** 297-308.

GROSS, K. & CIDLOWSKI, J. (2008) Tissue-specific glucocorticoid action: a family affair. *Trends Endocrinol Metab,* 19**,** 331-9.

GUHA, D. & CHATTERJEE, R. (2009) Cytokine levels in HIV infected and uninfected Indian women: correlation with other STAs. *Exp Mol Pathol,* 86**,** 65-8.

GUMBI, P. P., NKWANYANA, N. N., BERE, A., BURGERS, W. A., GRAY, C. M., WILLIAMSON, A.-L., HOFFMAN, M., COETZEE, D., DENNY, L. & PASSMORE, J.-A. S. (2008) Impact of mucosal inflammation on cervical HIV-1-specific CD8 T-cell responses in the female genital tract during chronic HIV infection. *Journal of Virology,* 82**,** 8529-8536.

GUO, W. X., ANTAKLY, T., CADOTTE, M., KACHRA, Z., KUNKEL, L., MASOOD, R. & GILL, P. (1996) Expression and cytokine regulation of glucocorticoid receptors in Kaposi's sarcoma. *Am J Pathol,* 148**,** 1999-2008.

HAASE, A. T. (2010) Targeting early infection to prevent HIV-1 mucosal transmission. *Nature,* 464**,** 217-223.

HAIDER, S. & DARNEY, F. (2007) Injectable contraception. *Clin Obstet Gynecol,* 50**,** 898-906.

HAMMES, S. R. & LEVIN, E. R. (2007) Extranuclear steroid receptors: nature and actions. *Endocr Rev,* 28**,** 726-741.

HAMMOND, G. L., LAHTEENMAKI, P. L., LAHTEENMAKI, P. & LUUKKAINEN, T. (1982) Distribution and percentage of non-protein bound contraceptive steroids in human serum. *J Steroid Biochem,* 17**,** 375-380.

HAMZA, T., BARNETT, J. B. & LI, B. (2010) Interleukin 12 a key immunoregulatory cytokine in infection applications. *International journal of Molecular Sciences,* 11**,** 789-806.

HAPGOOD, J. P. & TOMASICCHIO, M. (2010) Modulation of HIV-1 virulence via the host glucocorticoid receptor: towards further understanding the molecular mechanisms of HIV-1 pathogenesis. *Arch Virol,* 155**,** 1009-1019.

HARRISON, R. F. & BARRY-KINSELLA, C. (2000) Efficacy of medroxyprogesterone treatment in infertile women with endometriosis: a prospective, randomized, placebo-controlled study. *Fertil Steril,* 74**,** 24-30.

HASKO, G. & SZABO, C. (1999) IL-12 as a therapeutic target for pharmacological modulation in immune-mediated and inflammatory diseases: regulation of T helper 1/T helper 2 responses. *British Journal of Pharmacology,* 127**,** 1295-304.

HATTORI, N., MICHAELS, F., FARGNOLI, K., MARCON, L., GALLO, R. C. & FRANCHINI, G. (1990) The human immunodeficiency virus type 2 vpr gene is essential for productive infection of human macrophages. *Proc Natl Acad Sci U S A,* 87**,** 8080-4.

HAYES, M. P., MURPHY, F. J. & BURD, P. R. (1998) Interferon-gamma-dependent inducible expression of the human interleukin-12p35 gene in monocytes initiates from a TATA-containing promoter distinct from the CpG-rich promoter active in Epstein-Barr-Virus transformed lymphoblastoid cells. *Blood,* 91**,** 4645-4651.

HEFFRON, R., DONNELL, D., CELUM, C., MUGO, N., WERE, E., DE BRUYN, G., NAKKA-JOLOBA, E., NGURE, K., KIARIE, J. & COOMBS, R. W. (2012) Use of hormonal contraceptives and risk of HIV-1 transmission: a prospective cohort study. *Lancet Infect Dis,* 12**,** 19-26.

HEIKINHEIMO, O. & LA, P. (2008) Contraception and HIV infection in women. *Human Reproduction Update***,** 1-11.

HEINLEIN, C. A. & CHANG, C. (2002) Androgen receptor (AR) coregulators: an overview. *Endocr Rev,* 23**,** 175-200.

HEINZINGER, N. K., BUKRINSKY, M. I., HAGGERTY, S. A., RAGLAND, A. M., KEWALRAMANI, V., LEE, M. A., GENDELMAN, H. E., RATNER, L., STEVENSON, M. & EMERMAN, M. (1994) The Vpr protein of human immunodeficiency virus type 1 influences nuclear translocation of viral nucleic acids in non-dividing host cells *Proc Natl Acad Sci U S A,* 91**,** 7311-7315.

HEL, Z., STRINGER, E. & MESTECKY, J. (2010) Sex steroid hormones, hormonal contraception, and the immunobiology of human immunodeficiency virus-1 infection. *Endocrine reviews,* 31**,** 79-97.

HICKEY, D. K., FAHEY, J. V. & WIRA, C. R. (2012) Mouse estrous cycle regulation of vaginal versus uterine cytokines, chemokines, alpha/beta-defensins and TLRs. *Innate Immunity,* 0**,** 1-11.

HICKEY, D. K., PATEL, M. V., FAHEY, J. V. & WIRA, C. R. (2011) Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *J Reprod Immunol,* 88**,** 185-94.

HIROI, M., STANCZYK, F. Z., GOEBELSMANN, U., BRENNER, P. F., LUMKIN, M. E. & MISHELL, D. R., JR. (1975) Radioimmunoassay of serum medroxyprogesterone acetate (Provera) in women following oral and intravaginal administration. *Steroids,* 26**,** 373-86.

HLADIK, F. & HOPE, T. J. (2009) HIV Infection of the Genital Mucosa in Women. *Current HIV/AIDS Reports,* 6**,** 20-28.

HLADIK, F. & MCELRATH, M. J. (2008) Setting the stage: host invasion by HIV. *Nat Rev Immunol,* 8**,** 447-57.

HOLMSTROM, S. R., CHUPRETA, S., SO, A. Y. & INIGUEZ-LLUHI, J. A. (2008) SUMO-mediated inhibition of glucocorticoid receptor synergistic activity depends on stable assembly at the promoter but not on DAXX. *Mol Endocrinol,* 22**,** 2061-75.

HOWARD, G., WARREN, R. J. & FOTHERBY, K. (1975) Plasma levels of norethisterone in women receiving norethisterone oenanthate intramuscularly. *Contraception,* 12.

HOWELL, A., ANDERSON, E., BLAMEY, R., CLARKE, R. B., DIXON, J. M., DOWSETT, M., JOHNSTON, S. R., MILLER, W. R., NICHOLSON, R. & ROBERTSON, J. F. (1998) The primary use of endocrine therapies. *Recent Results Cancer Res,* 152**,** 227-44.

HOWELL, A., ASIN, S. N., YEAMAN, G. R. & WIRA, C. R. (2005) HIV-1 infection of the female reproductive tract. *Current HIV/AIDS Reports,* 2**,** 35-38.

HOWELL, A. L., EDKINS, R. D., RIER, S. E., YEAMAN, G. R., STERN, J. E., FANGER, M. W. & WIRA, C. R. (1997) Human immunodeficiency virus type 1 infection of cells and tissues from the upper and lower human female reproductive tract. *J Virol,* 71.

HU, J., ZHANG, Z., SHEN, W. J. & AZHAR, S. (2010) Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutrition and Metabolism,* 7**,** 1-25.

HUANG, P., CHANDRA, V. & RASTINEJAD, F. (2010) Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. *Annu Rev Physiol,* 72**,** 247-72.

HUGHES, G. C., THOMAS, S., LI, C., KAJA, M. & CLARK, E. A. (2008) Cutting edge: Progesterone regulates IFN-alpha production by plasmacytoid dendritic cells. *The Journal of Immunology,* 180**,** 2029-2033.

ILDGRUBEN, A. K., SJO, I. M. & HAMMARSTRO, M.-L. K. C. (2003) Influence of Hormonal Contraceptives on the Immune Cells and Thickness of Human Vaginal Epithelium. *Obstetrics & Gynecology,* 102**,** 571-582.

IRAHARA, M., UEMURA, H., YASUI, T., KINOSHITA, H., YAMADA, M., TEZUKA, M., KIYOKAWA, M., KAMADA, M. & AONO, T. (2001) Efficacy of every-other-day administration of conjugated equine estrogen and medroxyprogesterone acetate on

gonadotropin-releasing hormone agonists treatment in women with endometriosis. *Gynecol Obstet Invest,* 52**,** 217-22.

JARVINEN, A., KAINULAINEN, P., NISSILA, M., NIKKANEN, H. & KELA, M. (2004) Pharmacokinetics of estradiol valerate and medroxyprogesterone acetate in different age groups of postmenopausal women. *Maturitas,* 47**,** 209-17.

JOHANSSON-HAQUE, K., PALANICHAMY, E. & OKRET, S. (2008) Stimulation of MAPK-phosphatase 1 gene expression by glucocorticoids occur through a tethering mechanim involving C/EBP. *Journal of Molecular Endocrinology,* 41**,** 239-249.

JOHN, M., LIM, S., SEYBOLD, J., JOSE, P., ROBICHAUD, A., O'CONNOR, B., BARNES, P. J. & CHUNG, F. K. (1998) Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1alpha, granulocyte-macrophage colony stimulating factor and interferone-gamma release from alveolar macrophages in astma. *Am J Respir Crit Care Med,* 157**,** 256-262.

JONES, K. A. & TJIAN, R. (1985) Sp1 binds to promoter sequences and activates herpes simplex virus 'immediate-early' gene transcription in vitro. *Nature,* 317**,** 179-82.

JONES, L. A., ANTHONY, J.-P., HENRIQUEZ, F. L., LYONS, R. E., NICKDEL, M. B., CARTER, K. C., ALEXANDER, J. & ROBERTS, C. W. (2008) Toll-like receptor-4-mediated macrophage activation is differentially regulated by progesterone via the glucocorticoid and progesterone receptors. *Immunology,* 125**,** 59-69.

KALKHOVEN, E., WISSINK, S., SAAG, P. T. V. D. & BURG, B. V. D. (1996) Negative Interaction between the RelA (p65) Subunit of NF- □ B and the Progesterone Receptor *. *Biochemistry,* 271**,** 6217-6224.

KAMISCHKE, A., DIEBACKER, J. & NIESCHLAG, E. (2000a) Potential of norethisterone enanthate for male contraception: pharmacokinetics and suppression of pituitary and gonadal function. *Clin Endocrinol,* 53**,** 351-358.

KAMISCHKE, A., VENHERM, S., PLOGER, D., VON ECKARSTEIN, S. & NIESCHLAG, E. (2000b) Intramuscular testosterone undecanoate and norethisterone enanthate in a clinical trial for male contraception. *J Clin Endocrinol Metab,* 86**,** 303-309.

KARPOVA, T. & MCNALLY, J. G. (2006) Detecting protein-protein interactions with CFP-YFP FRET by acceptor photobleaching. *Curr Protoc Cytom,* Chapter 12**,** Unit12 7.

KASSEL, O. & HERRLICH, P. (2007) Crosstalk between the glucocorticoid receptor and other transcription factors: Molecular aspects. *Molecular and Cellular Endocrinology,* 275**,** 13-29.

KASTNER, P., BOCQUEL, M.-T., TURCOTTE, B., GARNIER, J.-M., HORWITZ, K. B., CHAMBON, P. & GRONEMEYER, H. (1990) Transient Expression of Human and Chicken Progesterone Receptors Does Not Support Alternative Translational Initiation from a Single mRNA as the Mechanism Generating Two Receptor Isoforms. *Journal of Biological Chemistry,* 265**,** 12163-12167.

KAUNITZ, A. M. (1998) Injectable depot medroxyprogesterone acetate contraception: an update for U.S. clinicians. *Int J Fertil Womens Med,* 43**,** 73-83.

KAUNITZ, A. M. (2000) Injectable contraception. New and existing options. *Obstet Gynecol Clin North Am,* 27**,** 741-80.

KAUSHIC, C. (2009) The role of the local microenvironment in regulating susceptibility and immune responses to sexually trasmitted virusses in the female genital tract. *Journal of Reproductive Immunology,* 83**,** 168-172.

KAUSHIC, C., ASHKAR, A. A., REID, L. A. & ROSENTHAL, K. L. (2003) Progesterone Increases Susceptibility and Decreases Immune Responses to Genital Herpes Infection. *Society,* 77**,** 4558-4565.

KAUSHIC, C., FERREIRA, V. H., KAFKA, J. K. & NAZLI, A. (2010) HIV Infection in the Female Genital Tract : Discrete Influence of the Local Mucosal Microenvironment. *American Journal of Reproductive Immunology,* 63**,** 566-575.

KAYISLI, U. A., MAHUTTE, N. G. & ARICI, A. (2002) Uterine chemokines in reproductive physiology and pathology. *Am J Reprod Immunol,* 47**,** 213-21.

KEIGHTLEY, M.-C. (1998) Steroid receptor isoforms : exception or rule ? *Molecular and Cellular Endocrinology,* 137**,** 1 - 5.

KELLY, R. W., CARR, G. G. & CRITCHLEY, H. O. D. (1997) A cytokine switch induced by human seminal plasma: an immune modulation with implications for sexually transmitted disease. *Human Reproduction,* 12**,** 677-681.

KEMPPAINEN, J. A., LANGLEY, E., WONG, C.-I., BOBSEINE, K., KELCE, W. R. & WILSON, E. M. (1999a) Distinguish androgen receptor agonist and antagonists: Distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. *Molecular Endocrinology,* 13**,** 440-454.

KEMPPAINEN, J. A., LANGLEY, E., WONG, C. I., BOBSEINE, K., KELCE, W. R. & WILSON, E. M. (1999b) Distinguishing androgen receptor agonists and antagonists: distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. *Mol Endocrinol,* 13**,** 440-54.

KIM, M. H., MINTON, A. Z. & AGRAWAL, V. (2009) C/EBPbeta regulates metastatic gene expression and confers TNF-alpha resistance to prostate cancer cells. *Prostate,* 69**,** 1435-47.

KIM, M. K., SEONG, S. J., LEE, T. S., KIM, J. W., NAM, B. H., HONG, S. R. & SUH, K. S. (2012) Treatment with Medroxyprogesterone Acetate Plus Levonorgestrel-releasing Intrauterine System for Early-stage Endometrial Cancer in Young Women: Single-arm, Prospective Multicenter Study: Korean Gynecologic Oncology Group Study (KGOG2009). *Jpn J Clin Oncol,* 42**,** 1215-8.

KIMMICK, G. G. & MUSS, H. B. (1998) Endocrine therapy in metastatic breast cancer. *Cancer Treat Res,* 94**,** 231-54.

KINO, B. T., GRAGEROV, A., KOPP, J. B., STAUBER, R. H., PAVLAKIS, G. N. & CHROUSOS, G. P. (1999) The HIV-1 Virion-associated Protein Vpr Is a Coactivator of the Human Glucocorticoid Receptor. *Journal of Experimental Medicine,* 189.

KINO, T., GRAGEROV, A., SLOBODSKAYA, O., TSOPANOMICHALOU, M., CHROUSOS, G. P. & PAVLAKIS, G. N. (2002) Human Immunodeficiency Virus Type 1 (HIV-1) Accessory Protein Vpr Induces Transcription of the HIV-1 and Glucocorticoid-Responsive Promoters by Binding Directly to p300/CBP Coactivators. *Journal of Virology,* 76**,** 9724-9734.

KINO, T., MARTINO, M. U., CHARMANDARI, E., MIRANI, M. & CHROUSOS, G. P. (2003) Tissue glucocorticoid resistance/hypersensitivity syndromes. *J Steroid Biochem Mol Biol,* 85**,** 457-467.

KLEINSCHMIDT, I., REES, H., DELANY, S., SMITH, D., DINAT, N., NKALA, B. & MCINTYRE, J. A. (2007) Injectable progestin contraceptive use and risk of HIV infection in a South African family planning cohort. *Contraception,* 75**,** 461-467.

KLEYNHANS, L., DU PLESSIS, N., BLACK, G. F., LOXTON, A. G., KIDD, M., VAN HELDEN, P. D., WALZL, G. & RONACHER, K. (2011) Medroxyprogesterone acetate alters Mycobacterium bovis BCG-induced cytokine production in peripheral blood mononuclear cells of contraceptive users. *Plos ONE,* 6**,** e24639.

KLINGE, C. M. (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res,* 29**,** 2905-19.

KOBAYASHI, M., FITZ, L., RYAN, M., HEWICK, R. M., CLARK, S. C., CHAN, S., LOUDON, R., SHERMAN, F., PERUSSIA, B. & TRINCHIERI, G. (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *Journal of Experimental Medicine,* 170**,** 827-845.

KOBAYASHI, S., STICE, J. P., KAZMIN, D., WITTMANN, B. M., KIMBREL, E. A., EDWARDS, D. P., CHANG, C. Y. & MCDONELL, D. P. (2010) Mechanisms of progesterone receptor inhibition of inflammatory responses in cellular models of breast cancer. *Mol Endocrinol,* 24**,** 2292-2302.

KOLLET, J., WITEK, C., GENTRY, J. D., LIU, X., SCHWARTZBACH, S. D. & PETRO, T. M. (2001) Deletional analysis of the murine IL-12 p35 promoter comparing IFN-gamma and lipopolysaccharide stimulation. *J Immunol,* 167**,** 5653-63.

KONG, E. H., PIKE, A. C. & HUBBARD, R. E. (2003) Structure and mechanism of the oestrogen receptor. *Biochem Soc Trans,* 31**,** 56-9.

KONTULA, K., PAAVONEN, T., LUUKKAINEN, T. & ANDERSSON, L. C. (1983) Binding of progestins to the glucocorticoid receptor. Correlation to their glucocorticoid-like effects on in vitro functions of human mononuclear leukocytes. *Biochem Pharmacol,* 32**,** 1511-8.

KOUBOVEC, D., RONACHER, K., STUBSRUD, E., LOUW, A. & HAPGOOD, J. P. (2005) Synthetic progestins used in HRT have different glucocorticoid agonist properties. *Mol Cell Endocrinol,* 242**,** 23-32.

KOUBOVEC, D., VANDEN BERGHE, W., VERMEULEN, L., HAEGEMAN, G. & HAPGOOD, J. P. (2004) Medroxyprogesterone acetate downregulates cytokine gene expression in mouse fibroblast cells. *Molecular and Cellular Endocrinology,* 221**,** 75-83.

KRAMAROVA, T. V., WRIGHT, K. D. & PONGRATZ, I. (2009) The role of the estrogen receptors in obesity. *Drug Discovery Today: Disease Mechanisms* xxx**,** 1-6.

KRATTENMACHER, R. (2000) Drospirenone: pharmacology and pharmacokinetics of a unique progestogen. *Contraception,* 62**,** 29-38.

KREISS, J., WILLERFORD, D. M., HENSEL, M. & AL., E. (1994) Association between cervical inflammation and cervical shedding of human immunodeficiency virus DNA. *Journal of Infectious Diseases,* 170.

KREMER, K. N., KUMAR, A. & HEDIN, K. E. (2007) Haplotype-independent costimulation of IL-10 secretion by SDF-1/CXCL12 proceeds vir AP-1 binding to the human IL-10 promoter. *The Journal of Immunology,* 178**,** 1581-1588.

KROZOWSKI, Z. S. & FUNDER, J. W. (1983) Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. *Proc Natl Acad Sci U S A,* 80**,** 6056-60.

KUBE, D., PLATZER, C., VON KNETHEN, A., STRAUB, H., BOHLEN, H., HAFNER, M. & TESCH, H. (1995) Isolation of the human interleukin 10 promoter. Characterization of the promoter activity in burkitt's lymphoma cell lines. *Cytokine,* 7**,** 1-7.

KUBIN, M., KAMOUN, M. & TRINCHIERI, G. (1994b) Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J Exp Med,* 180**,** 211-22.

KUHL, H. (2005) Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric,* 8 Suppl 1**,** 3-63.

KUHL, H. (2011) Pharmacology of progestens. *J Reproduktionsmed Endokrinol,* 8 157-176.

KUMWENDA, J. J., MAKANANI, B., TAULO, F., NKHOMA, C., KAFULAFULA, G., LI, Q., KUMWENDA, N. & TAHA, T. E. (2008) Natural history and risk factors associated with early and established HIV type 1 infection among reproductive-age women in Malawi. *Clin Infect Dis,* 46**,** 1913-20.

KUREBAYASHI, J., OTSUKI, T., TANAKA, K., YAMAMOTO, Y., MORIYA, T. & SONOO, H. (2003) Medroxyprogesterone acetate decreases secretion of interleukin-6 and parathyroid hormone-related protein in a new anaplastic thyroid cell line, KTC-2. *Thyroid,* 13**,** 249-258.

LANGE, C. A. (2008) Challenges to defining a role for progesterone in breast cancer. *Steroids,* 73**,** 914-21.

LANNAN, E. A., GALLIHER-BECKLEY, A. J., SCOLTOCK, A. B. & CIDLOWSKI, J. A. (2012) Proinflammatory actions of glucocorticoids: Glucocorticoids and TNF coregulate gene expression in vitro and in vivo. *Endocrinology,* 153.

LARREA, F., VILCHIS, F., CHAVEZ, B., PEREZ, A. E., GARZA-FLORES, J. & PEREZ-PALACIOS, G. (1987) The metabolism of 19-Nor contraceptive progestins modulates their biological activity at the neuroendocrine level. *Journal of Steroid Biochemistry,* 27.

LE DOUCE, V., HERBEIN, G., ROHR, O. & SCHWARTZ, C. (2010) Molcular mechanisms of HIV-1 persistance in the monocyte-macrophage lineage. *Retrovirology,* 7**,** 1-16.

LE ROUZIC, E. & BENICHOU, S. (2005) The Vpr protein from HIV-1: distinct roles along the viral life cycle. *Retrovirology,* 2**,** 1-14.

LEE, E. B., KIM, A., KANG, K., KIM, H. & LIM, J. S. (2010) NDRG2-mediated modulation of SOCS3 and STAT3 activity inhibits IL-10 production. *Immune Network,* 10**,** 219-229.

LEIFELD, L., CHENG, S., RAMAKERS, J., DUMOULIN, F.-L., TRAUTWEIN, C., SAUERBRUCH, T. & SPENGLER, U. (2002) Imbalanced intrahepatic expression of interleukin 12, interferon gamma, and interleukin 10 in fulminant hepatitis B. *Hepatology,* 36**,** 1001-8.

LEMUS, A. E., ENRIQUEZ, J., HERNANDEZ, A., SANTILLAN, R. & PEREZ-PALACIOS, G. (2009) Bioconversion of norethisterone, a progesterone receptor agonist into estrogen receptor agonist in osteoblastic cells. *Journal of Endocrinology,* 200**,** 199-206.

LEONHARDT, S. A., BOONYARATANAKORNKIT, V. & EDWARDS, D. P. (2003) Progesterone receptor transcription and non-transcription signaling mechanisms. *Steroids,* 68**,** 761-70.

LEONHARDT, S. A. & EDWARDS, D. P. (2002) Mechanism of action of progesterone antagonists. *Experimental Biology and Medicine,* 227**,** 969-80.

LEVY, D. N., REFAELI, Y. & WEINER, D. B. (1995) Extracellular Vpr protein increases cellular permissiveness to human immunodeficiency virus replication and reactivates virus from latency. *Journal of Virology,* 69**,** 1243-1252.

LI, C., WILAMAN, K., LIDFELT, J., NERBRAND, C., DAVID, C. & SCHERSTEN, B. (2000) Effects of norethisterone acetate addition to estradiol in long term HRT. *Maturitas,* 36**,** 139 - 152.

LI, G., ELDER, R. T., DUBROVSKY, L., LIANG, D., PUSHKARSKY, T., CHIU, K., FAN, T., SIRE, J., BUKRINSKY, M. & ZHAO, R. Y. (2010) HIV-1 replication through hHP23A-mediated interaction of Vpr with 26S proteasome. *Plos ONE,* 5**,** 1-11.

LI, J. C. B. & LAU, A. S. Y. (2007) A role for mitogen-activated protein kinase and Ets-1 in the induction of interleukin-10 transcription by human immunodeficiency virus-1 Tat. *Immunology,* 121**,** 337-348.

LI, L., H.S., L., PAUZA, D., BUKRINSKY, M. & ZHAO, R. Y. (2005) Roles of HIV-1 auxilliary proteins in viral pathogenesis and host-pathogen interactions. *Cell Research,* 15**,** 923-934.

LIBERMAN, A. C., DRUKER, J., PERONE, M. J. & ARZT, E. (2007) Glucocorticoids in the regulation of transcription factors that control cytokine synthesis. *Cytokine & growth factor reviews,* 18**,** 45-56.

LIM, C. S., BAUMANN, C. T., HTUNT, H., XIAN, W., IRIE, M., SMITH, C. L. & HAGER, G. L. (1999) Differential localization and activity of the A- and B-forms of the human progesterone receptor using green fluorescent protein chimeras. *Molecular Endocrinology,* 13**,** 366-375.

LITTLE, R. F., PLUDA, J. M., WYVILL, K. M., RODRIGUEZ-CHAVEZ, I. R., TOSATO, G., CATANZARO, A. T., STEINBERG, S. M. & YARCHOAN, R. (2006) Activity of subcutaneous interleukin-12 in AIDS-related Kaposi sarcoma. *Blood,* 107**,** 4650-4657.

LU, N. Z., WARDELL, S. E., BURNSTEIN, K. L., DEFRANCO, D., FULLER, P. J., GIGUERE, V., HOCHBERG, R. B., MCKAY, L., RENOIR, J. M., WEIGEL, N. L., WILSON, E. M., MCDONNELL, D. P. & CIDLOWSKI, J. A. (2006) International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. *Pharmacol Rev,* 58**,** 782-97.

LUO, Y., BATALAO, A., ZHOU, H. & ZHU, L. (1997) Mammalian two-hybrid system: a complementary approach to the yeast two-hybrid system. *BioTechniques,* 22**,** 350-352.

MA, H., SHANG, Y., LEE, D. Y. & STALLCUP, M. R. (2003) Study of nuclear receptor-induced transcription complex assembly and histone modification by chromatin immunoprecipitation assays. *Methods Enzymol,* 364**,** 284-96.

MA, W., GEE, K., LIM, W., CHAMBERS, K., ANGEL, J. B., KOZLOWSKI, M. & KUMAR, A. (2004) Dexamethasone inhibits IL-12p40 production in lipopolysaccharide-stimulated

human monocytic cells by down-regulating the activity of c-Jun N-terminal kinase, the activation protein-1, and NF-kappa B transcription factors. *J Immunol,* 172**,** 318-30.

MA, W., LIM, W., GEE, K., AUCOIN, S., NANDAN, D., KOZLOWSKI, M., DIAZ-MITOMA, F. & KUMAR, A. (2001) The p38 Mitogen-activated Kinase pathways regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. *The Journal of biological chemistry,* 276**,** 13664-13674.

MA, X. & MONTANER, L. J. (2000) Proinflammatory response and IL-12 expression in HIV-1 infection. *Journal of Leukocyte Biology,* 68**,** 383-90.

MAGLIANI, W., CONTI, S., SALATI, A., ARSENI, S., FRAZZI, R., RAVANETTI, L. & POLONELLI, L. (2002) New strategies for treatment of Candida vaginal infections. *Rev Iberoam Micol,* 19**,** 144-8.

MAHER, D., WU, X., SCHACKER, T., HORBUL, J. & SOUTHERN, P. (2005) HIV binding , penetration , and primary infection in human cervicovaginal tissue. *Review Literature And Arts Of The Americas*.

MANGELSDORF, D. J., THUMMEL, C., BEATO, M., HERRLICH, P., SCHUTZ, G., UMESONO, K., BLUMBERG, B., KASTNER, P., MARK, M., CHAMBON, P. & EVANS, R. M. (1995) The nuclear receptor superfamily: the second decade. *Cell,* 83**,** 835-9.

MANSOUR, D. (2005) Progestogen-only contraceptives. *Women's health medicine,* 2**,** 6-12.

MARKIEWICZ, L. & GURPIDE, E. (1994) Estrogenic and progestagenic activities coexisting in steroidal drugs: quantitative evaluation by in vitro bioassays with human cells. *J Steroid Biochem Mol Biol,* 48**,** 89-94.

MARTIN, H. L., NYANGE, P. M., RICHARDSON, B. A., LAVREYS, L., MANDALIYA, K., JACKSON, D. J., NDINYA-ACHOLA, J. O. & KREISS, J. (1998) Hormonal contraception, sexually transmitted diseases, and risk of heterosexual transmission of human immunodeficiency virus type 1. *The Journal of Infectious Diseases,* 178**,** 1053-1059.

MATHRUBUTHAM, M. & FOTHERBY, K. (1981) Medroxyprogesterone acetate in human serum. *Journal of Steroid Biochemistry,* 14**,** 783.

MATSUO, R., KOBAYASHI, M., HERNDON, D. N., POLLARD, B. & SUZUKI, F. (1996) Interleukin 12 protects thermally injured mice from herpes simplex virus type 1 infection. *Journal of leukocyte biology,* 59**,** 623-630.

MAUCK, C. K., CALLAHAN, M. M., BAKER, J., ARBOGAST, K., VEAZEY, R., STOCK, R., PAN, Z., MORRISON, C. S., CHEN-MOK, M., ARCHER, D. F. & GABELNICK, H. L. (1999) The effect of one injection of Depo-Provera on the human vaginal epithelium and cervical ectopy. *Contraception,* 60**,** 15-24.

MCDONNELL, D. P. & GOLDMAN, M. E. (1994) RU486 exerts antiestrogenic activities through a novel progesterone receptor A form-mediated mechanism. *J Biol Chem,* 269**,** 11945-9.

MCDONNELL, D. P., SHAHBAZ, M. M., VEGETO, E. & GOLDMAN, M. E. (1994) The human progesterone receptor A-form functions as a transcriptional modulator of mineralocorticoid receptor transcriptional activity. *J Steroid Biochem Mol Biol,* 48**,** 425-32.

MCEWAN, I. J. (2009) Nuclear receptors: one big family. *Methods Mol Biol,* 505**,** 3-18.

MCEWAN, I. J., WRIGHT, A. P. & GUSTAFSSON, J. A. (1997) Mechanism of gene expression by the glucocorticoid receptor: role of protein-protein interactions. *BioEssays,* 19**,** 153-60.

MCKAY, L. I. & CIDLOWSKI, J. A. (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr Rev,* 20**,** 435-459.

MCKENNA, N. J., LANZ, R. B. & O'MALLEY, B. W. (1999) Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev,* 20**,** 321-44.

MCPAUL, M. J. (2008) Mechanisms of prostate cancer progression to androgen independence. *Best Practice and Research Clinical Endocrinology & Metabolism,* 22**,** 373-388.

MEDICAL RESEARCH COUNCIL, D. O. H. (2003) South Africa Demographic and Health Survey 2003.

MELCHJORSEN, J., SORENSEN, L. N. & PALUDAN, S. R. (2003) Expression and function of chemokines during viral infections: from molecular mechanisms to in vivo function. *Journal of leukocyte biology,* 74**,** 331-343.

MERTENS, H. J. M. M., HEINEMAN, M. J. & THEUNISSEN, P. H. M. H. (2001) Androgen , estrogen and progesterone receptor expression in the human uterus during the menstrual cycle $. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 98**,** 58-65.

MIAN, M. & ASHKOR, A. A. (2011) Induction of innate immune responses in the female genital tract: Friend or foe of HIV-1 infection? *Am J Reprod Immunol,* 65**,** 344-351.

MIRANI, M., ELENKOV, I. J., VOLPI, S., HIROI, N., CHROUSOS, G. P. & KINO, T. (2002) HIV-1 Protein Vpr Suppresses IL-12 Production from Human Monocytes by Enhancing Glucocorticoid Action: Potential Implications of Vpr Coactivator Activity for the Innate and Cellular Immunity Deficits Observed in HIV-1 Infection. *The Journal of Immunology,* 169**,** 6361-6368.

MISCIA, S., MARCHISIO, M., GRILLI, A., DI VALERIO, V., CENTURIONE, L., SABATINO, G., GARACI, F., ZAULI, G., BONVINI, E. & DI BALDASSARRE, A. (2002) Tumor necrosis factor alpha (TNF-alpha) activates Jak1/Stat3-Stat5B signaling through TNFR-1 in human B cells. *Cell Growth Differ,* 13**,** 13-8.

MISHELL, D. R., JR. (1996) Pharmacokinetics of depot medroxyprogesterone acetate contraception. *J Reprod Med,* 41**,** 381-90.

MOCELLIN, S., PANELLI, M. C., WANG, E., NAGORSEN, D. & MARINCOLA, F. M. (2003) The dual role of IL-10. *Trends in immunology,* 24**,** 36-43.

MOORE, K. W., MALEFYT, R. D. W., COFFMAN, R. L. & O'GARRA, A. (2001) Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology,* 19**,** 683-765.

MOORE, K. W., O'GARRA, A., DE WAAL MALEFYT, R., VIEIRA, P. & MOSMANN, T. R. (1993) Interleukin-10. *Annu. Rev. Immunol.,* 11**,** 165-.

MOORE, N. L., BUCHANAN, G., HARRIS, J. M., SELTH, L. A., BIANCO-MIOTTO, T., HANSON, A. R., BIRRELL, S. N., BUTLER, L. M., HICKEY, T. E. & TILLEY, W. D. (2012) An androgen receptor mutation in the MDA-MB-453 cell line model of molecular apocrine breast cancer compromises receptor activity. *Endocrine related cancer,* 19**,** 599-613.

MORAS, D. & GRONEMEYER, H. (1998) The nuclear receptor ligand-binding domain: structure and function. *Curr Opin Cell Biol,* 10**,** 384-91.

MORELLET, N., ROQUES, B. P. & BOUAZIZ, S. (2009) Structure-function relationship of Vpr: Biological implications. *Current HIV Research,* 7**,** 184-210.

MORRISON, C., RICHARDSON, B., MMIRO, F., CHIPATO, T., CELENTANO, D., LUOTO, J., MUGERWA, R., PADIAN, N., RUGPAO, S., BROWN, J., CORNELISSE, P. & SALATA, R. (2007) Hormonal contraception and the risk of HIV acquisition. *AIDS,* 21**,** 85-95.

MORRISON, C. S., BRIGHT, P., WONG, E. L., KWOK, C., YACOBSON, I., GAYDOS, C. A., TUCKER, H. T. & BLUMENTHAL, P. D. (2004) Hormonal contraceptive use, cervical ectopy and the acquisition of cervical infection. *Sex Transm Dis,* 31**,** 561-567.

MORRISON, C. S., CHEN, P. L., KWOK, C., RICHARDSON, B. A., CHIPATO, T., MUGERWA, R., BYAMUGISHA, J., PADIAN, N., CELENTANO, D. D. & SALATA, R. (2010) Hormonal contraception and HIV acquisition: reanalysis using margincal structural modeling. *AIDS,* 24**,** 1777-1778.

MORRISON, C. S., SKOLER-KARPOFF, S., KWOK, C., CHEN, P. L., VAN DE WIJGERT, J., GEHRET-PLAGIANOS, M., PATEL, S., AHMED, K., RAMJEE, G., FRIEDLAND, B. & LAHTEENMAKI, P. (2012) Hormonal contraception and the risk of HIV acquisition among women in South Africa. *AIDS,* 26**,** 497-504.

MORRISON, C. S., TURNER, A. N. & JONES, L. B. (2009) Highly effective contraception and acquisition of HIV and other sexually transmitted infections. *Best Pract Res Clin Obstet Gynaecol,* 23**,** 263-84.

MOSSER, D. M. & ZHANG, X. (2008) Interleukin-10: new perspectives on an old cytokine. *Immunological Reviews,* 226**,** 205-218.

MOSTAD, S. B., KREISS, J. K., RYNCARZ, A. J., MANDALIYA, K., CHOHAN, B., NDINYA-ACHOLA, J., BWAYO, J. J. & COREY, L. (2000) Cervical Shedding of Herpes Simplex Virus in Human Immunodeficiency Virus-Infected Women: Effects of Hormonal Contraception, Pregnancy, and Vitamin A Deficiency. *The Journal of Infectious Diseases,* 181**,** 58-63.

MOSTAD, S. B., OVERBAUGH, J., DEVANGE, D. M., WELCH, M. J., CHOHAN, B., MANDALIYA, K., NYANGE, P., MARTIN, H. L., JR., NDINYA-ACHOLA, J., BWAYO, J. J. & KREISS, J. K. (1997) Hormonal contraception, vitamin A deficiency, and other risk factors for shedding of HIV-1 infected cells from the cervix and vagina. *Lancet,* 350**,** 922-7.

MOTE, P. A., TRAN, S. B. & CLARKE, C. L. (2002) Loss of co-ordinate expression of progesteron receptor A and B is an early event in breast carcinogenesis. *Breast Cancer Research and Treatment,* 72**,** 163-172.

MOZO, L., SUAREZ, A. & GUTIERREZ, C. (2003) Glucocorticoids up-regulate constitutive interleukin-10 production by human monocytes. *Clinical and Experimental Allergy,* 34**,** 406-412.

MUNEYYIRCI-DELALE, O. & KARACAN, M. (1998) Effect of norethindrone acetate in the treatment of symptomatic endometriosis. *Int J Fertil Womens Med,* 43**,** 24-7.

MURPHY, T. L., CLEVELAND, M. G., KULESZA, P., MAGRAM, J. & MURPHY, K. M. (1995) Regulation of interleukin 12p40 expression through an NF-kB half-site. *Molecular and Cellular Biology,* 15**,** 5258-5267.

MUTHUMANI, K., CHOO, A. Y., ZONG, W.-X., MADESH, M., HWANG, D. S., PREMKUMAR, A., THIEU, K. P., EMMANUEL, J., KUMAR, S., THOMPSON, C. B. &

WEINER, D. B. (2006) The HIV-1 Vpr and glucocorticoid receptor complex is a gain-of-function interaction that prevents the nuclear localization of PARP-1. *Nature Cell Biology*.

MUTHUMANI, K., DESAI, B. M., HWANG, D. S., CHOO, A. Y., LADDY, D. J., THIEU, K. P., RAO, R. G. & WEINER, D. B. (2004) HIV-1 Vpr and anti-inflammatory activity. *DNA Cell Biology,* 23**,** 239-47.

MYER, L., DENNY, L., WRIGHT, T. C. & KUHN, L. (2007) Prospective study of hormonal contraception and women's risk of HIV infection in South Africa. *International Journal of Epidemiology,* 36**,** 166-174.

NAICKER, D. D., WERNER, L., KORMUTH, E., PASSMORE, J.-A., MLISANA, K., ABDOOL KARIM, S., NDUNG'U, T. & TEAM, C. A. I. S. (2009) Interleukin-10 promoter polymorphism influence HIV-1 susceptibility and primary HIV-1 pathogenesis. *The Journal of infectious diseases,* 200**,** 448-452.

NAZLI, A., CHAN, O., DOBSON-BELAIRE, W. N., OUELLET, M., TREMBLAY, M. J., GRAY-OWEN, S. D., ARSENAULT, A. L. & KAUSHIC, C. (2010) Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. *PLoS Pathog,* 6**,** e1000852.

NEMETH, Z. H., LUTZ, C. S., CSAKO, B., DEITCH, E. A., LEIBOVICH, S. J., GAUSE, W. C., TONE, M., PACHER, P., VIZI, E. S. & HASKO, G. (2005) Adenosine augments IL-10 production by macrophages through an A2B receptor mediated posttranscriptional mechanism. *The Journal of Immunology,* 175**,** 8260-8270.

NETTLES, K. W. & GREENE, G. L. (2005) Ligand control of coregulator recruitment to nuclear receptors. *Annu Rev Physiol,* 67**,** 309-33.

NEWTON, R. (2000) Molecular mechanisms of glucocorticoid action: what is important? *Thorax,* 55**,** 603-613.

NKWANYANA, N. N., GUMBI, P. P., ROBERTS, L., DENNY, L., HANEKOM, W., SOARES, A., ALLAN, B., WILLIAMSON, A., COETZEE, D., OLIVIER, A. J., BURGERS, W. A. & PASSMORE, J. A. (2009) Impact of human immunodeficiency virus 1 infection and inflammation on the composition and yield of cervical mononuclear cells in the female genital tract. *Immunology,* 128**,** e746-e757.

NOETH, D. (2012) Regulation of chemokine gene expression by synthetic progestins in a human vaginal epithelial cell line. *Biochemistry.* Stellenbosch, University of Stellenbosch, South Africa.

OCHIEL, D. O., FAHEY, J. V., GHOSH, M., HADDAD, S. N. & WIRA, C. R. (2008) Innate immunity in the female reproductive tract: Role of sex hormones in regulating uterine epithelial cell protection against pathogens. *Current Womens Health Review,* 4**,** 102-117.

OJULE, J. D., ORIJI, V. K. & OKONGWU, C. (2010) A five year review of the complications of progestogen only injectable contraceptive at the University of Port-Harcourt Teaching Hospital. *Niger J Med,* 19**,** 87-95.

OPAL, S. M. & DEPALO, V. A. (2000) Anti-inflammatory cytokines. *Chest,* 117**,** 1162-72.

OROPEZA, M. V., CAMPOS, M. G., LEMUS, A. E., GARCIA, G., PEREZ-PALACIOS, G. & PONCE-MONTER, H. Estrogenic action of norethisterone and its A-ring reduced metabolites. Induction of in vitro uterine sensitivity to serotonin.

OSBORNE, C. K., ZHAO, H. & FUQUA, S. A. W. (2000) Selective Estrogen Receptor Modulators : Structure , Function , and Clinical Use. *J Clin Oncol,* 18**,** 3172-3186.

OTTESEN, B. & PEDERSEN, A. T. (1996) Physiological effects of ovarian hormones : Clinical aspects and compliance. *Heart,* 17**,** 20-26.

PALVIMO, J. J., REINIKAINEN, P., IKONEN, T., KALLIO, P. J., MOILANEN, A. & JANNE, O. A. (1996) Mutual transcriptional interference between RelA and androgen receptor. *J Biol Chem,* 271**,** 24151-6.

PAR, G., GELI, J., KOZMA, N., VARGA, P. & SZEKERES-BARTO, J. (2003) Progesterone regulates IL-12 expression in pregnancy lymphocytes by inhibiting phospholipase A2. *American Journal of Reproductive Immunology,* 49**,** 1-5.

PASCUAL, G. & GLASS, C. K. (2006) Nuclear receptors versus inflammation : mechanisms of transrepression. *Trends in Endocrinology and Metabolism,* 17.

PAXTON, W., CONNOR, R. I. & LANDAU, N. R. (1993) Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirements for the p6 region of gag and mutational analysis. *J Virol,* 67**,** 7229-7237.

PEEK, E. J., RICHARDS, D. F., FAITH, A., LAVENDER, P., LEE, T. H., CORRIGAN, C. J. & HAWRYLOWICZ, C. M. (2005) Interleukin-10-secreting "regulatory" T cells induced by glucocorticoids and beta2-agonists. *Am J Respir Crit Care Med,* 33**,** 105-111.

PESTKA, S., KRAUSE, C. D., SARKAR, D., WALTER, M. R., SHI, Y. & FISHER, P. B. (2004) Interleukin-10 and related cytokines and receptors. *Annual review of immunology,* 22**,** 929-79.

PFAFFL, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research,* 29**,** 2002-2007.

PHILIBERT, D., BOUCHOUX, F., DEGRYSE, M., LECAQUE, D., PETIT, F. & GAILLARD, M. (1999) The pharmacological profile of a novel norpregnance progestin (trimegestone). *Gynecol Endocrinol,* 13**,** 316-326.

PHILLIPS, A., DEMAREST, K., HAHN, D. W., WONG, F. & MCGUIRE, J. L. (1990) Progestational and androgenic receptor binding affinities and in vivo activities of norgestimate and other progestins. *Contraception,* 41**,** 399-410.

PLEVY, S. E., GEMBERLING, J. H., HSU, S., DORNER, A. J. & SMALE, S. T. (1997) Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Molecular and Cellular Biology,* 17**,** 4572-88.

POLLOW, K., JUCHEM, M., ELGER, W., JACOBI, N., HOFFMANN, G. & MOBUS, V. (1992) Dihydrospirorenone (ZK30595): a novel synthetic progestagen--characterization of binding to different receptor proteins. *Contraception,* 46**,** 561-74.

POPE, M. & HAASE, A. T. (2003) Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. *Nature Medicine,* 9**,** 847-52.

PRATT, W. B. & TOFT, D. O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev,* 18**,** 306-60.

PRETOLANI, M. (1999) Interleukin-10: an anti-inflammatory cytokine with therpeutic potential. *Clinical and Experimental Allergy,* 29**,** 1164-1171.

PUDNEY, J., QUAYLE, A. J. & ANDERSON, D. J. (2005) Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol Reprod,* 73**,** 1253-63.

QUAYLE, A. J. (2002) The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells. *Journal of Reproductive Immunology,* 57**,** 61-79.

QUINKLER, M., BUMKE-VOGT, C., GROSSMANN, C., GRUBER, U., OELKERS, J., DIEDERICH, S. & BAHR, V. (2002) Agonistic and antagonistic properties of progesterone metabolites at the human mineralocorticoid receptor. *Eur J Endocrinol,* 146**,** 789-790.

REFAELI, Y., LEVY, D. N. & WEINER, D. B. (1995) The glucocorticoid receptor type II complex is a target of the HIV-1 Vpr gene product. *Proc. Natl. Acad. Sci. USA,* 92**,** 3621-3625.

RICHARDS, D. F., FERNANDEZ, M., CAULFIELD, J. & HAWRYLOWICZ, C. M. (2000) Glucocorticoids drive human CD8(+) T cell differentiation towards a phenotype with high IL-10 and reduced IL-4, IL-5 and IL-13 production. *Eur J Immunol,* 30**,** 2344-2354.

RICHARDSON, B. A., OTIENO, P. A., MBORI-NGACHA, D., OVERBAUGH, J., FARQUHAR, C. & JOHN-STEWART, G. C. (2007) Hormonal contraception and HIV-1 disease progression among postpartum Kenyan women. *AIDS,* 21**,** 749-753.

RICHER, J. K., JACOBSEN, B. M., MANNING, N. G., ABEL, M. G., WOLF, D. M. & HORWITZ, K. B. (2002) Differential Gene Regulation by the Two Progesterone Receptor Isoforms in Human Breast Cancer Cells *Biochemistry,* 277**,** 5209 -5218.

ROBERTS, L., LIEBENBERG, L., BARNABAS, S. & PASSMORE, J. A. (2012a) Vaginal microbicides to prevent human immunodeficiency virus infection in women: Perspectives on the female genital tract, sexual maturity and mucosal inflammation. *Best Pract Res Clin Obstet Gynaecol,* 26**,** 441-9.

ROBERTS, L., PASSMORE, J. A., MLISANA, K., WILLIAMSON, C., LITTLE, F., BEBELL, L. M., WALZL, G., ABRAHAMS, M. R., WOODMAN, Z., ABDOOL KARIM, Q. & ABDOOL KARIM, S. S. (2012b) Genital tract inflammation during early HIV-1 infection predicts higher plasma viral load set point in women. *J Infect Dis,* 205**,** 194-203.

ROBERTSON, S. E. (2011) Receptor concentration affects glucocorticoid action. *Department of Biochemistry.* Stellenbosch, University of Stellenbosch.

ROBINSON, K., VONA-DAVIS, L., RIGGS, D., JACKSON, B. & MCFADDEN, D. (2006) Peptide YY attenuates STAT1 and STAT3 activation induced by TNF-alpha in acinar cell line AR42J. *J Am Coll Surg,* 202**,** 788-96.

ROGATSKY, I., LUECKE, H. F., LEITMAN, D. C. & YAMAMOTO, K. R. (2002) Alternate surfaces of transcriptional coregulator GRIP1 function in different glucocorticoid receptor activation and repression contexts. *Proc Natl Acad Sci U S A,* 99**,** 16701-6.

ROMANI, B. & ENGELBRECHT, S. (2009) Human immunodeficiency virus type 1 Vpr : functions and molecular interactions. *Journal of General Virology***,** 1795-1805.

ROMERO, R., MAZOR, M., MUNOZ, H., GOMEZ, R., GALASSO, M. & SHERER, D. M. (1994) The preterm labor syndrome. *Ann N Y Acad Sci,* 734**,** 414-429.

RONACHER, K., HADLEY, K., AVENANT, C., STUBSRUD, E., SIMONS, S. S., LOUW, A. & HAPGOOD, J. P. (2009) Ligand-selective transactivation and transrepression via the

glucocorticoid receptor: role of cofactor interaction. *Molecular and Cellular Endocrinology,* 299**,** 219-31.

ROOS, A. B. & NORD, M. (2012) The emerging role of C/EBPs in glucocorticoid signaling: lessons from the lung. *J Endocrinol,* 212**,** 291-305.

ROSSOUW, J. E., ANDERSON, G. L., PRENTICE, R. L., LACROIX, A. Z., KOOPERBERG, C., STEFANICK, M. L., JACKSON, R. D., BERESFORD, S. A. A., HOWARD, B. V., JOHNSON, K. C., KOTCHEN, J. M. & OCKENE, J. (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the Women's Health Initiative randomized controlled trial. *JAMA,* 288**,** 321-333.

SAMBROOK, J., MANIATUS, T. & FRITSCH, E. F. (Eds.) (1989) *Molecular cloning: A Labroratory Manual,* New York, Cold Spring Harbor Laboratory Press.

SASAGAWA, S., SHIMIZU, Y., KAMI, H., TAKEUCHI, T., MITA, S., IMADA, K., KATO, S. & MIZUGUCHI, K. (2008) Dienogest is a selective progesterone receptor agonist in transactivation analysis with potent oral endometrial activity due to its efficient pharmacokinetic profile. *Steroids,* 73.

SASHA, A., BUTTS, C. L. & STERNBERG, E. M. (2008) The role of glucocorticoids and progestins in inflammatory, autoimmune, and infectious disease. *Journal of leukocyte biology,* 84**,** 924-931.

SCARPIN, K. M., GRAHAM, J. D., MOTE, P. A. & CLARKE, C. L. (2009) Progesterone action in human tissues : regulation by progesterone receptor (PR) isoform expression , nuclear positioning and coregulator expression. *Review Literature And Arts Of The Americas,* 7**,** 1-13.

SCHAFER, E. A., VENKATACHARI, N. J. & AYYAVOO, V. (2006) Antiviral effects of mifepristone on human immunodeficiency virus type-1 (HIV-1): targeting Vpr and its cellular partner, the glucocorticoid receptor (GR). *Antiviral Research,* 72**,** 224-232.

SCHINDLER, A. E., CAMPAGNOLI, C., DRUCKMANN, R., HUBER, J., PASQUALINI, J. R., SCHWEPPE, K. W. & THIJSSEN, J. H. (2003) Classification and pharmacology of progestins. *Maturitas,* 46 Suppl 1**,** S7-S16.

SCHOONEN, W. G., DECKERS, G. H., DE GOOIJER, M. E., DE RIES, R. & KLOOSTERBOER, H. J. (2000) Hormonal properties of norethisterone, 7alpha-methyl-norethisterone and their derivatives. *J Steroid Biochem Mol Biol,* 74**,** 213-22.

SCHOTTELIUS, A. J., MAYO, M. W., SARTOR, R. B. & BALDWIN, A. S. (1999) Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *The Journal of biological chemistry,* 274**,** 31868-74.

SCHWEPPE, K. W. (2001) Current place of progestins in the treatment of endometriosis-related complaints. *Gynecol Endocrinol,* 15**,** 22-28.

SELMAN, P. J., WOLFSWINKEL, J. & MOL, J. A. (1996) Binding specificity of medroxyprogesterone acetate and proligestone for the progesterone and glucocorticoid receptor in the dog. *Steroids,* 61**,** 133-7.

SELZMAN, C. H., SHAMES, B. D., MILLER, S. A., PULIDO, E. J., MENG, X., MCINTYRE, R. C. & HARKEN, A. H. (1998) Therapeutical implications of interleukin-10 in surgical disease. *Shock,* 10**,** 309-318.

SHERMAN, M. P., NORONHA, C. M. C. D. E. & PEARCE, D. (2000) Human Immunodeficiency Virus Type 1 Vpr Contains Two Leucine-Rich Helices That Mediate Glucocorticoid Receptor Coactivation Independently of Its Effects on G 2 Cell Cycle Arrest. *Journal of Virology,* 74**,** 8159-8165.

SHIELDS-BOTELLA, J., DUC, I., DURANTI, E., PUCCIO, F., BONNET, P., DELANSORNE, R. & PARIS, J. (2003) An overview of nomegestrol acetate selective receptor binding and lack of estrogenic action on hormone-dependent cancer cells. *Journal of Steroid Biochemistry and Molecular Biology,* 87**,** 111-122.

SHRIER, L. A., BOWMAN, F. P., LIN, M. & CROWLEY-NOWICK, P. A. (2003) Mucosal immunity of the adolescent female genital tract. *The Journal of adolescent health : official publication of the Society for Adolescent Medicine,* 32**,** 183-6.

SHRIMANKER, K., SAXENA, B. N. & FOTHERBY, K. (1978) A radioimmunoassay for serum medroxyprogesterone acetate. *J Steroid Biochem,* 9**,** 359-63.

SIEBURTH, D., JABS, E. W., WARRINGTON, J. A., LI, X., LASOTA, J., LAFORGIA, S., KELLEHER, K., HUEBNER, K., WASMUTH, J. J. & WOLF, S. F. (1992) Assignment of genes encoding a unique cytokine (IL12) composed of two unrelated subunits to chromosomes 3 and 5. *Genomics,* 14**,** 59-62.

SIMON, A. J. (2007) Uses of progesterone throughout a women's life, Introduction: An overview of progesterone and progestins. IN MEDICINE, A. S. O. R. (Ed.) *A supplement to The Journal of Family Practice.* New Orlands, Louisiana, Solvay Pharmaceuticals Inc.

SITRUK-WARE, R. (2004a) New Progestogens: A review of their effects in perimenopausal and postmenopausal women. *Drugs Aging,* 21**,** 865-883.

SITRUK-WARE, R. (2004b) Pharmacological profile of progestins. *Maturitas,* 47**,** 227-283.

SITRUK-WARE, R. (2006) New progestagens for contraceptive use. *Human Reproduction,* 12**,** 169-178.

SKOUBY, S. O. & JESPERSEN, J. (2009) Progestins in HRT: sufferance or desire? *Maturitas,* 62**,** 371-5.

SLADEK, F. M. (2010) What are nuclear receptor ligands? *Mol Cell Endocrinol,* 334**,** 3-13.

SMIT, J., GRAY, A., MCFADYEN, L. & ZUMA, K. (2001) Counting the costs: comparing depot medroxyprogesterone acetate and norethisterone enanthate utilization patterns in South Africa. *BMC Health Serv Rese,* 1.

SMOAK, K. A. & CIDLOWSKI, J. A. (2004) Mechanisms of glucocorticoid receptor signaling during inflammation. *Mechanisms of ageing and development,* 125**,** 697-706.

SOLTER, C. (1999) Comprehensive Reproductive Health and Family Planning Training Curriculum, Module 4: Combined oral contraceptives and progestin-only pills. IN HEALTH, I. F. R. (Ed.). Pathfinder International.

SPEROFF, L. (1996) A Clinical Guide for Contraception. 2nd Edition ed., Baltimore: Williams & Wilkins, Baltimore, MD.

SPINILLO, A., CAPUZZO, E., NICOLA, S., BALTARO, F., FERRARI, A. & MONACO, A. (1995) The impact of oral contraception on vulvovaginal candidiasis. *Contraception,* 51**,** 293-7.

STADBERG, E., WESTLUND, P., LANDGREN, B. M., AEDO, A. R., CEKAN, S. Z. & MATTSSON, L. A. (1999) Bioavailability of norethisterone acetate alone and in

combination with estradiol administered in single or multiple oral doses to postmenopausal women. *Maturitas,* 33**,** 59-69.

STAHLBERG, C., PEDERSEN, A. T., LYNGE, E., ANDERSEN, Z. J., KEIDING, N., HUNDRUP, Y. A., OBEL, E. B. & OTTESEN, B. (2004) Increased risk of breast cancer following different regimens of hormone replacement therapy frequently used in Europe. *Int J Cancer,* 109**,** 721-7.

STANCZYK, F. Z. (2003) All progestins are not created equal. *Steroids,* 68**,** 879-890.

STANCZYK, F. Z. & ROY, S. (1990) Metabolism of levonorgestrel, norethindrone, and structurally related contraceptive steroids. *Contraception,* 42**,** 67-96.

STEELE, C. & FIDEL, P. L. (2002) Cytokine and Chemokine Production by Human Oral and Vaginal Epithelial Cells in Response to Candida albicans. *Infection and Immunity,* 70**,** 577-583.

STEINKE, J. W., BAREKZI, E., HAGMAN, J. & BORISH, L. (2004) Functional analysis of -571 IL-10 promoter polymorphism reveals a repressor element controlled by Sp1. *The Journal of Immunology,* 173**,** 3215-3222.

STERN, A. S., PODLASKI, F. J., HULMES, J. D., PAN, Y. C., QUINN, P. M., WOLITZKY, A. G., FAMILLETTI, P. C., STREMLO, D. L., TRUITT, T. & CHIZZONITE, R. (1990) Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proceedings of the National Academy of Sciences of the United States of America,* 87**,** 6808-12.

STRINGER, E. & ANTONSEN, E. (2008) Hormonal contraception and HIV disease progression. *Clinical infectious diseases,* 47**,** 945-951.

SUI, X., BRAMLETT, K. S., JORGE, M. C., SWANSON, D. A., VON ESCHENBACH, A. C. & JENSTER, G. (1999) Specific androgen receptor activation by an artificial coactivator. *J Biol Chem,* 274**,** 9449-54.

SUSLOV, O. & STEINDLER, D. A. (2005) PCR inhibition by reverse transcriptase leads to an overestimation of amplification efficiency. *Nucleic Acids Res,* 33**,** e181.

SUZUKI, K., NAKAJI, S., YAMADA, M., TOTSUKA, M., SATO, K. & SUGAWARA, K. (2002) Systemic inflammatory response to exhaustive exercise. Cytokine kinetics. *Exerc Immunol Rev,* 8**,** 6-48.

SZALMAS, A., BANATI, F., KOROKNAI, A., LASZLO, B., FEHER, E., SALAMON, D., GERGELY, L., MINAROVITS, J. & KONYA, J. (2008) Lineage-specific silencing of human IL-10 gene expression by promoter mehtylation in cervical cancer cells. *European Journal of Cancer,* 44**,** 1030-1038.

TAITEL, H. F. & KAFRISSEN, M. E. (1995) Norethindrone--a review of therapeutic applications. *Int J Fertil Menopausal Stud,* 40**,** 207-23.

TANG, Y., GETZENBERG, R. H., VIETMEIER, B. N., STALLCUP, M. R., EGGERT, M., RENKAWITZ, R. & DEFRANCO, D. B. (1998) The DNA-binding and tau2 transactivation domains of the rat glucocorticoid receptor constitute a nuclear matrix-targeting signal. *Mol Endocrinol,* 12**,** 1420-31.

TATSUMI, H., KITAWAKI, J., TANAKA, K., HOSODA, T. & HONJO, H. (2002) Lack of stimulatory effect of dienogest on the expression of intercellular adhesion molecule-1 and

vascular cell adhesion molecule-1 by endothelial cell as compared with other synthetic progestins. *Maturitas,* 42**,** 287-94.

TEULINGS, F. A., VAN GILSE, H. A., HENKELMAN, M. S., PORTENGEN, H. & ALEXIEVA-FIGUSCH, J. (1980) Estrogen, androgen, glucocorticoid, and progesterone receptors in progestin-induced regression of human breast cancer. *Cancer Res,* 40**,** 2557-61.

TRINCHIERI, A. & SCOTT, P. (1995a) Interleukin 12: a proinflammatory cytokine with immunoregulatory functions. *Res Immunology,* 146**,** 423-431.

TRINCHIERI, G. (1997) Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, INF-gamma). *Current opinion in immunology,* 9**,** 17-23.

TRINCHIERI, G. (1998) Proinflammatory and lmmunoregulatory Functions of lnterleukin-12. *Internation Reviews of Immunology,* 16**,** 365-396.

TRINCHIERI, G. (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature Reviews Immunology,* 3**,** 133-146.

TRINCHIERI, G. & GEROSA, F. (1996) Immunoregulation by interleukin-12. *Journal of leukocyte biology,* 59**,** 505-511.

TRINCHIERI, G. & SCOTT, P. (1995b) Immunoregulation by interleukin-12. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. *Res Immunol (62nd Forum in Immunology),* 146**,** 423-431.

TRISTEM, M., MARSHALL, C., KARPAS, A. & HILL, F. (1992) Evolution of the primate lentiviruses: evidence from vpx and vpr. *EMBO J,* 11**,** 3405-12.

TRUITT, S. T., FRASER, A. B., GRIMES, D. A., GALLO, M. F. & SCHULZ, K. F. (2003) Combined hormonal versus nonhormonal versus progestin-only contraception in lactation. *Cochrane Database Syst Rev***,** CD003988.

TRUNOVA, N., TSAI, L., TUNG, S. & AL., E. (2006) Progestin-based contraceptive suppresses cellular immune responses in SHIV-infected rhesus macaques. *Virology,* 352.

UNAIDS (2007) AIDS epidemic update.

UNTERBERGER, C., STAPLES, K. J., SMALLIE, T., WILLIAMS, L., FOXWELL, B., SCHAEFER, A., KEMPKES, B. & ZIEGLER-HEITBROCK, L. (2008) The role of STAT3 in glucocorticoid-induced expression of the human IL-10 gene. *Molecular immunology,* 45**,** 3230-3237.

VARIN, A., DECRION, A., SABBAH, E. S., QUIVY, V., SIRE, J., VAN LINT, C., AGGARWAL, B. B. & HERBEIN, G. (2005) Synthetic Vpr protein activates activator protein-1, c-Jun N-terminal kinase, and NFkB and stimulates HIV-1 transcription in promonocytic cells and primary macrophages. *The Journal of biological chemistry,* 28**,** 42557-42567.

VATS, V., AGRAWAL, T., SALHAN, S. & MITTAL, A. (2007) Primary and secondary immune response of mucosal and peripheral lymphocytes during Chlamydia trachomatis infection. *FEMS Immunology and Medical Microbiology,* 49**,** 280-287.

VEGETO, E., SHAHBAZ, M. M., WEN, D. X., GOLDMAN, M. E., O'MALLEY, B. W. & MCDONNELL, D. P. (1993) Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. *Mol Endocrinol,* 7**,** 1244-55.

VERCELLINI, P., FEDELE, L., PIETROPAOLO, G., FRONTINO, G., SOMIGLIANA, E. & CROSIGNANI, P. G. (2003) Progestogens for endometriosis: forward to the past. *Hum Reprod Update,* 9**,** 387-396.

VERHOOG, N., DU TOIT, A., AVENANT, C. & HAPGOOD, J. P. (2011) Glucocorticoid-independent repression of TNF-alpha stimulated IL-6 expression by the glucocorticoid receptor: a potential mechanism for protection against an excessive inflammatory response. *Journal of Biological Chemistry,* 286**,** 19297-19310.

VERHOOG, N. J. D. (2010) Investigation of differential TNF-alpha-induced interleukin-6 gene regulation by synthetic progestins medroxyprogesterone aceate (MPA) and norethisterone acetate (NET-A) in human endocervical epithelial cells and the role of the unliganded glucocorticoid receptor. *Molecular and Cell Biology.* Cape Town, University of Cape Town.

VISSER, J., BOXEL-DEZAIRE, A. V., METHORST, D., BRUNT, T. & KLOET, E. R. D. (1998) Differential Regulation of Interleukin-10 (IL-10) and IL-12 by Glucocorticoids in vitro. *Blood,* 91**,** 4255-4264.

VOGEL, C. & MARCOTTE, E. M. (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature reviews Genetics,* 13**,** 227-232.

WAMBACH, G. & HIGGINS, J. R. (1978) Antimineralocorticoid action of progesterone in rat: Correlation of the effect on elecrolyte excretion and interaction with renal mineralocorticoid receptors. *Endocrinology,* 102**,** 1686.

WANG, C. C. E. A. (2004) The effect of hormonal contraception on genital tract shedding of HIV-1. *AIDS,* 18**,** 205-209.

WARREN, M. P. (2004) A comparative review of the risks and benefits of hormone replacement therapy regimens. *Am J Obstet Gynecol,* 190**,** 1141-67.

WATFORD, W. T., MORIGUCHI, M., MORINOBU, A. & O'SHEA, J. J. (2003) The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine and Growth Factor Reviews,* 14**,** 361-368.

WEATHERMAN, R. V., FLETTERICK, R. J. & SCANLAN, T. S. (1999) Nuclear-receptor ligands and ligand-binding domains. *Annu Rev Biochem,* 68**,** 559-581.

WEBSTER, J. C. & CIDLOWSKI, J. A. (1999) Mechanisms of Glucocorticoid- receptor-mediated Repression of Gene Expression. *TEM,* 10**,** 396-402.

WESTERVELT, P., HENKEL, T., TROWBRIDGE, D. B., ORENSTEIN, J., HEUSER, J., GENDELMAN, H. E. & RATNER, L. (1992) Dual regulation of silent and productive infection in monocytes by distinct human immunodeficiency virus type 1 determinants. *J Virol,* 66**,** 3925-31.

WESTHOFF, C. (2003) Depot-medroxyprogesterone acetate injection (Depo-Provera): a highly effective contraceptive option with proven long-term safety. *Contraception,* 68**,** 75- 87.

WHITEHEAD, M. I., KING, R. J., MCQUEEN, J. & CAMPBELL, S. (1979) Endometrial histology and biochemistry in climacteric women during oestrogen and oestrogen/progestogen therapy. *J R Soc Med,* 72**,** 322-7.

WILLIAMS, C. E. & CREIGHTON, S. M. (2012) Menstrual disorders in adolescents: Review of current practice. *Horm Res Paediatr,* 78**,** 135-143.

WINNEKER, R. C., BITRAN, D. & ZHANG, Z. (2003) The preclinical biology of a new potent and selective progestin: trimegestone. *Steroids,* 68**,** 915-20.

WINNEKER, R. C. & PARSONS, J. A. (1981) Glucocorticoid-like actions of medroxyprogesterone acetate upon MtTW15 rat mammosomatotropic pituitary tumors. *Endocrinology,* 109**,** 99-105.

WIRA, C., PATEL, M. V., GHOSH, M., MUKURA, L. & FAHEY, J. V. (2011) Innate immunity in the human female reproductive tract: Endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. *American Journal of Reproductive Immunology,* 65**,** 196-211.

WIRA, C. R., FAHEY, J. V., GHOSH, M., PATEL, M. V., HICKEY, D. K. & OCHIEL, D. O. (2010) Sex hormone regulation of innate immunity in the female reproductive tract: The role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. *American Journal of Reproductive Immunology,* 63**,** 544-565.

WIRA, C. R., FAHEY, J. V., SENTMAN, C. L., PIOLI, P. A. & SHEN, L. (2005a) Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunol. Rev,* 206**,** 306-335.

WIRA, C. R., GRANT-TSCHUDY, K. S. & CRANE-GODREAU, M. A. (2005b) Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection. *American Journal of Reproductive Immunology,* 53**,** 65-76.

WONG, M. L. & MEDRANO, J. F. (2005) Real-time PCR for mRNA quantitation. *BioTechniques,* 39**,** 75-85.

YAMASHITA, J., HIDESHIMA, T., SHIRAKUSA, T. & OGAWA, M. (1996) Medroxyprogesterone acetate treatment reduces serum interleukin-6 levels in patients with metastatic breast carcinoma. *Cancer,* 78**,** 2346-52.

YU, Z. Y., WRANGE, O., BOETHIUS, J., HATAM, A., GRANHOLM, L. & GUSTAFSSON, J. A. (1981) A study of glucocorticoid receptors in intracranial tumors. *J Neurosurg,* 55**,** 757-60.

ZAREMBER, K. A. & GODOWSKI, P. J. (2002) Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol,* 168**,** 554-61.

ZDRAVKOVIC, M., MULLER, M., LARSEN, S., DEGENKOLB, J. & PABST, G. (2001) Bioequivalence and relative bioavailability of three estradiol and norethisterone acetate-containing hormone replacement therapy tablets. *Int J Clin Pharmacol Ther,* 39**,** 41-6.

ZHANG, Z., JONES, S., HAGOODS, J. S., FUENTES, N. L. & FULLER, G. M. (1997) STAT3 acts as a co-activator of glucocorticoid receptor signaling. *The Journal of biological chemistry,* 272**,** 30607-30610.

ZHANG, Z., LUNDEEN, S. G., ZHU, Y., CARVER, J. M. & WINNEKER, R. C. (2000) In vitro characterization of trimegestone: a new potent and selective progestin. *Steroids,* 65**,** 637-43.

ZHAO, D., LEBOVIC, D. I. & TAYLOR, R. N. (2002) Long-Term Progestin Treatment Inhibits RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) Gene Expression in Human Endometrial Stromal Cells. *J Clin Endocrinol Metab,* 87**,** 2514-2519.

ZHAO, Q., PANG, J., FAVATA, M. F. & TRZASKOS, J. M. (2003) Receptor density dictates the behavior of a subset of steroid ligands in glucocorticoid receptor-mediated transrepression. *Int Immunopharmacol,* 3**,** 1803-17.

ZHOU, J. & CIDLOWSKI, J. A. (2005) The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. *Steroids,* 70**,** 407-17.

ZIEGLER-HEITBROCK, H. W. L., LOTZERICH, M., SCHAEFER, A., WERNER, T., FRANKENBERGER, M. & BENKHART, E. (2003) IFN-gamma induces the human IL-10 gene by recruiting both IFN regulatory factor 1 and Stat3. *The Journal of Immunology,* 171**,** 285-290.

ADDENDUM A

ADDITIONAL DATA

A.1: Mycoplasma-negative Ect1/E6E7 cells

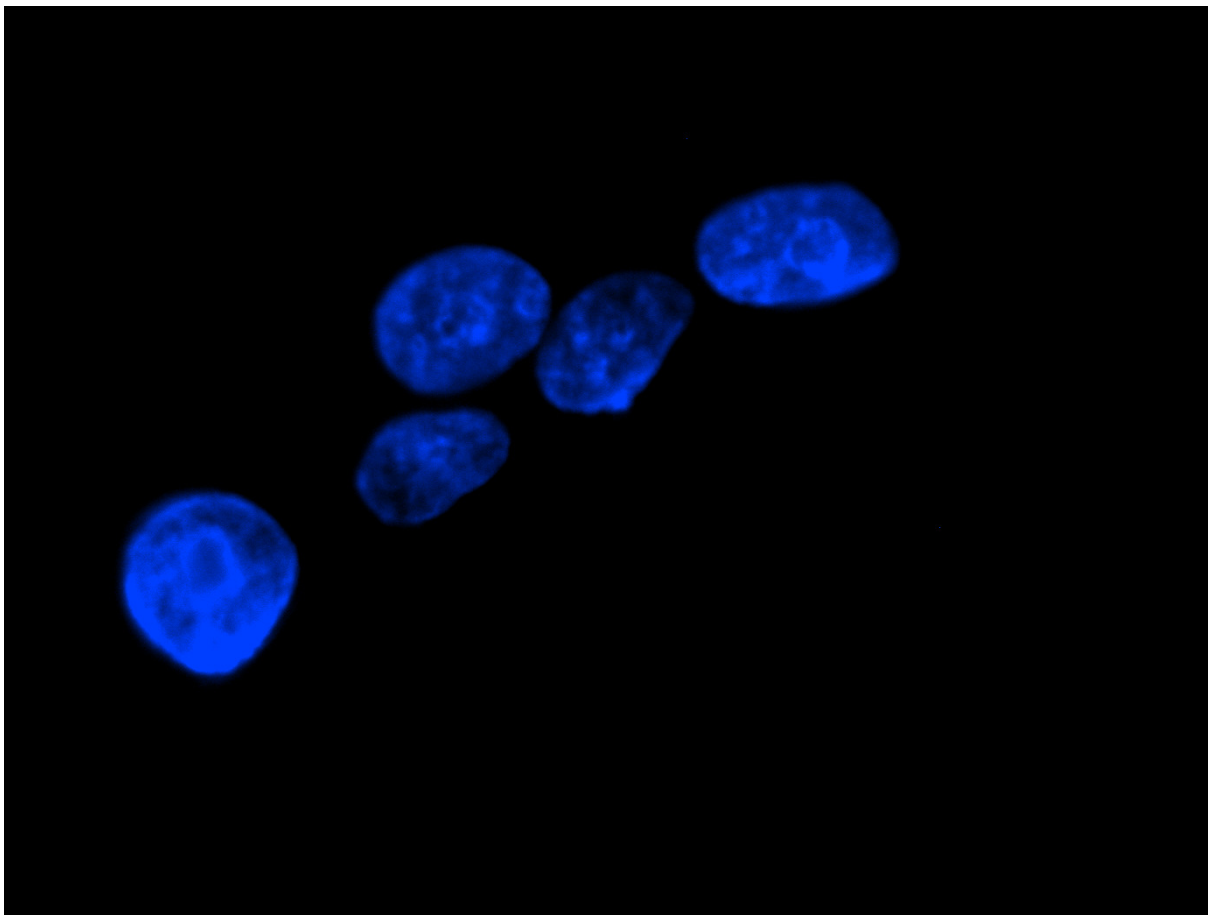

Figure A.1. Mycoplasma-negative Ect1/E6E7 cells. The human Ect1/E6E7 cell line was stained with the DNA Hoechst 33258 dye, and a photograph was taken using a fluorescence microscope. Only DNA-containing nuclei are stained with the Hoechst dye and fluoresce brightly. As shown in this photograph only mycoplasma-negative cells are present.

A.2: Time course to establish equilibrium time for binding of 10 nM [^{3}H]-Dex to endogenously expressed GR in the human Ect1/E6E7 cell line

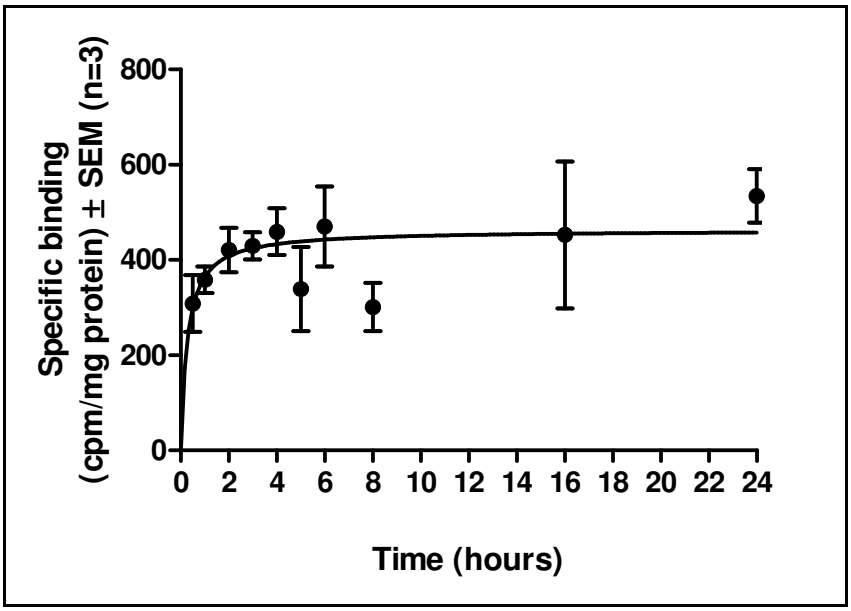

Figure A.2. The equilibrium time for 10 nM [^{3}H]-Dex to bind to the endogenously expressed GR in the Ect1/E6E7 cell line is 6 hours. Human Ect1/E6E7 cells (1x10^5 cells per well in a 24-well plate) were incubated with 10 nM [^{3}H]-Dex in the absence (total binding) and presence of 10 µM unlabelled (non-specific binding) Dex for varying times. Specific binding (total binding minus non-specific binding) normalised to total protein is plotted.

A.3: Low detectable IL-12p40 and IL-10 protein levels in the Ect1/E6E7 cell line

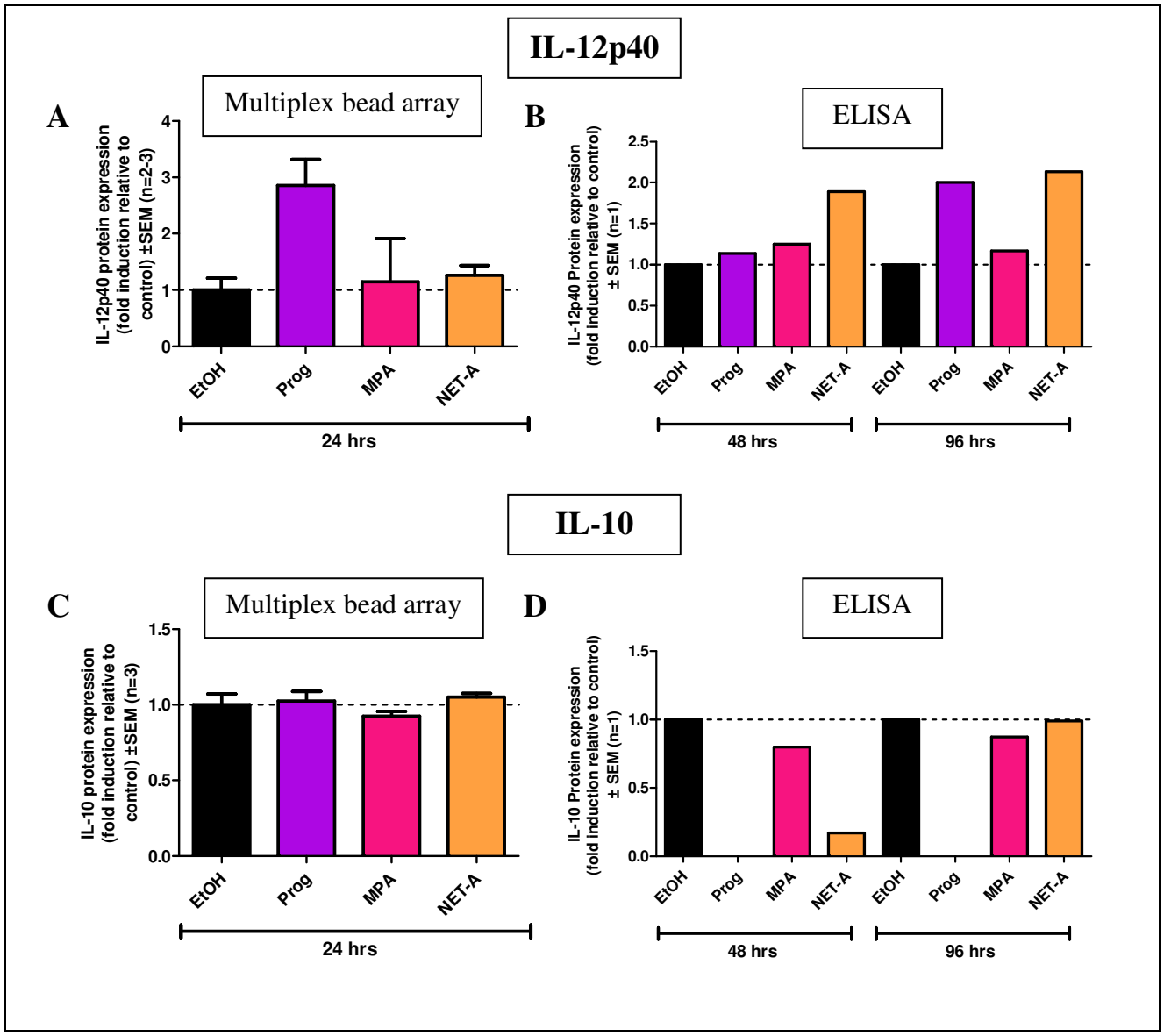

Figure A.3. Effect of Prog, MPA and NET-A on the TNF-α induced protein levels of IL-12p40 and IL-10 in the human ectocervical cell line. The human Ect1/E6E7 cell line was incubated with 0.02 µg/ml TNF-α in the presence of 0.1% EtOH (control) or 1 µM Prog, MPA or NET-A for 24, 48 or 96 hours. The supernatants were collected and analysed by using **(A and C)** a Multiplex bead array to simultaneously quantify the protein levels of **(A)** IL-12p40 and **(C)** IL-10 in the cell culture supernatants according to the manufacturer's instructions. All samples, as well as two quality controls included in the kit, were analysed in duplicate. The levels of all the analytes in the quality controls were within the expected ranges. A standard curve ranging from 3.2 pg/ml to 10 000 pg/ml was used for all the cytokines. Bio-Plex Manager Software, version 4.1.1., was used to analyse the data. **(B and D)** Commercial ELISA kits were used to measure the protein levels of **(B)** IL-12p40 and **(D)** IL-10. The standard curve ranged from 62.5 pg/ml to 4 000 pg/ml for IL-12p40 and from 2 pg/ml to 300 pg/ml for IL-10. The relative protein levels of the treated samples were calculated relative to vehicle control (EtOH), which was set as one.

A.4: The effect of the progestogens on the pro-inflammatory cytokines/chemokines IL-6, IL-8 and RANTES protein expression

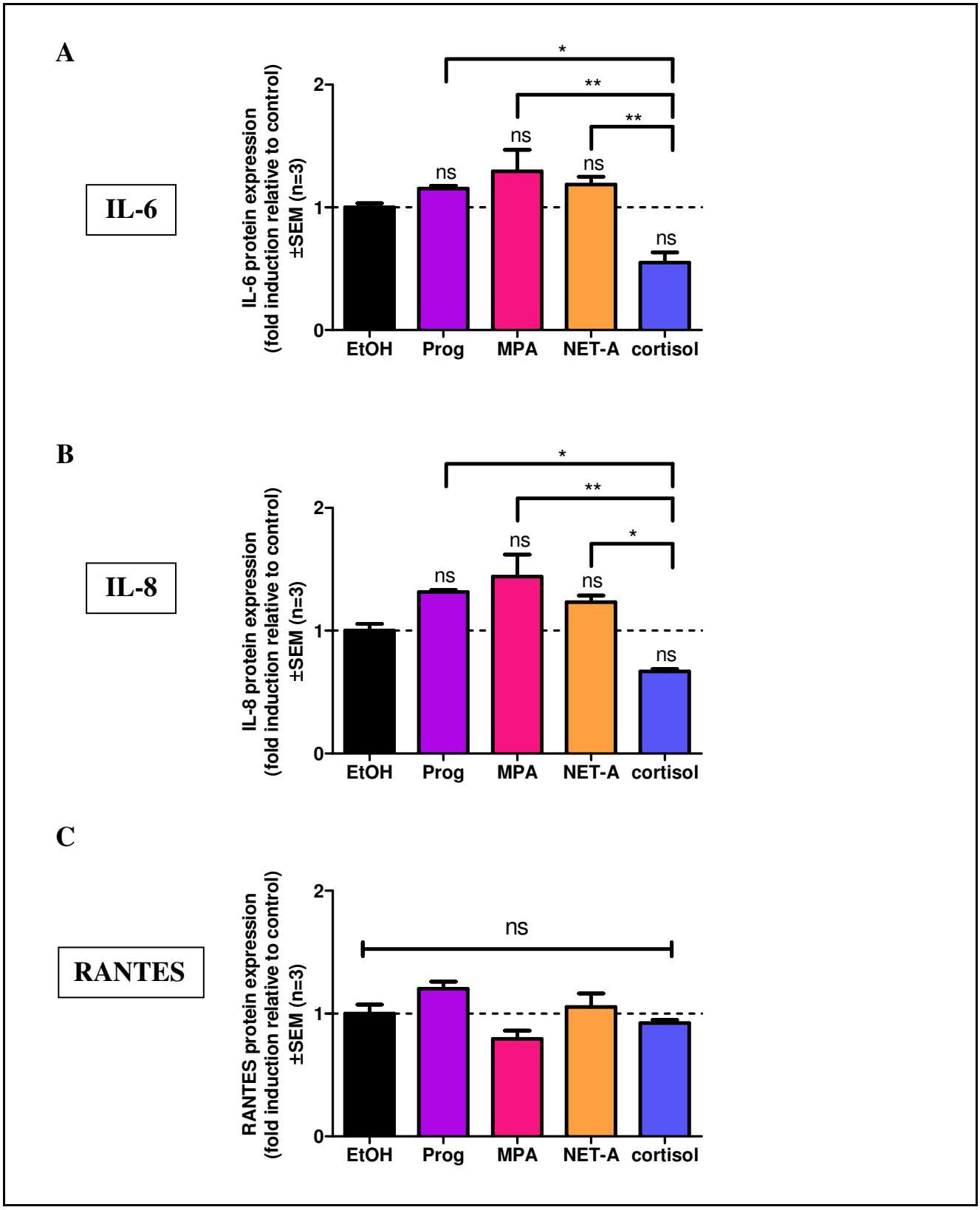

Figure A.4 legend on next page.

Figure A.4. Protein expression of the pro-inflammatory cytokine IL-6 and the chemokines, IL-8 and RANTES as determined by Multiplex bead array. The Human Ect1/E6E7 cell line was treated with 0.02 µg/ml TNF-α and 0.1% EtOH (control) or 1 µM Prog, MPA, NET-A or cortisol for 24 hours. The human 8-plex Luminex assay (cat# MPXHCYTO-60K, Millipore, USA) was used to simultaneously quantify the protein levels of **(A)** IL-6, **(B)** IL-8, and **(C)** RANTES in the cell culture supernatants according to the manufacturer's instructions. All samples, as well as two quality controls included in the kit, were analysed in duplicate. The levels of all the analytes in the quality controls were within the expected ranges. A standard curve ranging from 3.2 pg/ml to 10 000 pg/ml was used for all the cytokines. Bio-Plex Manager Software, version 4.1.1., was used to analyse the data. One-way ANOVA and Bonferonni post-test were used for statistical analysis. Statistical significance of difference is indicated by *, **, ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively, while no statistical significance ($p>0.05$) is indicated by ns.

ADDENDUM B

EXPERIMENTAL OPTIMISATION

B1: Realtime quantitative polymerase chain reaction (qPCR) optimisation

The relative mRNA gene expression levels of the human cytokines IL-12 and IL-10 were quantitatively measured using real-time qPCR. The first crucial step in the analysis of gene expression is the isolation of high quality, intact RNA, as poor quality RNA can compromise experimental results. The RNA quality was therefore assessed firstly by the optical density (OD) of the RNA and secondly by analysing the RNA on a 1% denaturing formaldehyde agarose gel. The OD was measured with a NanoDrop (ND-100 Spectrophotometer) at 260 nm (specific for nucleic acids) and 280 nm (specific for proteins) and the 260/280 ratio was determined. A ratio of greater than 1.9 is generally considered acceptable (Sambrook et al., 1989). The denaturing gel was used to determine the RNA integrity, as the 260 nm OD reading can be compromised by the presence of genomic DNA. On the gel intact total RNA from eukaryotic samples will show sharp, clear 28S and 18S RNA bands. If the 28S RNA band is approximately twice as intense as the 18S RNA band (2:1 ratio), it is a good indication that the RNA is completely intact. Figure B1.1 is an example of a 1% denaturing formaldehyde agarose gel showing intact RNA.

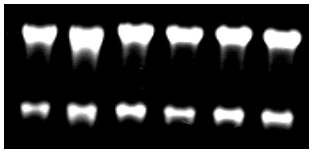

Figure B1.1. A representative 1% denaturing formaldehyde agarose gel showing intact RNA. RNA was isolated from the human Ect1/E6E7 cell line using Tri-reagent (Sigma-Aldrich, South Africa) according to the manufacturer's instructions, and 1 µg RNA was loaded onto an agarose gel.

The intact total RNA was subsequently used to synthetise complementary DNA (cDNA) as described in Chapter 2. The next step in this technique involves the rapid amplification of the short DNA sequences in a realtime PCR thermal cycler (Roche LightCycler). Theoretically the amount of DNA in the reaction should double at each cycle, thus leading to the exponential amplification of the initial target DNA (Fraga et al., 2008). The PCR reaction can be divided into 4 major phases:

linear ground phase, early exponential phase, log-linear phase and plateau phase (figure B1.2.) (Wong and Medrano, 2005, Fraga et al., 2008). The first phase, linear ground phase, refers to the initiation of the PCR reaction (first 10 – 15 cycles), where the emitted fluorescence is below background level. During the early exponential phase the amount of fluorescence reaches a threshold which is significantly higher than the background levels. The point at which the fluorescence exceeds the background fluorescence is called the crossing point (CP). The log-linear phase is defined as the phase where the amplicon increases exponentially until an optimal amplification period is reached, thus the PCR product doubles after every cycle due to ideal reaction conditions. After a while, the PCR reaction will reach a plateau as the PCR reaction components become depleted.

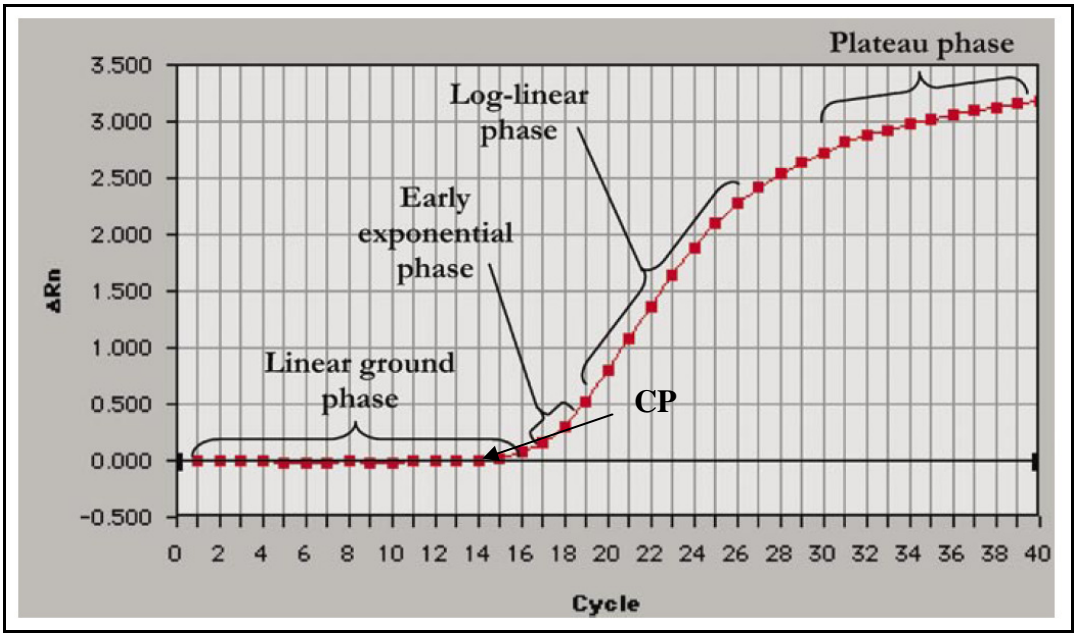

Figure B1.2. A PCR amplification curve indicating the four major phases of real-time qPCR. An increase in the PCR product (amount of DNA) is indicated as an increase in the fluorescence. Figure taken from Wong and Medrano, 2005.

B1.1. Determination of primer pair efficiency

In order to validate the PCR results obtained, the amplification efficiency of each of the primers first had to be determined. As the method by Pfaffl was used to determine relative gene expression,

the primer efficiencies were determined. Standard curves were created by making serial dilutions of a single cDNA sample (EtOH+TNF-α) with each dilution analysed by qPCR in triplicate. A no template control was used to confirm that no contamination occurred. A standard curve is generated by plotting the CP for each dilution (Y-axis) against the log cDNA concentration (X-axis) as shown in figure B.1.3. The slope of the standard curve is used to calculate the exponential amplification value (PCR efficiency) (*E*) for each of the primers according to the following equation (1) (Pfaffl, 2001):

$$E = 10^{[-1/\text{slope}]} \quad (1)$$

Table B1.1 summarises the average primer efficiencies for IL-12p40, IL-12p35, IL-10 and GAPDH determined by pooling the primer efficiencies from two independent experiments, and show efficiencies of >2 for IL-12p40, IL-12p35 and IL-10. It has been suggested that the primer efficiencies for IL-12p40 and IL-10 are acceptable, while the primer efficiency for IL-12p35 is considered high (Realtime qPCR guide, Eurogentec). Primer efficiencies exceeding a value of two have been suggested to be due to the formation of primer-dimers and non-specific amplicons (Realtime qPCR guide, Biorad). As the determination of primer efficiencies were from only two independent experiments, it would be ideal to repeat these experiments. Particular care should be given to accurate pipetting, removal of inhibitors of the qPCR reaction e.g. reverse transcriptase inhibitors (Suslov and Steindler, 2005), decreasing the primer concentrations as well as adjusting PCR conditions such as adjusting the cycle number.

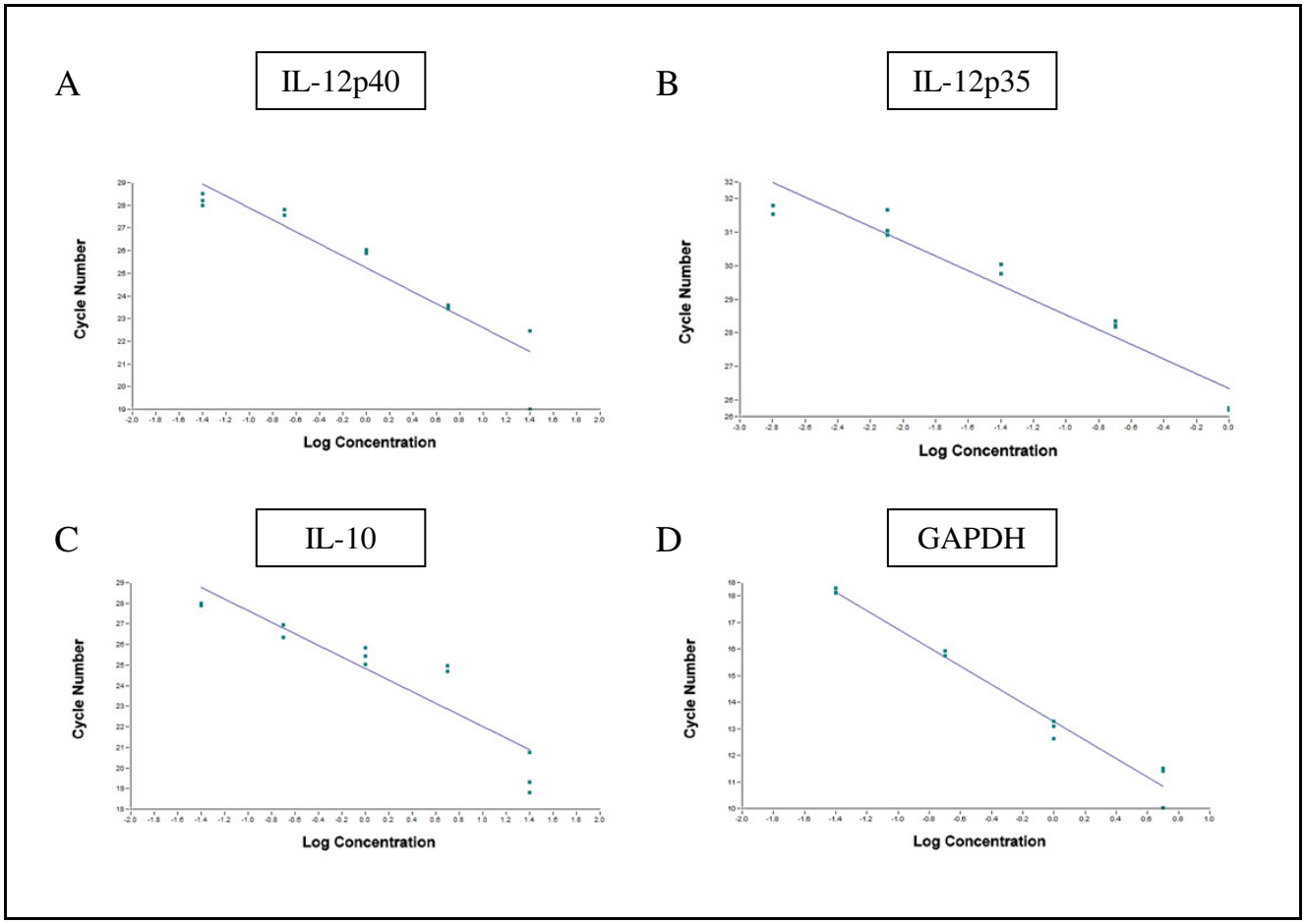

Figure B.1.3. Representative standard curves showing the cycle number versus the log concentration of the amplified cDNA. The standard curves shown in A, B, C and D were generated for the human IL-12p40, IL-12p35, IL-10 and GAPDH primers, respectively.

Table B1.Primer efficiency for each primer used in this thesis

Primer Set	Exponential amplification values (*E*)
IL-12p40	2.39
IL-12p35	2.80
IL-10	2.27
GAPDH	1.86

B1.2. Determination of relative expression values

Following the determination of the primer efficiency, the relative expression levels of the target gene could be calculated by normalising to the levels of a housekeeping gene (Giulietti et al., 2001) to correct for amplification efficiencies. Housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are used for normalisation because they are constantly expressed in different tissues and are not affected by experimental treatment. Using the mathematical model of Pfaffl, the relative expression of a target gene was calculated as a relative expression ratio (*R*) of target gene to reference gene (Pfaffl, 2001):

$$R = \frac{E_{target\,gene}^{\Delta CP\,(control-sample)}}{E_{reference\,gene}^{\Delta CP\,(control-sample)}} \qquad (2)$$

The expression ratio (*R)* of a target gene is calculated based on *E* (PCR efficiency as determined from equation 1) and a change in the crossing point (ΔCP). The value for ΔCP is obtained by subtracting the CP value for the test compound treated sample from the CP value for the EtOH (solvent; control). A relative expression ratio (R) of =1 indicates that there is no difference between samples treated with test compounds and the solvent (EtOH). If R > 1 the mRNA expression of the target gene of interest is upregulated, whereas R < 1 indicates that the mRNA expression of the target gene is inhibited.

B1.3. Melting point analysis

It should be noted that prior to the calculation of the relative expression levels, the conditions for optimal mRNA expression of each target gene was optimised. Also, melting point analysis was performed for each qPCR reaction to confirm amplicon specificity, as the melting curve generated is used to distinguish between the target amplicons, primer-dimers and any non-specific products (Fraga et al., 2008). Target amplicons generally have a higher melting temperature than PCR

artefacts. In addition, the optimal time for the relative expression levels of IL-12p40, IL-12p35, IL-10 and GAPDH had to be determined as different genes are expressed at different times. To determine the optimal time for expression of the target genes, as well as the $MgCl_2$ concentration, a time-course and $MgCl_2$ titration was performed. Optimal IL-12p40 gene expression in the presence of TNF-α was observed at 6 hours using a final $MgCl_2$ concentration of 3 mM (figure B1.4). Similar optimisations were performed for IL-12p35 (figure B1.5) and IL-10 (figure B1.6). From these results, the mRNA expression of all target genes (IL-12p40, IL-12p35 and IL-10) was analysed at 6 hours, due to time and financial constraints, using a final concentration of 3 mM $MgCl_2$ for IL-12p40 and IL-10, and 2 mM $MgCl_2$ for IL-12p35.

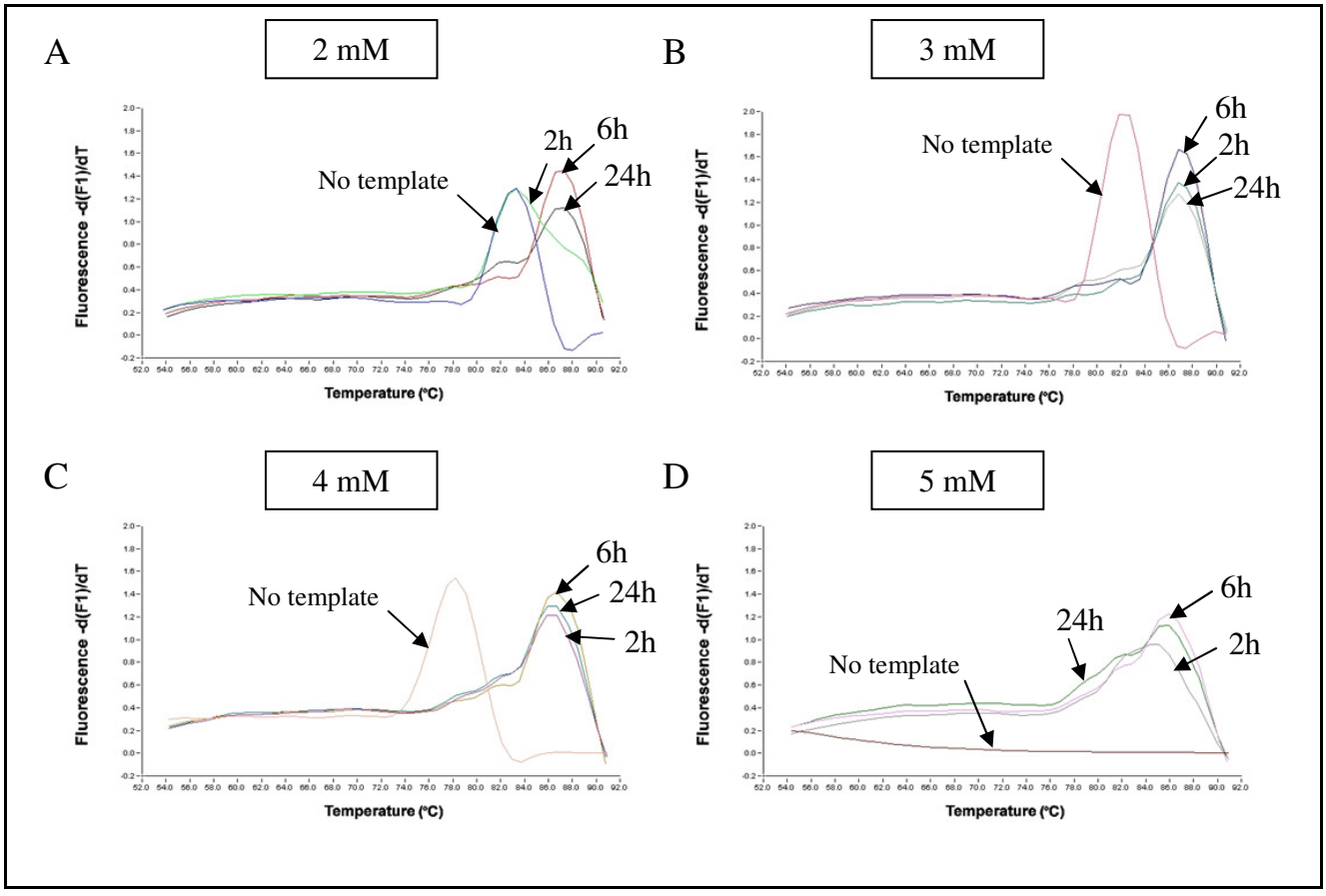

Figure B1.4. Melting curve analysis for human IL-12p40 at different times using varying $MgCl_2$ concentrations. Ect1/E6E7 cells were stimulated with TNF-α (0.02 µg/ml) and treated with 0.1% EtOH (vehicle control) for 2 hours, 6 hours and 24 hours. A $MgCl_2$ titration was performed to obtain a final concentration of **(A)** 2 mM, **(B)** 3 mM, **(C)** 4 mM and **(D)** 5 mM. A no template was included for each condition (primer-dimers formed).

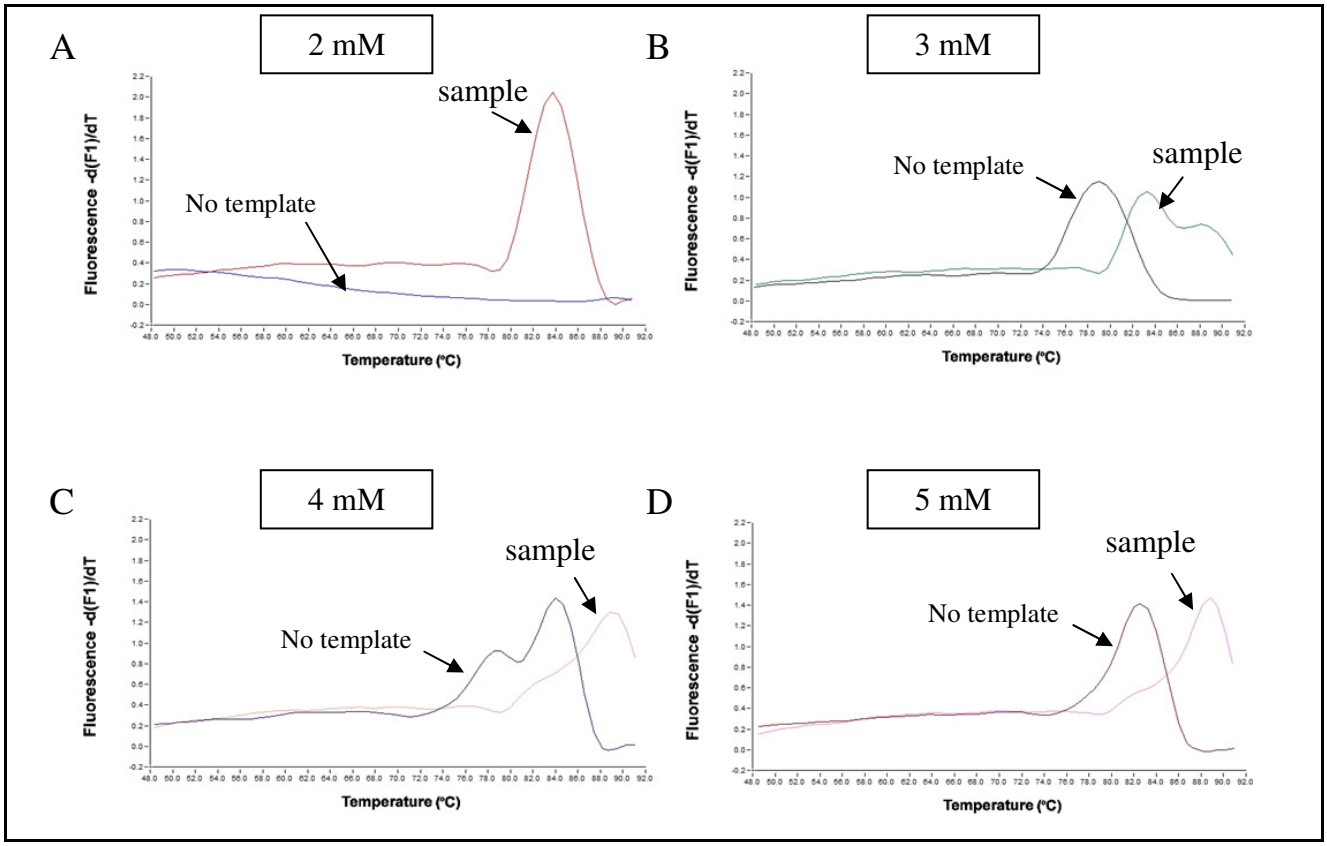

Figure B1.5. Melting curves for human IL-12p35 at 6 hours using varying $MgCl_2$ concentrations. Ect1/E6E7 cells were treated with 0.1% EtOH (solvent) and TNF-α (0.02 µg/ml) (denoted as a) for 6 hours. A $MgCl_2$ titration was performed to obtain a final concentration of **(A)** 2 mM, **(B)** 2.5 mM, **(C)** 3 mM and **(D)** 4 mM. A no template was included for each condition (primer-dimers formed).

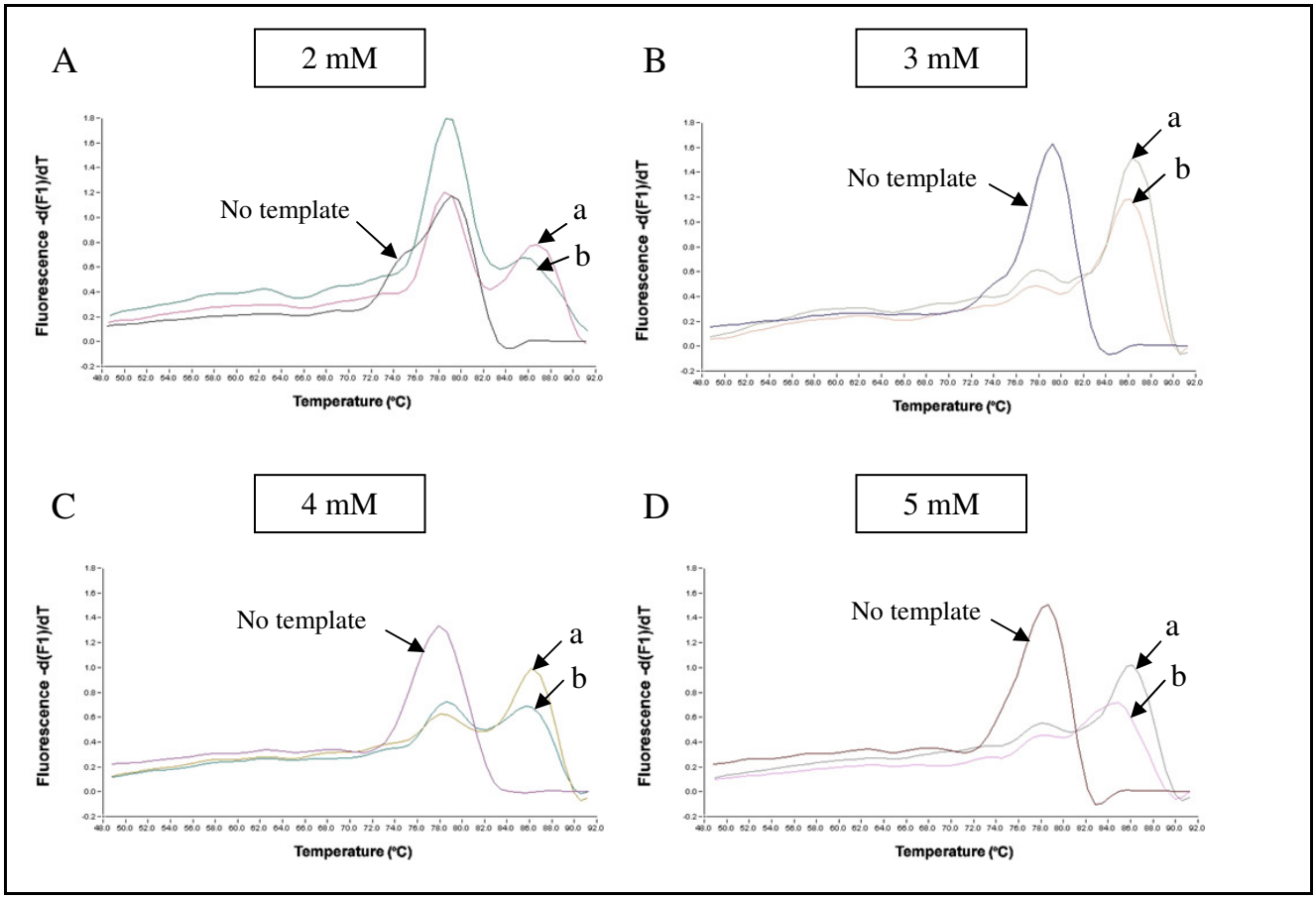

Figure B1.6. Melting curves for human IL-10 at 6 hours using varying $MgCl_2$ concentrations. Ect1/E6E7 cells were induced with (a) 0.1% EtOH (vehicle control) and (b) stimulated with TNF-α (0.02 µg/ml) for 6 hours. A $MgCl_2$ titration was performed to obtain a final concentration of **(A)** 2 mM, **(B)** 3 mM, **(C)** 4 mM and **(D)** 5 mM. A no template was included for each condition (primer-dimers formed).

B2: Chromatin immunoprecipitation (ChIP) assay optimisation

The recruitment of proteins to specific DNA *cis*-elements within the promoter area of target genes, i.e. protein-DNA interactions, was investigated by means of ChIP assays. This entailed (i) cross-linking the DNA-binding proteins to the chromatin, (ii) fragmenting the protein-DNA complex by sonication, (iii) immunoprecipitation with an antibody specific for the protein of interest, followed by the (iv) amplification and quantification of the specific DNA sequence associated with the protein of interest using qPCR. Optimal sonication of the chromatin is crucial as it aids in further lysing of the formaldehyde cross-linked cells, as well as allowing the determination of the average size of the DNA fragments. As very small fragments may be lost during the DNA purification step and large DNA fragments may cause inefficient immunoprecipitation, the optimal DNA fragment size should range between 300 and 800 bp (Ma et al., 2003). Thus, the sonification step needs to be optimised for different cell lines. For the Ect1/E6E7 epithelial cell line, we used varying cell numbers, power settings and times, and evaluated the size of the fragmented chromatin by agarose gel electrophoresis. As shown in figure B2.1 chromatin fragments with an average size of 300 bp were produced, when the human Ect1/E6E7 cells were sonicated using the Misonix sonicator on 75% power for 50 cycles at 30 seconds per cycle, with 30 second intervals between pulses.

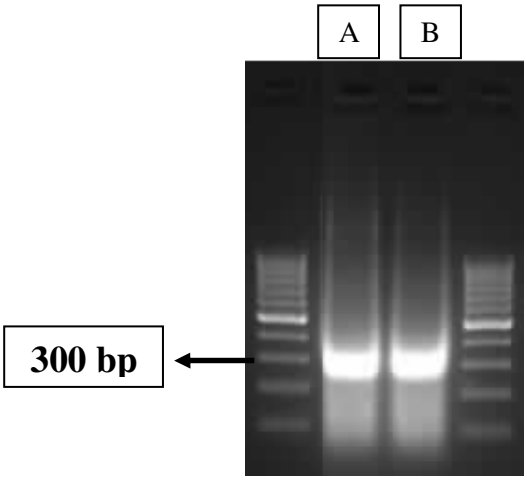

Figure B2.1. An example of an agarose gel electrophoresis analysis of chromatin after sonication. The human Ect1/E6E7 cell line was treated with 0.1% EtOH (vehicle control) in the (A) absence and (B) presence of 0.02 µg/ml TNF-α for 2 hours. Thereafter, the cells were cross-linked, harvested and sonicated at 75% power for 50 cycles at 30 seconds per cycle, with 30 second intervals between pulses using the Misonix sonicator.

ADDENDUM C

CONTRIBUTION TO PUBLICATION

(Africander et al., 2011a)

ELSEVIER

Contraception

Contraception 84 (2011) 423–435

Original research article

Differential regulation of endogenous pro-inflammatory cytokine genes by medroxyprogesterone acetate and norethisterone acetate in cell lines of the female genital tract

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Received 14 December 2010; revised 2 April 2011; accepted 13 June 2011

Abstract

Background: Medroxyprogesterone acetate (MPA) and norethisterone (NET) and its derivatives are widely used in female reproductive therapy, but little is known about their mechanisms of action via steroid receptors in the female genital tract. MPA used as a contraceptive has been implicated in effects on local immune function. However, the relative effects of progesterone (Prog), MPA and norethisterone acetate (NET-A) on cytokine gene expression in the female genital tract are unknown.
Study Design: Using two epithelial cell lines generated from normal human vaginal (Vk2/E6E7) and ectocervical (Ect1/E6E7) cells as in vitro cell culture model systems for mucosal immunity of the female cervicovaginal environment, we investigated steroid receptor expression and activity as well as regulation of cytokine/chemokine genes by MPA and NET-A, as compared to the endogenous hormone Prog.
Results: We show that the Prog, androgen, glucocorticoid and estrogen receptors (PR, AR, GR and ER, respectively) are expressed in both the Vk2/E6E7 and Ect1/E6E7 cell lines, and that the GR and AR are transcriptionally active. This study is the first to show ligand-, promoter- and cell-specific regulation of IL-6, IL-8 and RANTES (regulated-upon-activation, normal T cell expressed and secreted) gene expression by Prog, MPA and NET-A in these cell lines. Moreover, we show that the repression of the TNF-α-induced RANTES gene by MPA in the Ect1/E6E7 cell line is mediated by the AR.
Conclusion: Collectively, these data demonstrate that cell lines from different anatomical sites of the female genital tract respond differently to Prog and the synthetic progestins, most likely due to differential actions via different steroid receptors. The results highlight the importance of choice of progestins for immune function in the cervicovaginal environment. They further suggest that choice of progestins in endocrine therapy may have implications for women's risk of susceptibility to infections due to differential actions on genes involved in inflammation and immune function.

Keywords: Synthetic progestins; Injectable contraceptives; Female genital tract; Cytokines

1. Introduction

The mucosal surface of the lower female genital tract (cervicovaginal environment) is a complex system that provides a barrier against pathogens. Epithelial cells lining the cervicovaginal mucosa are the point of entry for many viral, bacterial and parasitic infections [1,2]. These cells thus have features enabling them to combat infections, such as the expression of cytokines, hormone receptors and genital tract-specific defensins [3–6] (and reviewed in Ref. [7]). Cervicovaginal epithelial cells constitutively express a wide variety of pro- and anti-inflammatory mediators, such as the cytokines, interleukin (IL)-1, IL-6, IL7, macrophage colony-stimulating factor, transforming growth factor beta and the chemoattractant cytokines, IL-8 and RANTES (regulated-upon-activation, normal T cell expressed and secreted), which are up-regulated in response to tumor necrosis factor (TNF)-α [1,2,8]. These cells thus play an important role in the innate and acquired immune systems present at the mucosal surfaces.

IL-8 and RANTES (also termed CCL5) are chemotactic cytokines, or chemokines, involved in the early inflammatory response by recruiting specific leukocytes, particularly macrophages, to sites of ongoing inflammation and injury, while IL-6 is responsible for neutrophil priming to chemotactic factors [9]. Interestingly, increased levels of the cytokines IL-

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doi:10.1016/j.contraception.2011.06.006

1, TNF-α, IL-6 and the chemokines RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β in cervicovaginal secretions have been associated with human immunodeficiency virus (HIV)-1 infection and bacterial vaginosis [10–12]. Moreover, decreased systemic $CD4^+$ cell counts during acute HIV infection has been associated with increased levels of IL-1β, IL-6 and IL-8 in genital tract secretions [13]. Thus, the cytokine milieu in the cervicovaginal mucosa is an important determinant of resistance and susceptibility to infections.

The transmission of and susceptibility to infections in women may be better understood if factors affecting the immune response in the vagina and cervix are more clearly defined. Research in animal models and in women indicates that local as well as regional immune responses affect the outcome of vaginal challenge with microbial pathogens (reviewed in Ref. [14]). Sex hormones are examples of factors that have been shown to influence susceptibility and disease predisposition to many genital tract infections [15]. Furthermore, there are indications that women using antibiotics, corticosteroids (immunosuppressive therapy), oral contraceptives and hormone replacement therapy (HRT) are more susceptible to fungal vaginal infections [16–18].

The synthetic progestins medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN) are the most widely used injectable female contraceptives, with at least 20 million current users of MPA worldwide [19]. MPA itself, rather than its metabolites, is the major progestogenic compound, while NET-EN and norethisterone acetate (NET-A) are hydrolyzed to norethisterone (NET) and its metabolites, which together have progestogenic action [20]. Both MPA and NET are also used for HRT in postmenopausal women. MPA used as contraceptive has been shown to increase HIV [21] and HSV cervical shedding in HIV-infected women [22]. Although Mostad et al. [21] did not investigate the molecular mechanism of these effects, they postulate that the effects may be mediated by factors such as direct effects on the virus, effects on local genital tract physiology or effects on immune modulation of viral replication, or a combination of these effects. Interestingly, an animal study showed that MPA increased susceptibility to vaginal simian–human immunodeficiency virus (SHIV) transmission and suppressed the antiviral cellular immune response in SHIV-infected rhesus macaques [23], indicating an immune- rather than a transmission-based mechanism. In another animal study, it was shown that MPA treatment at contraceptive doses rendered mice 100-fold more susceptible to genital HSV-2 infection compared to untreated mice [24]. In addition, the use of MPA has been associated with increased acquisition of cervical chlamydial and gonococcal infections [25]. Whether or not NET is associated with an increased risk of HIV/HSV acquisition and shedding, viral load and viral diversity remains to be determined. In this regard, two recent studies in a cohort of South African women showed no association between risk of HIV infection and NET-EN at contraceptive doses [26,27]. However, a recent re-analysis of earlier data has shown that MPA used as an injectable contraceptive (referred to in this case as Depo-MPA or DMPA) is associated with an increase in HIV acquisition in women [28,29].

To date, very little is known about the molecular mechanisms of action of MPA and NET on immune function, in particular the target cells, target genes and dose responses. MPA has been reported to modulate transcription of a number of genes via the glucocorticoid receptor (GR), for example, IL-2 in normal human lymphocytes [30], IL-6 and IL-8 in a mouse fibroblast cell line [31] and the *nm-23* tumor suppressor gene in a breast cancer cell line [32]. In contrast, MPA's suppression of the RANTES gene in endometrial cells was progesterone receptor (PR) mediated [33]. Interestingly, NET-A is not an agonist for transactivation via the GR and only marginally (22%) transrepressed an IL-8 reporter at 10 μM [34]. Furthermore, MPA has been shown to regulate a number of genes via the PR and the androgen receptor (AR) in human breast cancer cell lines [35]. In contrast to the data available for MPA, much less is known about the biological activity of NET via the AR. However, the recent study by Sasagawa et al. [36] has now characterized both MPA and NET pharmacologically in terms of potency for transactivation via the AR. Furthermore, both MPA and NET-A have been reported to bind to the mineralocorticoid receptor (MR) with low relative affinity and do not display any agonist or antagonist properties towards the MR [37,38]. It is thus apparent that even though MPA and NET were developed for the similarity of their biological actions to those of progesterone (Prog), mediating their effects by binding to the PR, they can also initiate a diverse range of biological effects by cross-reacting with other members of the steroid receptor family such as the GR, the AR and possibly the MR [34,39–42].

An important question is thus whether, and to what extent, MPA, and also NET-A, regulates known pro-inflammatory mediators such as IL-6, IL-8 and RANTES, in the cervicovaginal mucosa. Understanding the mechanisms of this regulation and the receptors involved would further our understanding of differential gene regulation by different progestins and assist in the design of new progestins with fewer side effects. Investigating these mechanisms at a site relevant to infections, such as the cervicovaginal environment, is likely to be relevant to mucosal immunity. Factors that affect immunity in the cervicovaginal environment may be important determinants of transmission risk of pathogens such viruses, and understanding these factors may shed light on molecular events occurring during infections. In the light of the above, our strategy was to investigate the effects of MPA and NET-A relative to Prog on mucosal immunity in an in vitro cell culture model of the female cervicovaginal environment, by comparing their effects on the regulation of the endogenous pro-inflammatory cytokine/chemokine genes IL-6, IL-8 and RANTES. Two epithelial cell lines generated from normal human vaginal (Vk2/E6E7) and ectocervical (Ect1/E6E7) cells, immortalized by expression of the E6 and E7 genes of human papillomavirus type 16 [42], were used as model systems.

2. Materials and methods

2.1. Inducing compounds

4-Pregnene-3,20-dione (Prog), 6α-methyl-17α-hydroxyprogesterone acetate (MPA), 17α-ethynyl-19-nortestosterone 17β-acetate (NET-A), 5α-androstan-17β-ol-3-one [dihydrotestosterone (DHT)], 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one [mifepristone (RU486)], 11β,16α-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione [dexamethasone (Dex)], 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al [aldosterone (Ald)], 17β-estra-1,3,5 (10)-triene-3,17-diol [β-estradiol (E_2)] and recombinant TNF-α were obtained from Sigma-Aldrich (South Africa). NET-A, the acetate, was used as it is soluble in water compared with the insoluble ester of NET-EN. In vivo, both NET-EN and NET-A undergo hydrolysis and are converted to NET and its metabolites [20]. Hydroxyflutamide (OHF) was obtained from Dr. C. Tendler (Schering Plough Research Institute, USA). Unlabelled 17β-hydroxy-7α,17α-dimethylestr-4-en-3-one [mibolerone (MIB)], [^{3}H]-MIB (76.8 Ci/mmol), unlabelled R5020, ^{3}[H]-R5020 (84.6 Ci/mmol) and [^{3}H]-Ald (87.9 Ci/mmol) were purchased from PerkinElmer Life and Analytical Science (South Africa). [^{3}H]-Dex (89 Ci/mmol) and ^{3}[H]-E_2 (110 Ci/mmol) were purchased from AEC-Amersham (South Africa). All unlabelled test compounds were dissolved in absolute ethanol (1 and 10 mM stock concentrations) and stored at −20°C. These compounds were then added to serum-free culturing medium such that the final concentration of ethanol was 0.1%. Control incubations (no test compounds) were performed in the presence of 0.1% ethanol.

2.2. Cell culture

Ect1/E6E7 and Vk2/E6E7 cell lines (human ectocervical and vaginal epithelial cell lines, respectively) were purchased from American Type Culture Collection (ATCC, USA) and maintained in keratinocyte serum-free medium (GibcoBRL, Paisley, UK) supplemented with 50 mcg/mL bovine pituitary extract (GibcoBRL), 0.1 ng/mL epidermal growth factor (GibcoBRL), 0.4 mM $CaCl_2$, 50 IU/mL penicillin and 50 mcg/mL streptomycin (GibcoBRL). These cells have been validated as a model for epithelial immune function by comparisons with primary cell cultures, tissues, animal models and clinical findings, and show similar toll-like receptor and cytokine profiles to primary cells [1,3,43]. Cultures were maintained in 75-cm^2 culture flasks (Greiner Bio-One International, Austria) at 37°C, in an atmosphere of 90% humidity and 5% CO_2. All cultures were regularly tested for mycoplasma infection by means of Hoechst staining [44], and only mycoplasma-negative cell lines were used in experiments.

2.3. Plasmids

Plasmids expressing the human mineralocorticoid receptor pRShMR and the human glucocorticoid receptor pRS-hGRα were a kind gift from Prof. R. Evans (Howard Hughes Medical Institute, La Jolla, CA, USA). A plasmid expressing the human androgen receptor pSVARo [45] was obtained from Frank Claessens, (University of Leuven, Leuven, Belgium). The plasmid expressing the human progesterone receptor (isoform B) pSG5hPR-B [46] was obtained from Stoney Simons, Jr. (NIH, Bethesda, USA), while the human estrogen receptor-α (pSG5-ERα) and -β (pSG5-ERβ) expression vectors were received from Prof. Frank Gannon, European Molecular Biology Laboratory (EMBL, Heidelberg, Germany). The pGL2basic empty vector and the pSV-β-galactosidase expression vector (pSV-β-gal) were obtained from Promega (Madison, WI, USA). The plasmid pTAT-GRE-E1b-luc, driven by the E1b promoter and containing two copies of the rat TAT-GRE was obtained from Dr. G. Jenster (Erasmus University of Rotterdam, The Netherlands). The 5xNFκB-luc plasmid and the cytomegalovirus (CMV)-driven-β-galactosidase expression vector (pCMV-β-gal) were from Stratagene (Houston, TX, USA).

2.4. Isolation of total RNA and real-time quantitative RT-PCR

Ect1/E6E7 and Vk2/E6E7 cell lines were maintained as described above. Cells were induced with 0.02 mcg/mL TNF-α and 1 μM of the test compounds, in the absence and presence of 10 μM receptor-specific antagonists, for 24 h. Total RNA was isolated from cells using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. The integrity of the RNA (presence of intact 18S and 28S ribosomal bands) was confirmed by denaturing agarose gel electrophoresis. Total RNA (1 mcg) was reverse transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, South Africa) according to the manufacturer's instructions. Real-time PCR was performed by using a LightCycler, rapid thermal cycler system (Roche Applied Science) according to the manufacturer's instructions. Nucleotides, TaqDNA polymerase and buffer used in the reaction were those included in the LightCycler-FastStart DNA Master^{PLUS} SYBR Green I system (Roche Diagnostics, South Africa). Agarose gel electrophoresis and melting curve analysis were performed to confirm the generated amplicon in each sample. The amplification efficiency for each primer was determined by generating a standard curve from a cDNA dilution series. The efficiencies were 1.99, 1.92, 1.98 and 2.0 for IL-6, IL-8, RANTES and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Relative IL-6, IL-8 and RANTES transcript levels were calculated with the Fit Points method described by Pfaffl [47] and were normalized to relative GAPDH transcript levels. The specific primers used were as follows: for RANTES, 5′-TACCATGAAGGTCTCCGC-3′ (forward primer) and 5′-GACAAAGACGACTGCTGG-3′ (reverse primer) [48]; for IL-6, 5′-TCTCCACAAGCGCCTTCG-3′ (forward primer) and 5′-CTCAGGGCTGAGATGCCG-3′ (reverse primer) [48]; for IL-8, 5′-TGCCAAGGAGTGC-TAAAG-3′ (forward primer) and 5′-CTCCACAA-CCCTCTGCAC-3′ (reverse primer) [48]; for GAPDH,

5′- TGAACGGGAAGCTCACTGG-3′ (forward primer) and 5′- TCCACCACCCTGTTGCTGTA-3′ (reverse primer [49]. The product sizes were 199, 193, 197 and 307 bp for RANTES, IL-6, IL-8 and GAPDH, respectively.

2.5. Western blotting

Ect1/E6E7 and Vk2/E6E7 cell lines were plated at 1×10^6 cells per well in six-well plates and allowed to grow to confluency. Cells were washed with phosphate-buffered saline (PBS) and lysed in 100 μL SDS-sample buffer [50]. Lysates were boiled and subjected to electrophoresis on an 8% SDS polyacrylamide gel using β-actin expression as a loading control. Following electrophoresis, proteins were transferred to nitrocellulose membranes. Blots were probed with anti-AR (H-280, 1:1000), anti-GR (H300, 1:3000), anti-PR-B (sc-811, 1:500), anti-PR-AB (sc-810, 1:500), anti-MR (H300, 1:1000), anti-ER-α (MC-20, 1:200) or anti-ER-β (H-150, 1:1000) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA), as well as the anti-β-actin (1:1500) antibody from Cell Signalling Technology (Massachusetts, USA), for 16 h at 4°C. Blots were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:10,000 for GR and β-actin, 1:2000 for MR, 1:5000 for ER-α, 1:1000 for ER-β; NA934VS, Amersham, South Africa) or goat anti-mouse secondary antibody (1:5000 for AR, 1:2000 for PR-A and PR-B; Santa Cruz Biotechnology) for 1 h. Proteins were visualized using enhanced chemiluminescence from Amersham, followed by exposure to Hyperfilm MP high-performance autoradiography film (Amersham, South Africa).

2.6. Whole cell binding assays

Competitive whole cell binding assays were performed essentially as described by Bamberger et al. [51], with the following modifications. Ect1/E6E7 and Vk2/E6E7 cell lines were maintained as described above. Cells were seeded into 12-well tissue culture plates (Nunc, Denmark) at 2×10^5 cells per well. On Day 3, the cells were washed with PBS and incubated for 2 h at 37°C with 10 nM [^{3}H]-MIB (76.8 Ci/mmol) for AR, [^{3}H]-Ald (87.9 Ci/mmol) for MR, [^{3}H]-R5020 (84.6 Ci/mmol) for PR ([^{3}H]-MIB, [^{3}H]-Ald and [^{3}H]-R5020 all from PerkinElmer Life and Analytical Science, South Africa), [^{3}H]-Dex (89 Ci/mmol) for GR (AEC-Amersham) or [^{3}H]-E_2 (110 Ci/mmol) for ER (AEC-Amersham), in the absence (total binding) or presence of 10 μM unlabelled MIB, Ald, R5020, Dex or E_2 (nonspecific binding), respectively. Working on ice at 4°C, cells were washed three times with ice-cold 1× PBS containing 0.2% (w/v) bovine serum albumin for 15 min. Cells were then lysed with a 200 μL reporter lysis buffer (Promega). Total binding was determined by scintillation counting as counts per minute (cpm), while specific binding was determined by subtracting nonspecific binding from total binding. Protein concentrations were determined using the Bradford protein assay method [52]. Specific binding was normalized to the protein concentration. The reported values are averages of at least three independent assays, with each condition performed in triplicate. The receptor numbers in femtomole per milligram of protein were determined as follows.

The specific activity of the tritium-labelled test compound (Ci/mmol) was converted to disintegrations per minute (dpm) per millimole, by multiplying by a factor of 2.22×10^{12} (1 Ci equals 2.22×10^{12} dpm). The disintegration per minute was multiplied by the counting efficiency to get the counts per minute per millimole and divided by 10^{12} to get the counts per minute per femtomole. Counting efficiency was 40% in our system. The specific activity was expressed as counts per minute per femtomole. Dividing the specific binding counts per minute value by this value gives specific binding in femtomoles, which was divided by the protein content of the sample in milligrams, to yield the number of binding sites in femtomoles per milligram of protein.

2.7. Luciferase reporter assays

For transactivation assays, cells were maintained as above and seeded into 24-well tissue culture plates at 5×10^4 cells per well. On Day 2, cells in each well were transiently transfected with 330 ng of the pTAT-GRE-E1b-luc construct and 33 ng of the expression vector for the hGR, hAR, hPR-B, hMR or the empty vector pGL2basic, using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. On Day 3, the cells were washed with PBS and induced with vehicle (0.1% EtOH) or 10 μM of either Dex, MIB, R5020 or Ald. For transrepression assays, cells were maintained as above and seeded into 24-well tissue culture plates at 5×10^4 cells per well. On Day 2, the cells were transiently transfected with (1) 100 ng of the 5xNF-κB-luc construct, either 50 ng of the expression vector for the hGR or the empty vector pGL2-basic, and 25 ng pSV-βgal or with (2) 400 ng of the 5xNF-κB-luc construct, either 70 ng of the expression vector for the hAR or pGL2-basic, and 70 ng pCMV-βgal, using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. On Day 3, the cells were stimulated with 0.02 mcg/mL TNF-α in the absence (0.1% ethanol) and presence of 10 μM Dex (for the GR) or MIB (for the AR). After 24 h, the cells were lysed with reporter lysis buffer (Promega). Luciferase activity in the lysate was measured using the Luciferase Assay System (Promega) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). The values obtained were normalized to expression of β-gal, which was measured using the GalactoStar Assay Kit from Tropix (Bedford, MA, USA). The reported values are averages of at least three independent experiments, with each condition performed in triplicate.

2.8. Data manipulation and statistical analysis

The GraphPad Prism software was used for data manipulations, graphical representations and statistical analysis. One-way ANOVA analysis of variance and Dunnett (compare all columns vs. control column) or Bonferroni post-

tests (compares all pairs of columns) were used for statistical analysis. In some figures, statistical significance of differences is indicated by *, ** or ***, to indicate $p<.05$, $p<.01$ or $p<.001$, respectively, whereas no statistical significance ($p>.05$) is indicated by 'ns'. In other figures, the letters 'a', 'b', 'c', etc., are also used to denote statistically significant differences, where all those values which differ significantly from others are assigned a different letter.

3. Results

3.1. MPA and NET-A, unlike Prog, exhibit differential patterns of gene regulation on pro-inflammatory chemokines

Human Ect1/E6E7 and Vk2/E6E7 cell lines were treated for 24 h with 0.02 mcg/mL TNF-α and 1 μM Prog, MPA or NET-A, followed by real-time quantitative RT-PCR (QPCR) analysis for expression of the IL-6, IL-8 and RANTES genes, respectively. In both the Ect1/E6E7 and Vk2/E6E7 cell lines, results show that unlike Prog which up-regulates IL-6 gene expression, both MPA and NET-A have no effect (Fig. 1A and 1B). In contrast, MPA and NET-A differentially regulate both the IL-8 (Fig. 2A) and RANTES (Fig. 3A) genes in the Ect1/E6E7 cell line. The results show that MPA, like Prog, significantly up-regulates IL-8 (10.3- vs. 12-fold, respectively), while NET-A has no significant effect (Fig. 2A). Although not statistically significantly different, Prog and NET-A seemed to up-regulate the IL-8 gene in the Vk2/E6E7 cell line to a similar extent, while MPA appeared to show the greatest level of up-regulation (Fig. 2B). Unlike Prog which significantly up-regulates RANTES gene expression in the Ect1/E6E7 cell line, MPA significantly down-regulates RANTES gene expression, while NET-A has no effect (Fig. 3A). Conversely, both MPA and NET-A, like Prog, appeared to slightly up-regulate the expression of the RANTES gene in the Vk2/E6E7 cell line (Fig. 3B). Taken together, MPA and NET-A exhibit differential patterns of gene regulation on expression of the IL-8 and RANTES genes, as compared to Prog, in a promoter- and cell-specific manner. In addition, unlike Prog, MPA and NET-A do not up-regulate IL-6 gene expression.

3.2. The PR, AR, GR and ER are expressed in both ectocervical and vaginal cell lines

As these progestins are known to interact with the PR, AR, GR and possibly the MR [36], we hypothesized that the differential regulation of genes by MPA and NET-A, particularly on the RANTES gene, was due to their action via different steroid receptors or to differing activities via the same steroid receptor. To test this hypothesis, we firstly investigated which members of the steroid receptor family were expressed in these cell lines using a combination of Western blotting (Fig. 4A) and whole cell binding (Fig. 4B and C).

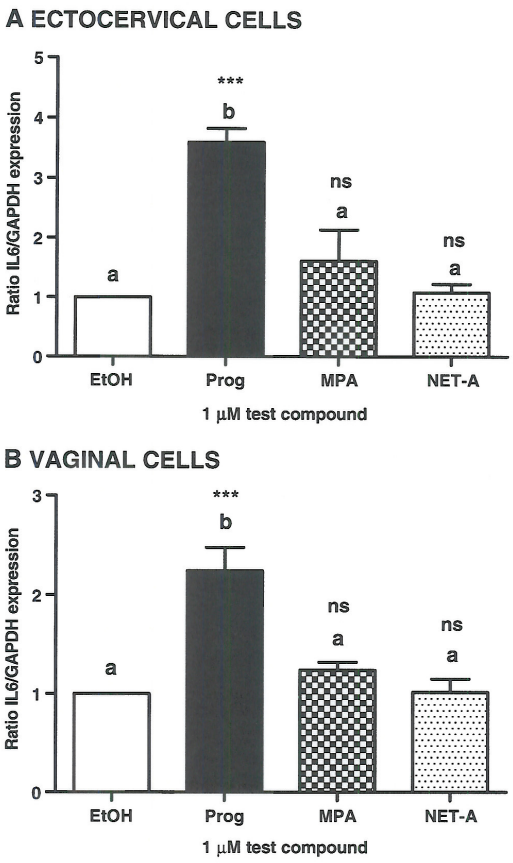

Fig. 1. Effect of MPA and NET-A on the TNF-α-induced expression of the IL-6 gene in human ectocervical and vaginal epithelial cell lines. (A) Human Ect1/E6E7 and (B) Vk2/E6E7 cell lines were incubated with 0.02 mcg/mL TNF-α in the presence of 0.1% EtOH or 1 μM Prog, MPA or NET-A for 24 h. Total RNA was extracted, cDNA synthesized and expression levels of IL-6 and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least three independent experiments. One-way ANOVA and both Bonferroni (compares all pairs of columns) and Dunnett [compares all columns vs. control (EtOH) column] post-tests were used for statistical analysis. The letters 'a', 'b', 'c', etc., are used to denote statistically significant differences, where all those values which differ significantly from others are assigned a different letter. Statistical significance of differences indicated by *, ** or *** indicates $p<.05$, $p<.01$ or $p<.001$, respectively, whereas no statistical significance ($p>.05$) is indicated by 'ns'.

Probing with an antibody specific for the PR-B isoform in the Western blot indicated that neither of these cell types expresses PR-B. We were unable to optimize the commercial antibody detecting the PR-A isoform [anti-PR-AB (sc-810), Santa Cruz Biotechnology] and thus could not determine by Western blotting whether the PR-A isoform is expressed in these cell lines. Similar levels of the AR and GR were detected in both cell lines by Western blotting (Fig. 4A).

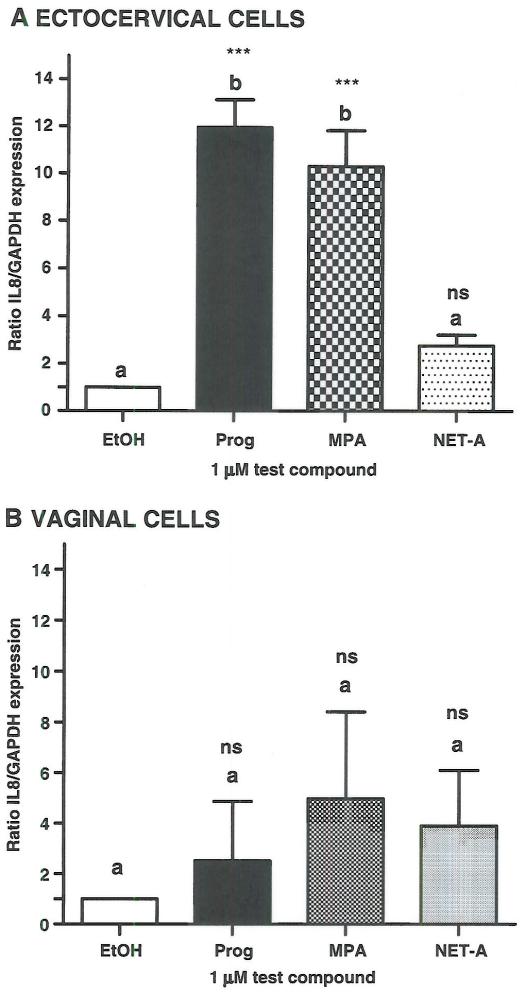

Fig. 2. Effect of MPA and NET-A on the TNF-α-induced expression of the IL-8 gene in human ectocervical and vaginal epithelial cell lines. (A) Human Ect1/E6E7 and (B) Vk2/E6E7 cell lines were incubated with 0.02 mcg/mL TNF-α in the presence of 0.1% EtOH or 1 µM Prog, MPA or NET-A for 24 h. Total RNA was extracted, cDNA synthesized and expression levels of IL-8 and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least three independent experiments. One-way ANOVA and both Bonferroni (compares all pairs of columns) and Dunnett [compares all columns vs. control (EtOH) column] post-tests were used for statistical analysis. The letters 'a', 'b', 'c', etc., are used to denote statistically significant differences, where all those values which differ significantly from others are assigned a different letter. Statistical significance of differences indicated by *, ** or *** indicates $p<.05$, $p<.01$ or $p<.001$, respectively, whereas no statistical significance ($p>.05$) is indicated by 'ns'.

Interestingly, we also detected low levels of endogenous GR in the untransfected COS-1 cell line, consistent with its ubiquitous expression (reviewed in Ref. [53]). We were unable to detect the presence of the MR by Western blotting in either the Ect1/E6E7 or the Vk2/E6E7 cell line, although the positive control indicated that the anti-MR antibody was

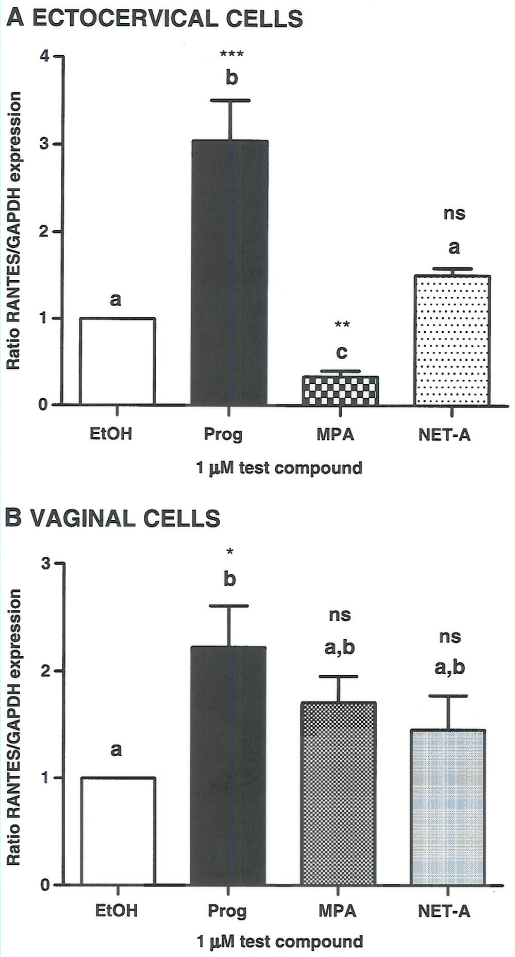

Fig. 3. Effect of MPA and NET-A on the TNF-α-induced expression of the RANTES gene in human ectocervical and vaginal epithelial cell lines. (A) Human Ect1/E6E7 and (B) Vk2/E6E7 cell lines were incubated with 0.02 mcg/mL TNF-α in the presence of 0.1% EtOH or 1 µM Prog, MPA or NET-A for 24 h. Total RNA was extracted, cDNA synthesized and expression levels of RANTES and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least three independent experiments. One-way ANOVA and both Bonferroni (compares all pairs of columns) and Dunnett [compares all columns vs. control (EtOH) column] post-tests were used for statistical analysis. The letters 'a', 'b', 'c', etc., are used to denote statistically significant differences, where all those values which differ significantly from others are assigned a different letter. Statistical significance of differences indicated by *, ** or *** indicates $p<.05$, $p<.01$ or $p<.001$, respectively, whereas no statistical significance ($p>.05$) is indicated by 'ns'.

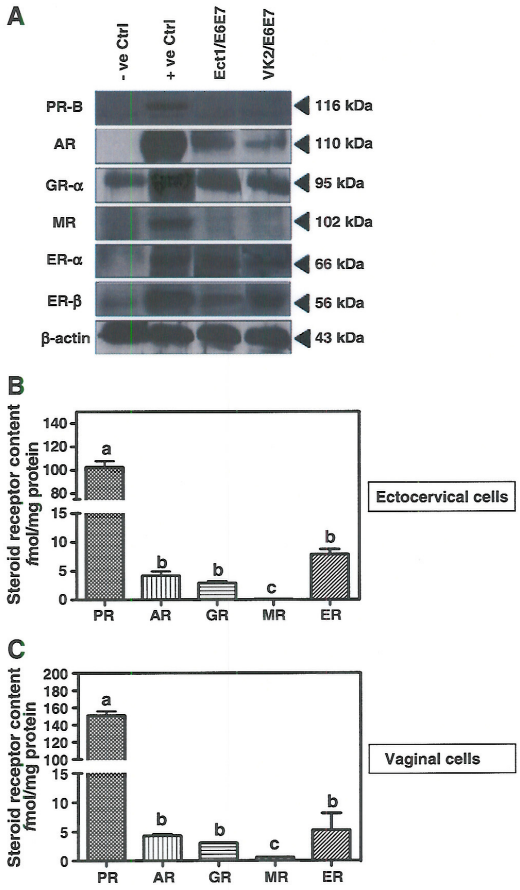

Fig. 4. PR, AR, GR and ER are expressed in human ectocervical and vaginal epithelial cell lines. (A) Whole cell extracts were prepared from human Ect1/E6E7 and Vk2/E6E7 cell lines; untransfected COS-1 cell line [negative control (−ve ctl)]; COS-1 cell line transfected with pSG5hPR-B, pSVARo, pRS-hGRα, pRS-hMR, pSG5-hERα or pSG5-hERβ expression vectors [positive controls (+ve ctl)]. Equal amounts of protein (20 mcg) were analyzed by Western blotting with the PR-B or AR or GR or MR or ERα or ERβ antibodies. (B) Human Ect1/E6E7 and (C) Vk2/E6E7 cell lines were incubated with 10 nM [^{3}H]-R5020 or [^{3}H]-MIB or [^{3}H]-Dex or [^{3}H]-Ald or [^{3}H]-E_2 in the absence (total binding) and presence of 10 μM unlabelled (nonspecific binding) R5020 or MIB or Dex or Ald or E_2, respectively. Specific binding (total binding minus nonspecific binding) is plotted. Results shown are the averages (±SEM) of at least three independent experiments with each condition performed in triplicate. The binding experiment for each receptor with its cognate ligand was performed independent of each other. Statistical significance was determined using a *t* test with a confidence level of .05, and the letters 'a', 'b', 'c', etc., are used to denote statistically significant differences ($p<.05$), where all those values which differ significantly from others are assigned a different letter. Statistical analysis showed the following results: PR vs. AR or GR or MR or ER ($p<.001$); MR vs. AR or GR or ER ($p<.05$); AR vs. GR vs. MR vs. ER ($p>.05$). The number of binding sites in femtomoles per milligram of protein (*f*mol/mg protein) was calculated from the specific activity of the radiolabelled ligands and from the specific binding counts per minute value, as described in the Materials and Methods section.

functional (Fig. 4A). Western blot analysis using antibodies specific for ER-α and ER-β indicated the presence of both isoforms in both cell lines.

Whole cell binding results with tritiated receptor-specific agonists confirmed that endogenous PR, AR, GR and ER are all expressed in both these cell lines (Fig. 4B and C). The results show that there is significantly ($p<.001$) more PR than AR, GR or ER in the Ect1/E6E7 and Vk2/E6E7 cell lines, respectively. Moreover, the levels of AR vs. GR vs. ER are similar to each other in both cell lines ($p>.05$), (Fig. 4B and C). These binding results are consistent with the detection of the GR, AR and ER in both cell lines by Western blotting (Fig. 4A). In contrast, the detection of relatively high amounts of PR in the binding assay appears to contradict the Western blot results. However, it is possible that the cells express the PR-A but not the PR-B isoform, and that the PR-A isoform is responsible for the binding activity observed in Fig. 4B and C. Although we did not detect any MR in these cells lines using Western blotting, the more sensitive whole cell binding assay indicated the expression of relatively very

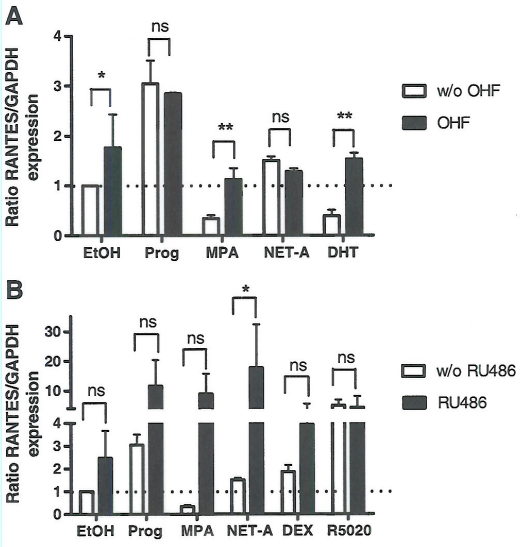

Fig. 5. Effect of the androgen receptor antagonist hydroxyflutamide (OHF) (A) or glucocorticoid/progesterone receptor antagonist (RU486) (B) on the MPA inhibition of the TNF-α-induced RANTES gene. The human Ect1/E6E7 cell line was incubated with 0.02 mcg/mL TNF-α in the presence of 1 μM Prog, MPA, NET-A, DHT, DEX or R5020, and absence or presence of 10 μM (A) OHF or (B) RU486 for 24 h. Total RNA was extracted, cDNA was synthesized and expression levels of RANTES and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least two independent experiments. Open bars are compounds and vehicle only, while the black bars are compounds plus antagonist. Two-way ANOVA and Bonferroni post-test (compares replicate means by row) were used for statistical analysis. Statistical significance of differences is indicated by *, ** or *** to indicate $p<.05$, $p<.01$ or $p<.001$, respectively, whereas no statistical significance ($p>.05$) is indicated by 'ns'.

low amounts of MR in the Vk2/E6E7 cell line (Fig. 4C). Taken together, the results show that the expression of the PR, AR, GR and ER in both the Ect1/E6E7 and Vk2/E6E7 cell lines is consistent with our hypothesis that the differential effects of MPA and NET-A could be a consequence of their action via different steroid receptors.

3.3. Receptor-specific antagonists indicate a role for the AR in the down-regulation of the RANTES pro-inflammatory chemokine gene by MPA in the ectocervical cell line

Having determined that these cell lines express PR, AR, GR and ER, our strategy was to determine which of these steroid receptors mediates the differential effects of MPA and NET-A on the RANTES genes in the Ect1/E6E7 cell line. Although differential effects were also observed for MPA and NET-A on the expression of the IL-8 gene in the Ect1/E6E7 cell line, it was decided to continue the investigation only for the RANTES gene, as MPA and NET-A exhibited differential effects on this gene, as compared not only to each other, but also to Prog. For this reason, Ect1/E6E7 cells were incubated with 0.02 mcg/mL TNF-α and 1 μM Prog, MPA or NET-A, in the absence or presence of 10 μM OHF, the classical androgen antagonist, or RU486, the PR and GR antagonist. As most of the literature indicates that MPA and NET-A do not bind to the ER [54–56], we did not investigate effects in the presence of an ER antagonist.

The addition of OHF prevented the inhibition by MPA, indicating that, in this cell line, the repression by MPA on the RANTES gene is mediated by the AR (Fig. 5A). Consistent with this result, DHT, the natural androgen, also repressed the RANTES gene in this cell line via the AR (Fig. 5A). Interestingly, the addition of OHF did not significantly change the response to Prog or NET-A, indicating that the effects of these compounds on the RANTES gene are not mediated via the AR. A similar experiment with OHF in the Vk2/E6E7 cell line indicated that the AR is not involved in any of the responses by Prog, MPA or NET-A on the RANTES gene (Supplementary Fig. S1).

However, in this cell line, RU486 did not inhibit the effects mediated by the positive controls — dexamethasone (Dex), a synthetic GR agonist, or promegestone (R5020), a synthetic PR agonist (Fig. 5B) — indicating that RU486 does not function as a GR or PR antagonist in the Ect1/E6E7 cell line, but rather as an agonist. This is consistent with previous results showing that RU486 may act as a GR agonist in some cells and as an antagonist in others [57]. The fact that the expression of the RANTES gene is enhanced when RU486 is added together with Prog, MPA or NET-A is

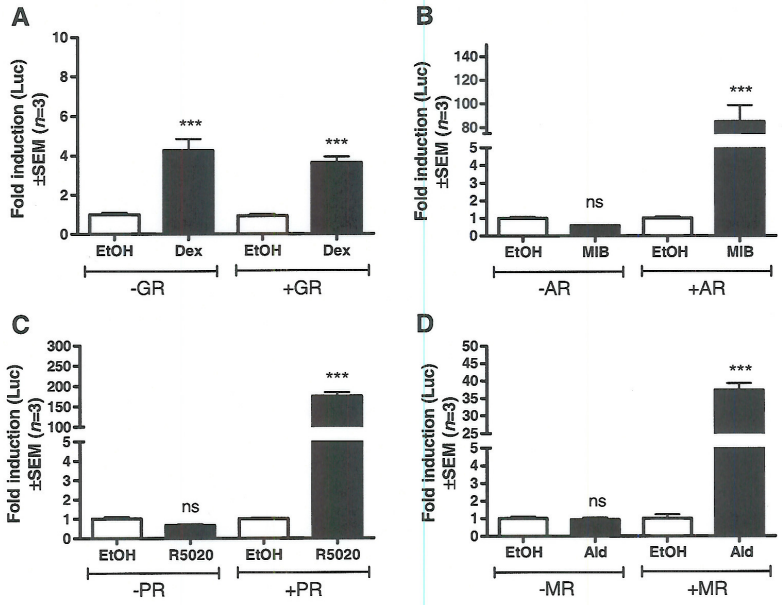

Fig. 6. Transactivation of a transiently transfected GRE-containing promoter–reporter construct via endogenous or expressed GR, AR, PR or MR. Ect1/E6E7 cells (5×10^4 cells per well in a 24-well plate) were transiently transfected with 330 ng pTAT-GRE2-Elb-luc and 33 ng pRS-hGRα, pSVARo, pSG5hPR-B, pRS-hMR or pGL2basic (empty vector control) as indicated, with FuGENE 6 transfection reagent according to the manufacturer's instructions. Twenty-four hours after transfection, cells were induced for 24 h with vehicle (EtOH), 10 μM Dex, MIB, R5020 or Ald (receptor agonists). One-way ANOVA and Dunnett post-test [compares all columns vs. control column (EtOH)] were used for statistical analysis. Statistical significance of differences is indicated by *, ** or *** to indicate $p<.05$, $p<.01$ or $p<.001$, respectively, whereas no statistical significance ($p>.05$) is indicated by 'ns'.

difficult to interpret, but may indicate additive or synergistic effects. Taken together, conclusions cannot be drawn as to the role of the GR or PR in mediating the effects of any of these ligands in this cell line. Using a PR antagonist, like Org31710, which has little anti-glucocorticoid activity compared to RU486 [58], or a GR-specific antagonist like DO6 [59], was not possible since these antagonists are not commercially available. Nevertheless, the results with the AR-specific antagonist showing a complete abrogation of the MPA response on the RANTES gene in the Ect1/E6E7 cell line suggest that the GR, PR, ER and MR are not involved in this MPA response.

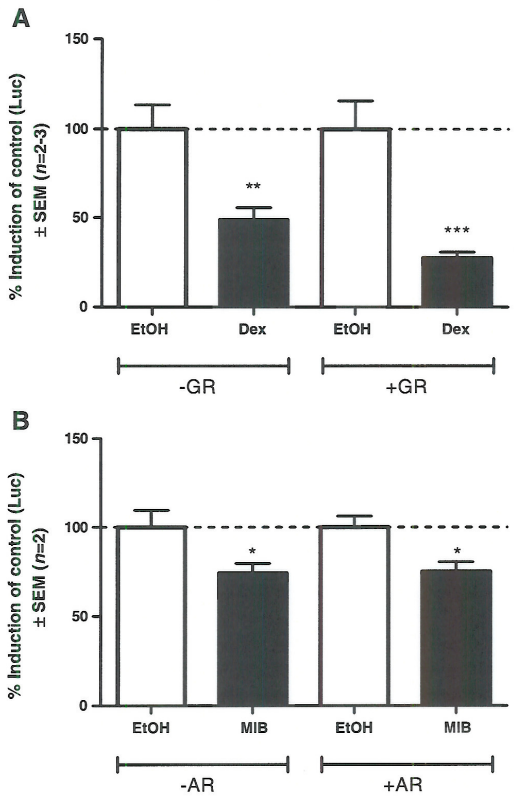

Fig. 7. Transrepression of a transiently transfected NFκB-containing promoter–reporter construct via endogenous or expressed GR or AR. Ect1/E6E7 cells (5×10^4 cells per well in a 24-well plate) were transiently transfected with (A) 100 ng NF-κB-luc, 50 ng pRS-hGRα or pGL2-basic and 25 ng pSV-βgal or with (B) 400 ng NF-κB-luc, 70 ng pSVARo or pGL2-basic and 70 ng pCMV-βgal, with FuGENE 6 transfection reagent according to the manufacturer's instructions. Twenty-four hours after transfection, cells were induced for 24 h with vehicle (0.1% EtOH) or 10 μM Dex or MIB (receptor agonists) and stimulated with 0.02 mcg/mL TNF-α. Percent induction by Dex or MIB, calculated as a percentage of EtOH+TNF-α (set as 100%), is plotted and indicated with dashed lines. Percent (%) repression was calculated by subtracting the induction in the presence of Dex or MIB from induction in the absence of ligand (EtOH=100%). One-way ANOVA and Dunnett post-test [compares all columns vs. control column (EtOH)] were used for statistical analysis. Statistical significance of differences is indicated by *, ** or *** to indicate $p<.05$, $p<.01$ or $p<.001$, respectively, whereas no statistical significance ($p>.05$) is indicated by 'ns'.

3.4. Evidence for steroid receptor functional activity in promoter-reporter assays in both ectocervical and vaginal cell lines

The antagonist results showing that the effects of MPA on RANTES gene expression appear to be mediated predominantly via the AR are surprising given the apparent high PR expression levels based on binding activity, as well as expression of the GR. Both the PR and GR have been shown to have agonist or partial agonist activity, respectively, when bound to MPA [30,34]. We thus investigated by reporter assays whether the AR, PR, GR and MR are transcriptionally active by transiently transfecting the Ect1/E6E7 and Vk2/E6E7 cells with a steroid response element (SRE)-driven reporter construct containing two copies of the rat tyrosine amino transferase (TAT) GRE. The cells were subsequently exposed to 10 μM of the receptor-specific agonist Dex for the GR, MIB for the AR, R5020 for the PR and Ald for the MR. The results show that only the endogenous GR is transcriptionally active on an SRE reporter gene in the Ect1/E6E7 (Fig. 6) and Vk2/E6E7 (Supplementary Fig. S2) cell lines. These results with transfected receptors also show that the agonists used are active in these cell lines. However, results from transrepression experiments via a synthetic NFκB-promoter reporter indicate the presence of transcriptionally active GR and AR in the ectocervical cells (Fig. 7A and B). The detection of this transrepressive activity in the absence of transactivation activity is consistent with previous reports showing that the potency of a ligand for transactivation by the GR via a GRE is less than the potency for transrepression via NFκB [60]. These results suggest that the endogenous AR-mediated transrepressive activity by MPA on the RANTES gene may be mediated via NFκB sites.

4. Discussion

Inflammation of the lower human female genital tract increases susceptibility to viral infections such as HIV [61,62] and human papilloma virus [63]. In addition, excessive release of proinflammatory cytokines may alter the mucosal immune function [64,65]. Thus, understanding factors that may influence the local mucosal immune response, such as endogenous hormones or hormonal contraception, is crucial, especially since the cervicovaginal mucosa is the primary site of HIV-1 infection during male-to-female transmission (reviewed in Ref. [66]). Although MPA and NET-A are widely used in both contraception and HRT, little is known about their effects on the cervicovaginal environment, and whether they interfere with local epithelial immune function. In this study, we investigated the effects of MPA, NET-A and Prog in two immortalized,

morphologically and functionally different epithelial cell lines, one from a normal human ectocervix and the other from a normal human vagina [42]. Although immortalized, the authors report that the morphological and immunocytochemical properties of these cell lines resemble not only their tissues of origin, but also those of primary cultures [42]. Notably, this is not the case for the HeLa cervical cell line, which is probably the most frequently used cell line derived from the human female lower genital tract mucosa [42]. The Ect1/E6E7 and Vk2/E6E7 cell lines express characteristics of stratified squamous nonkeratinizing epithelia and constitutively express IL-8, while IL-6 and RANTES are produced when the cell lines are stimulated with TNF-α [1].

The general trend that was observed for the effect of Prog, MPA and NET-A on endogenous cytokine genes in the cervicovaginal environment is that Prog up-regulates the expression of IL-6, IL-8 and RANTES in both the Ect1/E6E7 and Vk2/E6E7 cell lines, while NET-A either up-regulates these genes (Vk2/E6E7 cells) or has no effect (both cell lines). In contrast, MPA either has no effect (e.g., IL-6 gene in both cell lines) or up-regulates (e.g., IL-8 gene in both cell lines) or down-regulates (e.g., RANTES gene in Ect1/E6E7 cells) the expression of these genes. Interestingly, although MPA substantially and significantly down-regulates the expression of the pro-inflammatory RANTES gene in the Ect1/E6E7 cell line (Fig. 3A), it up-regulates the RANTES gene in the Vk2/E6E7 cell line (Fig. 3B). Thus, the same compound, MPA, exhibits differing effects on the same gene (RANTES), in different cell types, indicating cell-specific effects of MPA. In addition, NET-A has no statistically significant effect on the RANTES gene in both cell types, indicating differential regulation of RANTES by the two synthetic progestins thought to have similar biological activity. Furthermore, Prog shows regulation different to both MPA and NET-A on the RANTES gene in the Ect1/E6E7 cell line (Fig. 3A), as well as on the IL-6 gene in both the Ect1/E6E7 and Vk2/E6E7 cell lines (Fig. 1A and B). Taken together, these results show that cytokine genes in the cervicovaginal environment are regulated in a ligand-specific and cell-specific manner by Prog, MPA and NET-A.

The physiological implications of these results are potentially important. As IL-8 is a chemotactic factor for neutrophils and lymphocytes, the local concentration of IL-8 is important in determining recruitment of neutrophils and lymphocytes. Prog has previously been shown to induce a neutrophil response in the vagina of germ-free mice, even in the absence of bacterial infection [67], indicating a protective role for Prog in the vagina. Thus, our results showing that MPA and NET-A are similar to Prog in up-regulating IL-8, albeit to different extents, in the Ect1/E6E7 and Vk2/E6E7 cell lines, could indicate that these progestins may provide an enhanced local defense mechanism against infections in the vaginal mucosa. Interestingly, unlike Prog which up-regulates the expression of IL-6, MPA and NET-A had no effect on this gene. This may imply that the synthetic progestins would not be as protective as Prog in the cervicovaginal environment. However, it may also indicate that the progestins would not lead to chronic inflammation in this environment, which could be positive in terms of susceptibility to pathogens such as HIV.

In epithelial cells, RANTES recruits immune cells into the reproductive tract, in this manner contributing to inflammation. Inhibition of RANTES by MPA would thus be expected to suppress inflammation, thereby blocking infiltration of immune cells [68]. This lack of recruitment of immune cells into the reproductive tract may have implications for women using MPA. In terms of HIV-1 infection, which has been associated with increased RANTES expression in cervicovaginal secretions [69], these implications may be positive in terms of less HIV infection due to decreased inflammation [64]. However, RANTES also acts as a natural HIV-1 entry inhibitor by competing with HIV-1 for the chemokine receptor CCR5 [70] (reviewed in Ref. [71]). In fact, RANTES peptide mimetics that display anti-HIV-1 activity are currently being developed as an HIV-1 entry inhibitor [72]. The suppression of RANTES by MPA may thus result in reduced competition with HIV-1 for CCR5, thereby allowing entry of HIV-1 into host cells and consequently leading to increased HIV-1 infection. In addition, women using MPA in contraception and HRT are also at risk of other infections, and thus the suppression of RANTES may prevent the mounting of an effective response to combat these infections in the ectocervical environment. Moreover, it is important to remember that the defense function in the cervicovaginal environment is dependent on number of regulatory factors in different anatomical sites, with a constant release of pro- and anti-inflammatory mediators. Thus, the observed effects of MPA on RANTES should be considered in the light of the fact that there may be a balance between pro- and anti-inflammatory molecules produced in the cervicovaginal environment. It would be of interest to investigate the expression of RANTES in cervicovaginal samples from healthy women and HIV-1-infected women using MPA and NET as injectable contraception, as compared to women not using these contraceptives.

We hypothesized that the ligand-specific and cell-specific effects on the regulation of cytokine genes in the cervicovaginal environment are due to ligands acting via different steroid receptors or to differing activities via the same steroid receptor. Using a combination of whole cell binding and Western blotting, we showed that the PR, AR, GR and ER are expressed in both cell lines (Fig. 4A–C). Whole cell binding experiments showed that the amount of GR, AR and ER in the Ect1/E6E7 and Vk2/E6E7 cell lines is similar (Fig. 4B and C). Since the whole cell binding experiments showed the presence of a large amount of PR, one would expect that the progestins would predominantly act via this receptor. However, we did not observe any PR-mediated effects in our study. In fact, the potent synthetic PR agonist, R5020, displayed no activity for transactivation via the PR (Fig. 6C and Supplementary Fig. S2(C)). A possible explanation for this intriguing result may be explained by the presence of different PR isoforms. We were unable to detect the PR-B isoform in any of the cell lines, and

thus we postulate that it is the transcriptionally less active PR-A isoform that is present in these cell lines. Indeed, variation in the expression of PR-A and PR-B has previously been reported in the eutopic endometrium during the menstrual cycle [73], with no PR-B detectable during the secretory and early proliferative phases. Similarly, Attia et al. [74] did not detect any PR-B protein in endometriotic tissue and they ascribe the clinically observed resistance of endometriosis to treatment with progestins to this absence of PR-B and to the presence of PR-A.

We next determined which steroid receptor(s) are involved in the response to MPA on the RANTES gene in the Ect1/E6E7 cell line, by using receptor-specific antagonists. Down-regulation of RANTES gene expression by MPA has previously been reported in endometrial stromal cells [33], and the PR was shown to mediate the effect. In addition, MPA has previously been shown to repress the pro-inflammatory chemokine IL-8 via the GR [31]. As we had shown that the Ect1/E6E7 cell line expresses AR, GR, PR and ER (Fig. 4B), we investigated the expression of the RANTES gene in the presence of OHF, the AR-specific antagonist, as well as RU486, the PR and GR antagonist. We did not investigate a role for the ER as evidence for activity of both MPA and NET-A via the ER is contradictory, with most of the literature indicating that these ligands do not bind to the ER [54,55,75]. The results show that in the Ect1/E6E7 cell line, RU486 behaves as an agonist, rather than as an antagonist (Fig. 5B). RU486 had previously been shown to act as both an agonist and as an antagonist, depending on receptor density [76]. Furthermore, it has been reported that RU486 is a partial agonist for both PR and GR when selective coregulators are recruited [77,78]. This approach of using RU486 as a receptor antagonist in this cell line is thus limited. However, when using the AR-specific antagonist OHF, we showed that the repression of MPA on the RANTES gene was lifted when the AR is antagonised, revealing a possible role for the AR (Fig. 5A). Since we showed that the endogenous AR is transcriptionally active in repression of a TNF-α-stimulated synthetic NFκB-reporter gene in the Ect1/E6E7 cell line (Fig. 7B), and since the RANTES promoter contains two NFκB binding sites which are strongly activated by TNF-α, [79], it seems likely that transrepression by MPA via the AR occurs via these NFκB sites. The fact that OHF could lift the repression of MPA on the RANTES gene to similar levels as the control (EtOH) suggests that the PR, despite being the most abundant receptor, as well as the GR, is not involved in the MPA-mediated down-regulation of the RANTES gene in the Ect1/E6E7 cell line. Consistent with this result, the androgenic properties of MPA have previously been proposed to be responsible for the anti-inflammatory effects of MPA on plasma inflammatory markers, such as C-reactive protein and serum amyloid A protein [80].

Considering that both MPA and NET-A have previously been reported to have androgenic properties [30,39,54–56,75,81–84], it was surprising that NET-A did not act via the AR in this cell line. However, as it is known that NET-A can be metabolized [20], the possibility exists that NET-A is metabolized in this cell line and that the metabolite(s) are less efficacious AR agonists than NET-A itself. In addition, the repression of RANTES by MPA via the AR was not observed in the Vk2/E6E7 cell line, indicating that MPA acts in a cell-specific manner. As the levels of AR in the Ect1/E6E7 and Vk2/E6E7 cell lines are similar, it is unlikely that these differences in responses are due to different levels of steroid receptors. It may be due to other factors such as different extents of metabolism or even different levels of coregulators.

In summary, results from our study are the first to indicate not only differential regulation of the cytokine genes by Prog, MPA and NET-A, but also cell-specific effects. Moreover, we showed that the effect of MPA on the RANTES gene in the Ect1/E6E7 cell line is AR mediated. Further work is necessary to assess the impact of this novel finding. Moreover, the finding that MPA acts differently to NET-A, and does not mimic the effects of Prog, has important implications for women's health.

Finally, the immunoregulatory milieu of the genital mucosa is important in determining the response to antigenic challenge. Mucosal innate immune factors as well as pro-inflammatory cytokines are associated with elevated levels of HIV-1 shedding in the female genital tract [85]. Thus, a greater understanding of the immune responses at these different anatomical locations in the genital tract, and how MPA and NET used as contraceptives and in HRT regulate genes relevant to the cervicovaginal mucosal immune response, may have implications for women's risk of susceptibility to infections. Our results have important implications for choice of progestin [86] in contraception as well as the development of microbicides for the prevention of sexually transmitted diseases.

Supplementary materials related to this article can be found online at doi:10.1016/j.contraception.2011.06.006.

Acknowledgments

We thank Carmen Langeveldt for technical support. This work supported by grants to JPH and DA from the medical Research Council (MRC) and the National Research Foundation (NRF) in South Africa, and Stellenbosch University. Any option, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF does not accept any liability in regard thereto.

References

[1] Fichorova RN, Anderson DJ. Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells. Biol Reprod 1999;60:508–14.
[2] Cummins Jr JE, Doncel GF. Biomarkers of cervicovaginal inflammation for the assessment of microbicide safety. Sex Transm Dis 2009;36 (3 Suppl):S84–91.
[3] Fichorova RN, Desai PJ, Gibson III FC, Genco CA. Distinct proinflammatory host responses to *Neisseria gonorrhoeae* infection

in immortalized human cervical and vaginal epithelial cells. Infect Immun 2001;69:5840–8.
[4] Press MF, Nousek-Goebl NA, Bur M, Greene GL. Estrogen receptor localization in the female genital tract. Am J Pathol 1986;123:280–92.
[5] Kutteh WH, Moldoveanu Z, Mestecky J. Mucosal immunity in the female reproductive tract: correlation of immunoglobulins, cytokines, and reproductive hormones in human cervical mucus around the time of ovulation. AIDS Res Hum Retroviruses 1998;14(Suppl 1):S51–5.
[6] Quayle AJ, Porter EM, Nussbaum AA, Wang YM, Brabec C, Yip KP, et al. Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. Am J Pathol 1998;152:1247–58.
[7] Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. Immunol Rev 2005;206:306–35.
[8] van de Wijgert J, Shattock R. Vaginal microbicides: moving ahead after an unexpected setback. AIDS 2007;21:2369–76.
[9] Kunkel S, Lukacs N, Chensue S, Strieter R, editors. Chemokines and the inflammatory response, 2nd ed. New York, NY: Marcel Dekker Inc; 1997.
[10] Fichorova R. Guiding the vaginal microbicide trials with biomarkers of inflammation. J Acquir Immune Defic Syndr 2004;37(Suppl 3):S184–93.
[11] Borrow P, Shattock RJ, Vyakarnam A. Innate immunity against HIV: a priority target for HIV prevention research. Retrovirology 2010;7:84.
[12] Zara F, Nappi RE, Brerra R, Migliavacca R, Maserati R, Spinillo A. Markers of local immunity in cervico-vaginal secretions of HIV infected women: implications for HIV shedding. Sex Transm Infect 2004;80:108–12.
[13] Bebell LM, Passmore JA, Williamson C, et al. Relationship between levels of inflammatory cytokines in the genital tract and CD4+ cell counts in women with acute HIV-1 infection. J Infect Dis 2008;198:710–4.
[14] Rakasz E, Lynch RG. Female sex hormones as regulatory factors in the vaginal immune compartment. Int Rev Immuno 2002;21:497–513.
[15] Brabin L. Interactions of the female hormonal environment, susceptibility to viral infections, and disease progression. AIDS Patient Care STDs 2002;16:211.
[16] Spinillo A, Capuzzo E, Nicola S, Baltaro F, Ferrari A, Monaco A. The impact of oral contraception on vulvovaginal candidiasis. Contraception 1995;51:293–7.
[17] Geiger AM, Foxman B. Risk factors for vulvovaginal candidiasis: a case-control study among university students. Epidemiology 1996;7:182–7.
[18] Magliani W, Conti S, Salati A, et al. New strategies for treatment of *Candida* vaginal infections. Rev Iberoam Micol 2002;19:144–8.
[19] Affandi B. Long-acting progestogens. Best Pract Res Clin Obstet Gynaecol 2002;16:169–79.
[20] Stanczyk FZ, Roy S. Metabolism of levonorgestrel, norethindrone, and structurally related contraceptive steroids. Contraception 1990;42:67–96.
[21] Mostad SB, Overbaugh J, DeVange DM, Welch MJ, Chohan B, Mandaliya K, et al. Hormonal contraception, vitamin A deficiency, and other risk factors for shedding of HIV-1 infected cells from the cervix and vagina. Lancet 1997;35:922–7.
[22] Mostad SB, Kreiss JK, Ryncarz AJ, et al. Cervical shedding of herpes simplex virus in human immunodeficiency virus-infected women: effects of hormonal contraception, pregnancy, and vitamin A deficiency. J Infect Dis 2000;181:58–63.
[23] Trunova N, Tsai L, Tung S, et al. Progestin-based contraceptive suppresses cellular immune responses in SHIV-infected rhesus macaques. Virology 2006;352:169.
[24] Kaushic C, Ashkar AA, Reid LA, Rosenthal KL. Progesterone increases susceptibility and decreases immune responses to genital herpes infection. J Virol 2003;77:4558–65.
[25] Morrison C, Bright P, Wong E, et al. Hormonal contraceptive use, cervical ectopy, and the acquisition of cervical infections. Sex Transm Dis 2004;31:561–7.
[26] Myer L, Denny L, Wright TC, Kuhn L. Prospective study of hormonal contraception and women's risk of HIV infection in South Africa. Int J Epidemiol 2007;36:166–74.
[27] Kleinschmidt I, Rees H, Delany S, Smith D, Dinat N, Nkala B, et al. Injectable progestin contraceptive use and risk of HIV infection in a South African family planning cohort. Contraception 2007:461–7.
[28] Morrison CS, Chen PL, Kwok C, et al. Hormonal contraception and HIV acquisition: reanalysis using marginal structural modeling. AIDS 2010:1778–81. 10.097/QAD.0b013e32833a2537.
[29] Blish CA, Baeten JM. Hormonal contraception and HIV-1 transmission. Am J Reprod Immunol 2011;65:302–7.
[30] Bamberger CM, Else T, Bamberger AM, Beil FU, Schulte HM. Dissociative glucocorticoid activity of medroxyprogesterone acetate in normal human lymphocytes. J Clin Endocrinol Metab 1999;84:4055–61.
[31] Koubovec D, Berghe WV, Vermeulen L, Haegeman G, Hapgood JP. Medroxyprogesterone acetate downregulates cytokine gene expression in mouse fibroblast cells. Molec Cell Endocrinol 2004;221:75–85.
[32] Ouatas T, Halverson D, Steeg PS. Dexamethasone and medroxyprogesterone acetate elevate Nm23-H1 metastasis suppressor gene expression in metastatic human breast carcinoma cells: new uses for old compounds. Clin Cancer Res 2003;9(10 Pt 1):3763–72.
[33] Zhao D, Lebovic DI, Taylor RN. Long-term progestin treatment inhibits RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) gene expression in human endometrial stromal cells. J Clin Endocrinol Metab 2002;87:2514–9.
[34] Koubovec D, Ronacher K, Stubsrud E, Louw A, Hapgood JP. Synthetic progestins used in HRT have different glucocorticoid agonist properties. Mol Cell Endocrinol 2005;242:23–32.
[35] Ghatge R, Jacobsen B, Schittone S, Horwitz K. The progestational and androgenic properties of medroxyprogesterone acetate: gene regulatory overlap with dihydrotestosterone in breast cancer cells. Breast Cancer Res 2005;7:R1036–50.
[36] Sasagawa S, Shimizu Y, Kami H, Takeuchi T, Mita S, Imada K, et al. Dienogest is a selective progesterone receptor agonist in transactivation analysis with potent oral endometrial activity due to its efficient pharmacokinetic profile. Steroids 2008;73:222–31.
[37] Winneker RC, Bitran D, Zhang Z. The preclinical biology of a new potent and selective progestin: trimegestone. Steroids 2003;68:915–20.
[38] Palacios S, Foidart JM, Genazzani AR. Advances in hormone replacement therapy with drospirenone, a unique progestogen with aldosterone receptor antagonism. Maturitas 2006;55:297–307.
[39] Bentel JM, Birrell SN, Pickering MA, Holds DJ, Horsfall DJ, Tilley WD. Androgen receptor agonist activity of the synthetic progestin, medroxyprogesterone acetate, in human breast cancer cells. Mol Cell Endocrinol 1999;154:11–20.
[40] Philibert D, Bouchoux F, Degryse M, Lecaque D, Petit F, Gaillard M. The pharmacological profile of a novel norpregnance progestin (trimegestone). Gynecol Endocrinol 1999;13:316–26.
[41] Hapgood JP, Koubovec D, Louw A, Africander D. Not all progestins are the same: implications for usage. Trends Pharmacol Sci 2004;25:554–7.
[42] Fichorova RN, Rheinwald JG, Anderson DJ. Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biol Reprod 1997;57:847–55.
[43] Trifonova RT, Doncel GF, Fichorova RN. Polyanionic microbicides modify toll-like receptor-mediated cervicovaginal immune responses. Antimicrob Agents Chemother 2009;53:1490–500.
[44] Freshney RI, John W, Sons I. Culture of animal cells, a manual of basic technique; 1987.
[45] Brinkmann AO, Blok LJ, de Ruiter PE, et al. Mechanisms of androgen receptor activation and function. J Steroid Biochem Mol Biol 1999;69:307–13.
[46] Kastner P, Bocquel MT, Turcotte B, et al. Transient expression of human and chicken progesterone receptors does not support alternative translational initiation from a single mRNA as the mechanism generating two receptor isoforms. J Biol Chem 1990;265:12163–7.
[47] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.

[48] Wolf K, Schulz C, Riegger GA, Pfeifer M. Tumour necrosis factor-alpha induced CD70 and interleukin-7R mRNA expression in BEAS-2B cells. Eur Respir J 2002;20:369–75.
[49] Ishibashi H, Suzuki T, Suzuki S, Moriya T, Kaneko C, Takizawa T, et al. Sex steroid hormone receptors in human thymoma. J Clin Endocrinol Metab 2003;88:2309–17.
[50] Sambrook J, Maniatus T, Fritsch EF, editors. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press; 1989.
[51] Bamberger CM, Bamberger AM, de Castro M, Chrousos GP. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. J Clin Invest 1995;95:2435–41.
[52] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[53] Gross K, Cidlowski J. Tissue-specific glucocorticoid action: a family affair. Trends Endocrinol Metab 2008;19:331–9.
[54] Teulings FA, van Gilse HA, Henkelman MS, Portengen H, Alexieva-Figusch J. Estrogen, androgen, glucocorticoid, and progesterone receptors in progestin-induced regression of human breast cancer. Cancer Res 1980;40:2557–61.
[55] Bergink EW, van Meel F, Turpijn EW, van der Vies J. Binding of progestagens to receptor proteins in MCF-7 cells. J Steroid Biochem 1983;19:1563–70.
[56] Deckers GH, Schoonen WG, Kloosterboer HJ. Influence of the substitution of 11-methylene, delta(15), and/or 18-methyl groups in norethisterone on receptor binding, transactivation assays and biological activities in animals. J Steroid Biochem Mol Biol 2000;74:83–92.
[57] Bamberger CM, Chrousos GP. The glucocorticoid receptor and RU 486 in man. Ann N Y Acad Sci 1995;761:296–310.
[58] Kloosterboer HJ, Deckers GH, Schoonen WG. Pharmacology of two new very selective antiprogestagens: Org 31710 and Org 31806. Hum Reprod 1994;9(Suppl 1):47–52.
[59] Miner JN, Tyree C, Hu J, Berger E, Marschke K, Nakane M, et al. A nonsteroidal glucocorticoid receptor antagonist. Mol Endocrinol 2003;17:117–27.
[60] Ronacher K, Hadley K, Avenant C, Stubsrud E, Simons Jr SS, Louw A, et al. Ligand-selective transactivation and transrepression via the glucocorticoid receptor: role of cofactor interaction. Mol Cell Endocrinol 2009;299:219–31.
[61] Kreiss J, Willerford DM, Hensel M, et al. Association between cervical inflammation and cervical shedding of human immunodeficiency virus DNA. J Infect Dis 1994;170:1597.
[62] Hart CE, Lennox JL, Pratt-Palmore M, Wright TC, Schinazi RF, Evans-Strickfaden T, et al. Correlation of human immunodeficiency virus type 1 RNA levels in blood and the female genital tract. J Infect Dis 1999:179.
[63] Castle PE, Walker JL, Schiffman M, Wheeler CM. Hormonal contraceptive use, pregnancy and parity, and the risk of cervical intraepithelial neoplasia 3 among oncogenic HPV DNA-positive women with equivocal or mildly abnormal cytology. Int J Cancer 2005;117:1007–12.
[64] Cummins JE, Christensen L, Lennox JL, et al. Mucosal innate immune factors in the female genital tract are associated with vaginal HIV-1 shedding independent of plasma viral load. AIDS Res Hum Retroviruses 2006;22:788–95.
[65] Nazli A, Chan O, Dobson-Belaire W, Ouellet M, Tremblay M, Gray-Owen S, et al. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. PLoS Pathog 2010;6:e1000852.
[66] Pope M, Haase AT. Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. Nat Med 2003;9:847–52.
[67] Beaver DL. The hormonal induction of a vaginal leukocytic exudate in the germ-free mouse. Am J Pathol 1960;37:769–73.
[68] Cho ML, Min SY, Chang SH, Kim KW, Heo SB, Lee SH, et al. Transforming growth factor beta 1(TGF-beta1) down-regulates TNFalpha-induced RANTES production in rheumatoid synovial fibroblasts through NF-kappaB-mediated transcriptional repression. Immunol Lett 2006;105:159–66.
[69] Behbahani H, Walther-Jallow L, Klareskog E, Baum L, French AL, Patterson BK, et al. Proinflammatory and type 1 cytokine expression in cervical mucosa during HIV-1 and human papillomavirus infection. J Acquir Immune Defic Syndr 2007;45:9–19.
[70] Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science 1995;270:1811–5.
[71] Vangelista L, Secchi M, Lusso P. Rational design of novel HIV-1 entry inhibitors by RANTES engineering. Vaccine 2008;26:3008–15.
[72] Lusso P, Vangelista L, Cimbro R, Secchi M, Sironi F, Longhi R, et al. Molecular engineering of RANTES peptide mimetics with potent anti-HIV-1 activity. FASEB J 2011;25:1230–43.
[73] Mangal RK, Wiehle RD, Poindexter III AN, Weigel NL. Differential expression of uterine progesterone receptor forms A and B during the menstrual cycle. J Steroid Biochem Mol Biol 1997;63:195–202.
[74] Attia GR, Zeitoun K, Edwards D, Johns A, Carr BR, Bulun SE. Progesterone receptor isoform A but not B is expressed in endometriosis. J Clin Endocrinol Metab 2000;85:2897–902.
[75] Schoonen WG, Deckers GH, de Gooijer ME, de Ries R, Kloosterboer HJ. Hormonal properties of norethisterone, 7alpha-methyl-norethisterone and their derivatives. J Steroid Biochem Mol Biol 2000;74:213–22.
[76] Zhao Q, Pang J, Favata MF, Trzaskos JM. Receptor density dictates the behavior of a subset of steroid ligands in glucocorticoid receptor-mediated transrepression. Int Immunopharmacol 2003;3:1803–17.
[77] Jackson TA, Richer JK, Bain DL, Takimoto GS, Tung L, Horwitz KB. The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. Mol Endocrinol 1997;11:693–705.
[78] Schulz M, Eggert M, Baniahmad A, Dostert A, Heinzel T, Renkawitz R. RU486-induced glucocorticoid receptor agonism is controlled by the receptor N terminus and by corepressor binding. J Biol Chem 2002;277:26238–43.
[79] Moriuchi H, Moriuchi M, Fauci AS. Nuclear factor-kappa B potently up-regulates the promoter activity of RANTES, a chemokine that blocks HIV infection. J Immunol 1997;158:3483–91.
[80] Wakatsuki A, Okatani Y, Ikenoue N, Fukaya T. Effect of medroxyprogesterone acetate on endothelium-dependent vasodilation in postmenopausal women receiving estrogen. Circulation 2001;104:1773–8.
[81] Hackenberg R, Hawighorst T, Filmer A, Nia AH, Schulz KD. Medroxyprogesterone acetate inhibits the proliferation of estrogen- and progesterone-receptor negative MFM-223 human mammary cancer cells via the androgen receptor. Breast Cancer Res Treat 1993;25:217–24.
[82] Perez-Palacios G, Chavez B, Vilchis F, Escobar N, Larrea F, Perez AE. Interaction of medroxyprogesterone acetate with cytosol androgen receptors in the rat hypothalamus and pituitary. J Steroid Biochem 1983;19:1729–35.
[83] Perez-Palacios G, Chavez B, Escobar N, Vilchis F, et al. Mechanism of action of contraceptive synthetic progestins. J Steroid Biochem 1981;15:125–30.
[84] Perez-Palacios G, Fernandez-Aparicio MA, Medina M, Zacarias-Villareal J, Ulloa-Aguirre A. On the mechanism of action of progestins. Acta Endocrinol (Copenh) 1981;97:320–8.
[85] Gumbi PP, Nkwanyana NN, Bere A, et al. Impact of mucosal inflammation on cervical human immunodeficiency virus (HIV-1)-specific CD8 T-cell responses in the female genital tract during chronic HIV infection. J Virol 2008;82:8529–36.
[86] Africander D, Verhoog N, Hapgood JP. Molecular mechanisms of steriod receptor-mediated actions by synthetic progestins used in HRT and contraception. Steriods 2011;76:636–52.