Investigation of differential TNFα–induced interleukin-6 gene regulation by synthetic progestins medroxyprogesterone acetate (MPA) and norethindrone acetate (NET-A) in human endocervical epithelial cells and the role of the unliganded glucocorticoid receptor

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Thesis presented for the degree of
DOCTOR OF PHILOSOPHY
In the Department of Molecular and Cell Biology
University of Cape Town

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May 2010

Declaration

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

Signature……. **Date……..**

Abstract

The endocervical mucosae of the female reproductive tract (FRT) not only serve as a physical barrier against microbial infection, but they also express a wide variety of immune mediators. The endocervical epithelial cells express a distinct profile of immune-regulators, which is higher than vaginal and ectocervical epithelial cells. Constant cytokine production would ensure rapid responses to infections and maintenance of the sterility of the upper genital tract. However, overproduction of cytokines could inhibit normal reproductive processes and stimulate excess growth and cell proliferation. The synthetic progestins medroxyprogesterone acetate (MPA) and norethisterone (NET) and its derivatives (norethisterone enanthate (NET-EN); norethisterone acetate (NET-A)) are synthetic steroidal hormones designed to elicit progestational effects similar to those of the endogenous hormone progesterone (P4). They are extensively used as contraceptives and in hormone replacement therapy (HRT). Numerous studies, however, have reported that synthetic progestins affect immune function, increase the risk of sexually transmitted infections (STIs) and also change the morphology of the cervicovaginal mucosa. Despite these findings little is known about the molecular mechanisms of action of MPA and NET, in particular their differential effects on gene expression. The first part of this thesis examined the regulation of three immune-regulator genes namely interleukin (IL)-6, IL-8, and regulated upon activation, normal T cell expressed and secreted (RANTES), by MPA and NET-A. Quantitative real-time PCR analysis showed that both MPA and NET-A significantly augmented tumour necrosis factor alpha (TNFα)-induced IL-8 and RANTES expression, in contrast to P4. While both MPA and NET-A upregulated the TNFα–response, they did so to different extents. Using siRNA technology, as well as a glucocorticoid receptor (GR) antagonist, it was found that the responses to P4, MPA and NET-A on the IL-6 gene are mediated predominantly via the GR. Towards further understanding the mechanisms of differential regulation via the progestins, different mitogen activated protein kinase (MAPK) pathways were shown to be involved in MPA and NET-A regulation of IL-6 mRNA levels. Both extracellular signal-regulated kinase (ERK1/2) and p38 MAPK pathways are involved in NET-A-induced IL-6 gene expression, whereas ERK1/2 and JNK (c-Jun N-terminal kinase) signalling pathways appear to inhibit MPA-induced IL-6 gene expression.

The second part of this thesis investigated a novel and unexpected mechanism of IL-6 gene regulation involving the GR and TNFα in the absence of GR ligand. GR silencing by siRNA, as well as the presence of the GR antagonist RU486, potentiated TNFα–induced IL-6 gene expression. As shown by chromatin-immunoprecipitation (ChIP) analysis, TNFα also recruited the GR to the IL-6 promoter in intact cells, to the same extent as dexamethasone (DEX), a potent GR agonist. This is the first study to report GR-recruitment to an endogenous cytokine gene promoter in response to TNFα in the absence of GR ligand. Furthermore, it was shown that TNFα selectively induces phosphorylation of the GR at Ser-226 and not Ser-211, consistent with a different mechanism of activation as compared to DEX, which results in phosphorylation at both these residues. Consistent with these results, biochemical fractionation revealed that partial nuclear translocation of the GR was also induced by TNFα. These results strongly suggest that TNFα activates the GR in a ligand-independent manner. Moreover, ChIP as well as overexpression assays support a model in which the cofactor GR-interacting protein type 1 (GRIP-1) is recruited to the IL-6 promoter in response to TNFα in the capacity of co-repressor.

The results of this thesis provide insight into the immune response in the endocervix and the effect of synthetic progestins on the endocervix. Contrasting effects of P4 and progestins are clinically important as MPA and NET are widely used progestins in the regimen of HRT and contraceptives. Moreover, this thesis presents a novel mechanism of ligand-independent GR activation by TNFα and subsequent modulation of IL-6 gene expression, which suggests a role for the unliganded GR in modulating immune function via dampening TNFα responses at the level of gene expression.

I would like to dedicate this thesis to my mother, Olive Verhoog
Mommy, you are my inspiration

Acknowledgements

I would like to thank the following people and institutions, without whom this study would not have been possible. It was a trying experience with many obstacles along the way and with out the support of the following people I would not have come this far.

First and foremost, I would like to thank my supervisor, **Prof Janet Hapgood**, for her enthusiastic approach and dedication to science. I truly learned so much from you. Thank you for allowing me to take root and grow as a scientist and encouraging me to only give my best and to never give up. Your constructive criticism has been invaluable. You are a true inspiration

The NRF- and MRC- of South Africa for the funding of this project and DAAD and the NRF for scholarships.

My parents, my dad, the late **Errol Verhoog** and my mom **Olive**, for all the sacrifices you have made so that I can get the best possible education that was denied to you. Your faith, encouragement, and unconditional love allowed me to continue. I hope I have made you proud. Mommy, you have worked so hard and long to provide me the opportunity to be able to study and pursue my dreams, thank you. Daddy thank you for keeping an eye on me from above, I miss you.

I would like to thank my friends and fellow students in the Hapgood lab. **Kate Hadley**, my rock, without you I would not have come this far. Special thank you for your help with the editing of this thesis I will be eternally grateful for all your help, support, encouraging words and wisdom, your input was invaluable. Your dedication, insight and enthusiasm as a scientist are an inspiration. Most importantly, thank you for your friendship. **Donita Africander**, a wonderful friend and inspirational scientist, your guidance throughout this journey encouraged me to continue during the difficult times. You are one of my main motivations for starting a PhD and I learned such a lot from you. **Andrea Kotitschke**, thank you so much for your encouragement, kindness, and wisdom. I always appreciated your advice and insights. You are an amazing

person and talented scientist. **Chanel Avenant,** your dedication and talent as a scientist is truly inspirational.
I learned so much from all of you. It was a great privilege working with you and thank you for your friendship. It was very comforting knowing I could both laugh and cry in front of you, sharing the good and the bad.

To my family and friends, especially **Lauren**, **Mia**, and **Richenda**, thank you for your support and love. Knowing that I could always count on you when I needed a shoulder to cry on was very comforting.

Wally and **Max**, my four legged friends, for loving me unconditionally. You made the writing of this thesis bearable.

The Lord Almighty, my source of strength, Whom without, this would not have been possible.

I can do all things through Christ, which strengtheneth me

Philippians 4:13

Mother Teresa's *Anyway* Poem

People are often unreasonable, illogical and self centered;
Forgive them anyway.

If you are kind, people may accuse you of selfish, ulterior motives;
Be kind anyway.

If you are successful, you will win some false friends and some true enemies;
Succeed anyway.

If you are honest and frank, people may cheat you;
Be honest and frank anyway.

What you spend years building, someone could destroy overnight;
Build anyway.

If you find serenity and happiness, they may be jealous;
Be happy anyway.

The good you do today, people will often forget tomorrow;
Do good anyway.

Give the world the best you have, and it may never be enough;
Give the world the best you've got anyway.

You see, in the final analysis, it is between you and your God;
It was never between you and them anyway.

List of Abbreviations

AF	Activation function
AIDS	Acquired immune deficiency syndrome
Ald	Aldosterone
ANOVA	Analysis of variance
AP-1	Activating protein-1
AR	Androgen receptor
ARE	Androgen response element
ATF	Activating transcription factor
bp	Base pairs
BPE	Bovine pituitary extract
bZIP	Basic leucine zipper
C/EBP	CCAAT enhancer binding protein
CBP	CREB binding protein
CCR5	C-C chemokine receptor type 5
cDNA	Complementary DNA
CEE	Conjugated equine estrogens
c-FOS	Cellular FOS
c-Jun	Cellular Jun
ChIP	Chromatin immunoprecipitation
CRE	cAMP response element
CREB	cAMP response element binding protein
CTD	Carboxy-terminal domain
DBD	DNA binding domain
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
DHT	Dihydrotestosterone
DMPA	Depo medroxyprogesterone acetate
DNA	Deoxyribonucleic acid

DUSP	Dual specific (Thr/Tyr) phosphatase
E2	17-β-estradiol
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
ERα	Estrogen receptor alpha
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FSH	Follicule-stimulating hormone
FRT	Female reproductive tract
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GILZ	Glucocorticoid-induced leucine zipper
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid Response Element
GRIP-1	GR-interacting protein type 1
GST	Glutathione-S-transferase
H3	Histone 3
HDAC	Histone deacetylase
HIV	Human immune deficiency virus
HPV	Human papilloma virus
HRP	Horseradish peroxidase
hrs	Hrs
HRT	Hormone replacement therapy
HSV	Herpes simplex virus
IGF	Insulin-like growth factor
IκB	Inhibitory-kappa B
IKK	IκB kinase
IL-1β	Interleukin-1β
IL-6	Interleukin-6

IL-8	Interleukin-8
IRF	Interferon regulatory factor
Jak	Janus kinase
Jak/STAT	Janus kinase-signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
K_d	Dissociation binding constant
KSF	Keratinocyte serum free
LBD	Ligand binding domain
LH	Luteinizing hormone
LPS	Lipoploysaccharide
MAPK	Mitogen activated protein kinase
MEKK1	Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1
MHC	Major histocompatibility complex
MIB	Mibolerone
MKP-1	MAPK phosphatase-1
MPA	Medroxyprogesterone acetate
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NET	Norethisterone
NET-A	Norethisterone acetate
NET-EN	Norethisterone enathate
NFκB	Nuclear factor kappa B
NIK	NFκB-inducing kinase
NK	Natural killer
OHFL	Hydroxyflutamide
P4	Progesterone
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered Saline
PCR	Polymerase chain reaction

Penstrep	Penicillin/streptomycin
PI3K	Phosphatidylinositol-3'kinase
PKA	Cyclic AMP-dependent protein kinase
PKC	Protein kinase C
PMA	4α-phorbol 12-myristate 13-acetate
Pol II	Polymerase II
PR	Progesterone receptor
P-TEFb	Transcription elongation factor b
RANTES	Regulated upon activation, normal T cell expressed and secreted
RIP1	Receptor-interacting protein 1
Ser	Serine
siRNA	Small interference RNA
SOCS	Suppressors of cytokine signalling
SRC	Steroid receptor co-activator
SRE	Steroid response elements
STAT	Signal transducer and activator of transcription
STI	Sexually transmitted infection
TBS	Tris buffered saline
TBS-T	Tris buffered saline-tween
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRADD	TNFα receptor 1- associated death domain protein
TRAF2	TNFα receptor associated factor 2
WHI	World health initiative

Thesis outline

This thesis consists of five chapters. Chapters 3 and 4 are written up in manuscript format and will shortly be submitted for publication. The "Material and Methods" and "Reference" sections were excluded from Chapters 3 and 4 to prevent unnecessary repetition. A combined "Reference" section for all the chapters follows after the "Addendums".

Chapter 1: **Literature review.** This chapter gives a detailed overview of the relevant knowledge currently available in the literature, with a particular focus on the immune response pertaining to the lower female reproductive tract and the molecular mechanisms of action of medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN)/norethisterone acetate (NET-A).

Chapter 2: **Materials and Methods.** This chapter gives details regarding the experimental protocols and materials used to obtain the results presented in chapters 3 to 5.

Chapter 3: **Differential cytokine gene regulation by the synthetic progestins, medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A) in a human endocervical epithelial cell line**. This chapter contains the results of a study investigating the mechanisms of regulation of endogenous cytokine genes by Prog, MPA and NET-A in the End1/E6E7 (human endocervical epithelial) cell line. All experiments were performed by the candidate and the manuscript is in preparation for submission to the journal "Contraception". A brief introduction is included.

Chapter 4: **Ligand-independent GR-mediated repression of IL-6 in response to tumour necrosis factor-alpha (TNFα) in an endocervical epithelial cell line.** This chapter contains the results of a study investigating the ligand-independent activation of the GR by TNFα in the End1/E6E7 cell line and the subsequent modulation of IL-6 expression. All experiments were performed by the candidate and the manuscript is in preparation for submission to the journal "Molecular Endocrinology". A brief introduction is included.

Chapter 5: **Conclusions and Future Perspectives.** In this final chapter, the results of the overall study are discussed and conclusions drawn in the context of the larger body of work.

The appendices include (A) optimisation of various experimental protocols and (B) data not shown, but referred to as supplementary material within Chapter 3.

Table of Contents

Acknowledgements **iv**

List of Abbreviations **……...ix**

Thesis Outline **……xiii**

Chapter One
Literature Review……1

Introduction.....1

1.1. The female reproductive tract (FRT).....1
1.1.1 The lower female reproductive tract.....2
1.1.1.1 Vagina and ectocervix.....2
1.1.1.2 Endocervix.....3
1.1.2 Immunity in the lower FRT.....4
1.1.3 Cytokine and chemokine expression in the lower FRT.....5
1.1.3.1 Interleukin-6.....6
1.1.3.2 Interleukin-8 and RANTES.....8

1.2 Important signalling pathways involved in immunity.....10
1.2.1 TNF alpha (TNFα) signalling.....10
1.2.2 AP-1 signalling.....13
1.2.3 NF-κB signalling.....14

1.3 Progesterone and the synthetic progestins Medroxyprogesterone acetate & Norethisterone enanthate/acetate.....15
1.3.1 The natural hormone-Progesterone.....15
1.3.2 Medroxyprogesterone acetate & Norethisterone enanthate/acetate: Synthetic progestins.....16
1.3.2.1 Mode of contraceptive action.....18
1.3.2.2 HRT and other applications.....18
1.3.2.2.1 MPA and NET in HRT……...…...18
1.3.2.2.2 High dosage progestin therapy…… 19

1.4 Effect of synthetic progestins on immunity.....19

1.5 Molecular mechanism of progestin signalling.....22
1.5.1 Steroid receptor signalling.....22
1.5.2 Interaction of P4 and synthetic progestins with steroid receptors.....25

1.6. Glucocorticoid receptor-mediated repression of immune-regulators.....27
1.6.1 Direct interaction of the GR with pro-inflammatory transcription factors.....28
1.6.1.1 Co-factors involved in transrepression.....30
1.6.1.2 The effect of tethered GR on basal transcription machinery.....31
1.6.2 Co-factor competition model.....31
1.6.3 Indirect repression by GR.....32

1.6.3.1 Cross-talk with signalling pathways 32
1.6.3.2 Increased transcription of anti-inflammatory genes 33

1.7 Conclusion 38

1.8 Aims of thesis 39

Chapter Two
Materials and Methods…….. 40

2.1 Cell culture 40

2.2 Test compounds and antibodies 41

2.3 Plasmids 42

2.4 Transient transfections 42

2.5 Promoter reporter luciferase assays 43

2.6 Small interference RNA (siRNA) transfections 43

2.7 RNA isolation and cDNA synthesis 44

2.8 Real time PCR 45

2.9 Chromatin immunoprecipitation assay 46
2.9.1 Treatment of End1/E6E7 cells 46
2.9.2 Formaldehyde crosslinking 46
2.9.3 Preparation of the sonicated cell lysate for immunoprecipitation 47
2.9.4 Isolation and purification of DNA associated with the immunoprecipitated protein 48
2.9.5 Analysis of DNA coupled with the immunoprecipitated protein 48

2.10 Subcellular nuclear fractionation assay 49

2.11 Western blot analysis 50

2.12 Data and statistical analysis 51

Chapter Three
Results and Discussion

Differential cytokine gene regulation by the synthetic progestins, medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A) in a human endocervical epithelial cell line …… 52

Abstract **52**
Introduction **53**
Results **56**
Discussion **72**

Chapter Four
Results and Discussion

Ligand-independent GR-mediated repression of IL-6 in response to tumour necrosis factor-alpha (TNFα) in an endocervical epithelial cell line……..85

Abstract **85**
Introduction **86**
Results **88**
Discussion **100**

Chapter Five
Conclusion and Future Perspectives
Introduction **108**
Regulation of pro-inflammatory cytokine genes by the natural hormone P4 and the synthetic progestins MPA and NET-A **109**
Ligand-independent activation of the GR modulates IL-6 expression in response to TNFα **113**

Addendum A
A.1: Quantitative real time PCR (qPCR) optimisation **119**
A.2. ChIP assay optimisation **122**

Addendum B
Supplementary results pertaining to Chapter 3……125

List of References……127

CHAPTER ONE

LITERATURE REVIEW

Introduction

Sexually transmitted infections (STIs) are a worldwide health problem (Brabin 2002). Pathogens such as *Chlamydia trachomatis, Neisseria gonorrhoeae Candida albicans* and *Trichomona vaginalis* as well as Human papillomavirus (HPV) are responsible for most sexually transmitted diseases, and these pathogens increase susceptibility to Human immunodeficiency virus (HIV) infection. HIV infection and Acquired immune deficiency syndrome (AIDS) are most prominent in sub-Saharan Africa, and present a serious burden to the health sector as well as the economy (Wira *et al.*, 2005a). An understanding of the local immune response induced in the lower female reproductive tract (FRT) is therefore important, as this region is the first site of exposure to such pathogens in women. Epithelial cells of the FRT play a vital role in preventing infection and are influenced by hormonal treatment such as contraceptives and hormone replacement therapy (HRT). While a few reports regarding the immune response in the FRT are available, there still remain many unanswered questions, particularly regarding the molecular mechanisms involved.

This chapter provides an overview of the human FRT, specifically the anatomical compartments which include the vagina, ectocervix and endocervix, as well as the role of epithelial cells in the immune response. A brief outline of innate and adaptive immune responses will be given, followed by signalling pathways required for the immune response.

1.1. The female reproductive tract (FRT)

Epithelial cells lining the FRT are the first line of defence against STI's and are generally considered to be the gatekeepers of the FRT. The epithelial surface of the FRT not only provides an uninterrupted physical barrier against infection, but is also capable of both innate and adaptive immune responses against invading pathogens (Wira *et al.*, 2005a; Ochiel *et al.*, 2008; Schaefer *et al.*, 2005). The epithelial cells lining the FRT are capable of producing chemokines and cytokines that activate and recruit immune cells such as macrophages, natural killer (NK) cells, neutrophils and B-cells,

followed by the attraction and maturation of T-cells (Wira *et al.*, 2005a; Ochiel *et al.*, 2008; Schaefer *et al.*, 2005). In addition, epithelial cells are able to secrete anti-microbial peptides, such as defensins, that provide protection against invading microbes (Wira *et al.*, 2005a; Ochiel *et al.*, 2008; Schaefer *et al.*, 2005).

The FRT comprises different anatomical compartments, namely the ovaries, fallopian tubes, uterus, cervix, and vagina. The lower FRT consists of three regions: the ectocervical, endocervical and vaginal mucosa (Figure 1.1). The mainly sterile endocervical mucosa is separated from the ectocervix by the transformation zone. The vaginal and ectocervical musoca are usually exposed to a wide variety of microorganisms, and is thus not as sterile as the endocervix.

1.1.1 The lower female reproductive tract

1.1.1.1 Vagina and ectocervix

The vagina is the entry to the FRT and both the vaginal and ectocervical mucosae consist of aglandular, non-keratinised, stratified, squamous epithelial cells, which comprise multiple layers (Fichorova *et al.*, 1997). The vagina is commonly infected by *C. albicans* and *T. vaginalis* (Quayle, 2002), while HPV generally infects the ectocervix or more commonly the transformation zone separating the ecto-and endocervix (Quayle, 2002). Commensal microorganisms such as the *Lactobacillus* and *Enterococcus* species are present in the vaginal- and ectocervical mucosae and assist in vaginal defence (Hillier *et al.*, 1993; Reid *et al.*, 2001). *Lactobacilli* do this by restricting the vaginal flora allowing only commensals that grow in the acidic environment. This species also produce hydrogen peroxide that has anti-microbial activity (Hillier *et al.*, 1993; Reid *et al.*, 2001). The ability of certain micro-organisms to lower the pH is particularly important in limiting the infection of the FRT by *C. albicans* as it cannot grow at low pH conditions (Mahmoud *et al.*, 1995). The presence of commensal microorganisms in the vagina and ectocervix is probably due to the fact that both elicit a weak immune response. Consistent with this finding, immortalised vaginal and ectovaginal epithelial cell lines have been shown to have low basal and tumour necrosis factor-alpha (TNFα) and interleukin (IL)-1β stimulated immuno-regulator concentrations (Fichorova & Anderson, 1999).

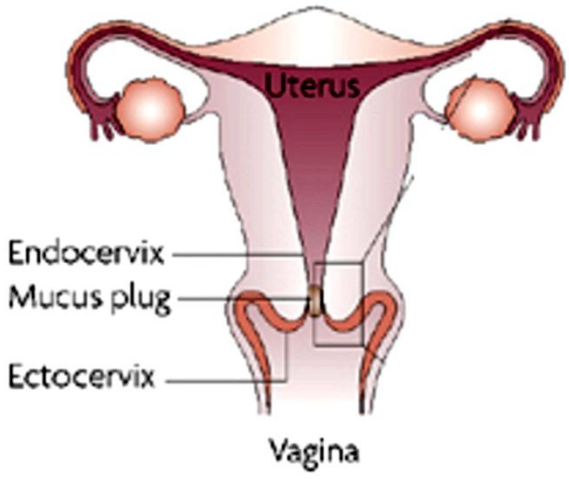


Figure 1.1: Schematic presentation of the lower female reproductive tract (Hladik & McElrath, 2008)

1.1.1.2 Endocervix

The epithelium of the endocervix consists of simple columnar epithelial cells with numerous glands, similar to that of the uterus and fallopian tubes (Fichorova *et al.,* 1997; Wira *et al.,* 2005a), unlike the ectocervix and vagina. The endocervix separates the sterile uterus from the microbe-filled lower FRT. The sterility status of the endocervix is dependent on hormone levels influenced by the menstrual cycle (Quayle, 2002). As the epithelium in this region consists of only a single layer of cells, the endocervix is more vulnerable to microbial infection and tissue injury than other regions of the FRT. *N. gonorrhoeae* and *C. trichomatis* are some of the main pathogens infecting the endocervix, with *C. trichomatis* being the most common cause of sexually transmitted diseases (Fenton *et al.,* 2001). It has been reported that chlymadial infection is more prevalent in women using oral contraceptives than those using barrier methods or non-users of contraception (Shafer *et al.,* 1984). In terms of HIV the exact site of infection remains unclear. However, it is believed to occur at epithelial cells of the lower female reproductive tract (al-Harthi & Landay, 2001), and indeed HIV has been isolated from the endocervix of HIV-infected women (Zhang *et al.,* 1999; Kaul *et al.,* 2000; Mostad *et al.,* 1997; Zorr *et al.,* 1994).

The endocervix, has been shown to have a higher constitutive and TNFα– and IL-1β–induced immune-regulator profile than the vagina and ectocervix, as well as

distinctly different immunological mediators (Fichorova & Anderson 1999; Castrilli *et al.,* 1997; Fahey *et al.,* 2005). This means that cytokines are constantly produced, which would ensure rapid responses to infections and maintenance of the sterility of the upper reproductive tract.

1.1.2 Immunity in the lower FRT

Mucosal immunity in the lower FRT is very similar to that found in the respiratory tract and intestine (Wira *et al.,* 2005a; Wira *et al.,* 2005b; MasCasullo *et al.,* 2005). The lower FRT lining, which consists mainly of mucus-secreting epithelial cells, forms a mucosal barrier protecting against invading pathogens. Tight junctions between columnar epithelial cells prevent transepithelial movement (Wira *et al.,* 2005a). The lower FRT is capable of both innate and acquired immune responses that are under hormonal control (Grossman 1984; Beagley & Gockel 2003; Franklin & Kutteh 1999; Lü *et al.,* 2002; Rakasz & Lynch 2002, Thongngarm *et al.,* 2003). These responses ultimately regulate the levels of cytokines, the distribution of different cell populations and the transport of immunoglobulins (Wira *et al.,* 2005a; Wira, *et al.,* 2005b), allowing the acquired immunity in the FRT to act in concert with the innate immune response (Wira *et al.,* 2005a; Wira *et al.,* 2005b).

Acquired immunity entails pathogen-specific defence responses, such as presentation of pathogenic antigens to T-cells by antigen-presenting cells, resulting in T-cell activation (indirect effect) (Wira et al., 2005a; Wira et al., 2005b). T-cell activation leads to cytokine production and subsequent antibody production and activation (Wira, et al., 2005a). Antibody production occurs via B-cell activation (humoral immunity). The immunoglobulin (Ig) G is the principal antibody secreted in the FRT (Wira et al., 2005a; Wira, et al., 2005). In addition, T-cells themselves are also capable of directly (cell mediated immunity) mediating an immune response against pathogens together with other immune cells e.g. macrophages, neutrophils etc.

Innate immunity involves mostly macrophages, dendritic cells, neutrophils and epithelial cells and as it is not antigen specific it elicits a much quicker response than acquired immunity (Janeway & Medzhitov 2002; Medzhitov & Janeway 2000; Quayle 2002). The epithelial cells of the FRT also release antimicrobials such as mucus, defensins, lactoferrin, and lysozyme that assist in the innate response. Secreted mucus

consists mainly of large mucin glycoproteins, which physically prevent microbial entrance and provide a medium for antibodies, peptides, proteins and organic molecules (Lamont, 1992). The presence of a wide variety of immune cells in the lower FRT has been identified. These include lymphocytes and antigen-presenting cells such as macrophages and dendritic cells (Wira *et al.,* 2005a; Wira *et al.,* 2005b). Additionally, Toll like receptors (TLRs), which are expressed mainly by macrophages and dendritic cells but also by vaginal, ectocervical and endocervical epithelial cells, are able to recognise pathogen proteins, or molecules such as lipoproteins, peptidoglycans, and repetitive protein structures (Fichorova *et al.,* 2002). Furthermore, the epithelial cells of the lower FRT have been reported to present antigen to T cells, an immune response under strict control by hormones such as estrogen and progesterone (De Buysscher, 1999; Prabhala & Wira, 1995). Both immortalised and primary vaginal, ectocervical and endocervical epithelial cells have also been shown to express a wide variety of cytokines and chemokines (Fahey *et al.,* 2005; Fichorova & Anderson, 1999). Differential cytokine/chemokine expression was observed between the endocervical epithelial cell lines when compared to the ectocervical and vaginal epithelial cell lines (Fichorova & Anderson, 1999). These immuno-regulators play a vital role in the immune response, by protecting against invading pathogens. Taken together, it is clear that epithelial cells play an important role in both innate and acquired immune responses in the FRT as they express TLRs on their surface and produce microbiocides as well as secreting immunoglobulins and immuno-regulatory chemokines and cytokines (Wira *et al.*, 2005b; De Buysscher, 1999).

1.1.3 Cytokine and chemokine expression in the lower FRT

Numerous cytokines and chemokines are constitutively expressed by the epithelial cells in the female reproductive tract e.g. interleukin (IL)-6, IL-8 and Regulated upon activation, normal T cell expressed and secreted (RANTES) (Fahey *et al.,* 2005; Fichorova & Anderson, 1999). Constitutive expression ensures the presence and activity of immune cells immediately on antigen presentation. In addition, immune-regulators produced by epithelial cells may also regulate the number and type of immune cells present in the FRT (Givan *et al*., 1997). Chemokines such as IL-8 and RANTES are mostly responsible for the attraction of immune cells (Baggiolini 1995; Kuna *et al.,* 1992; Kameyoshi *et al.,* 1992; Schall *et al.,* 1990), while cytokines like IL-6 are mainly involved in the activation and differentiation of T- and B-cells (Hodge *et*

al., 2005; Akira *et al.,* 1990; Akira *et al.,* 1992; Kishimoto, 2006). Some immune-regulators also influence cell proliferation and apoptosis, which may assist in maintaining normal FRT structure and surroundings (Wira *et al.,* 2005a; Wira *et al.,* 2005b). Cytokines and chemokines can also regulate each other by autocrine or paracrine mechanisms involving gene expression (Wira *et al.,* 2005b; Legrand-poels *et al.,* 2000; Shalaby *et al.,* 1989). Moreover, sex hormones have been shown to control the expression of many immuno-regulators (Critchley *et al.,* 2001). For example, progesterone (P4) withdrawal, as experienced during the follicular phase, has been implicated in the upregulation of IL-8 (Critchley *et al.,* 2001). In the next sections, immune-regulators IL-6, IL-8, and RANTES will be discussed in more detail.

1.1.3.1 Interleukin-6

IL-6 is a multifunctional pleiotropic glycoprotein, the most significant role of which is that of a pro-inflammatory mediator (Kishimoto, 1989). Some of its pro-inflammatory roles include B-cell differentiation, leukocytosis and acute phase protein synthesis and T-cell lymphocyte activation (Kishimoto, 1989). Moreover, IL-6 has been shown to be anti-apoptotic and induce proliferation of numerous cell types including tumour cells, by both autocrine and paracrine mechanisms (Wei *et al.,* 2001a; Wei *et al.,* 2001b; Eustace *et al.,* 1993; Kawano *et al.,* 1988; Okamoto *et al.,* 1997a; Okamoto *et al.,* 1997b; Iglesias *et al.,* 1995). For example, IL-6 has been implicated in cervical cancer progression and pathogenesis (Tartour *et al.,* 1994; Eustace *et al.,* 1993; Scambia *et al.,* 1994; Wei *et al.,* 2001a; Wei *et al.,* 2001b; Wei *et al.,* 2003; Tjiong *et al.,* 1999) due to chronic inflammation during infection (Richter *et al.,* 1999). In contrast, IL-6 has also been reported to inhibit cell proliferation of lung (Takizawa *et al.,* 1993) and breast cancer cells (Danforth & Sgagias, 1993; Klein *et al.,* 1995). IL-6 has been associated with many autoimmune and inflammatory diseases such as pelvic inflammatory disease (Richter *et al.,* 1999) and rheumatoid arthritis (Okamoto *et al.,* 1997b; Mihara *et al.,* 1995). Experimentally, IL-6 treatment induces collagen-induced arthritis (Alonzi *et al.,* 1998; Sasai *et al.,* 1999) as well as antigen-induced arthritis (Ohshima *et al.,* 1998) consistent with its pro-inflammatory effects.

IL-6 signalling entails binding of IL-6 to the α-chain of the IL-6 receptor, thereby recruiting the receptor β-chain (gp130). This in turn initiates homodimerization of gp130, which activates cytoplasmic tyrosine kinases. Consequently, various signalling

cascades including Janus kinase-signal transducer and activator of transcription (Jak/STAT), phosphatidyl inositol-3 kinase (PI3K)/protein kinase B (Akt) and Ras/Mitogen activated pathways kinase (MAPK) pathways are activated (Hirano *et al.,* 1997).

Regulation of IL-6 primarily occurs at the level of gene transcription and its expression is highly responsive to the early-response pro-inflammatory cytokines IL-1β and TNFα, (Benveniste *et al.,* 1990; Cromwell *et al.,* 1992; Iglesias *et al.,* 1995; Beyaert *et al.,* 1996; Zhang *et al.,* 1990). The promoter of IL-6 contains binding sites for several inducible transcription factors necessary for its induction by a variety of stimuli. These sites are situated in close proximity to each other as schematically represented in Figure 1.2A and include a nuclear factor-kappa (NFκB) binding element between positions –73 and –63, a CCAAT enhancer binding protein (C/EBPβ) binding site at position –173 and –145 and an activator protein-1 (AP-1) binding site located between –283 and –277, relative to the transcription start site (Ray *et al.,* 1988; Georganas *et al.,* 2000; Tanabe *et al.,* 1988; Libermann & Baltimore, 1990). The NFκB binding site binds protein dimers of the Rel/NFκB family of transcription factors, the C/EBPβ binding site binds protein dimers of the C/EBP basic-leuzine zipper family, while the distal AP-1 site interacts with protein dimers of the Fos and Jun families (Lee *et al.,* 1987). These *cis*-acting transcription factors all play a functional role in the regulation of the IL-6 promoter (Vanden Berghe *et al*., 1998). The transcription factor NFκB, the binding site of which is the most proximal of all the above-mentioned regulatory elements, plays a dominant role in IL-6 expression, and is responsible for basal IL-6 expression in various cell types (Vanden Berghe *et al.,* 1998; Zhang *et al.,* 1990; Shimizu *et al.,* 1990). Studies using promoter mutations and deletion mutants showed that the NFκB binding sequence is required for IL-6 promoter activity (Vanden Berghe *et al.,* 1998), while AP-1 and C/EBP binding sites are only required for maximal IL-6 promoter activity *(*Vanden Berghe *et al.,* 1998). In addition, the MAPKs, p38 and extracellular signal-regulated kinase (ERK1/2) have been implicated in the induction of IL-6 expression by TNFα and IL-1β (Blanque *et al.,* 1997; Ridley *et al.,* 1997; Miyazawa *et al.,* 1998; Vanden Berghe *et al.,* 1998; Wery-zennaro *et al.,* 2000). In contrast, steroids acting via their cognate receptors have been shown to repress IL-6 expression in a wide variety of cell

types (Bellido *et al.,* 1995; Kurebayashi *et al.,* 1997; Waage *et al.,* 1990; Koubovec *et al.,* 2004). Repression by steroid receptors is mainly due to their direct protein-protein interactions with NFκB and AP-1 transcription factors, which will be discussed later in this chapter. Interestingly, a negative feedback mechanism whereby IL-6 overproduction is prevented has previously been reported (Kishimoto, 2006). In this feedback process, IL-6 acting via its cognate receptor activates the expression of STAT3, which in turn activates the suppressors of cytokine signalling (SOCS). The IL-6 receptor gp130 β chain is subsequently inhibited by SOCS binding to the JAK tyrosine kinase (Kishimoto, 2006).

1.1.3.2 Interleukin-8 and RANTES

IL-8 and RANTES are both potent chemoattractant cytokines, although they belong to different chemokine families. IL-8 belongs to the CXC-chemokine family, while RANTES is a member of the CC chemokine family (Baggiolini, 2000; Song *et al.,* 2002). Similar to IL-6, both RANTES and IL-8 promoters contain a small region with similar *cis*-regulatory elements (Figure 1.2B & 1.2C). Both the RANTES- and IL-8 promoters contain a proximal NFκB binding site but unlike IL-8, RANTES contains two NFκB binding sites situated next to each other (Moriuchi *et al.,* 1997; Hiura *et al.,* 1999; Henriquet *et al.,* 2007). Both chemokine genes contain the *cis*-regulator element for C/EBPβ (Song *et al.,* 2000; Mukaida *et al.,* 1994) and also AP-1 binding sites. However, the RANTES promoter contains two distal AP-1 binding sites, while the IL-8 promoter contains only one (Moriuchi *et al.,* 1997; Henriquet *et al.,* 2007; Song *et al.,* 2000; Mukaida *et al.,* 1994). Similarly, to the IL-6 gene, the NFκB binding site plays a dominant role in both IL-8 and RANTES expression in a wide variety of cell types (Brasier *et al.,* 1998; Hoffmann *et al.,* 2002; Lebovic *et al.,* 2001; Melchjorsen & Paludan, 2003; Moriuchi *et al.,* 1997; Hiura *et al.,* 1999), and has been shown in numerous studies to be strongly activated by TNFα (Cromwell *et al.,* 1992; Brasier *et al.,* 1998). Brasier and colleagues demonstrated that NFκB recruitment to the IL-8 promoter in alveolar epithelial cells is required for IL-8 expression as it is the first transcription factor to associate with the IL-8 promoter on TNFα stimulation (Brasier *et al*., 1998). C/EBPβ, the binding site of which is situated in close proximity to the NFκB binding site, has been proposed to form a dimer with NFκB, and these two transcription factors thereby cooperatively affect transcription of IL-8 (Mukaida *et al.,* 1990; Kunsch

& Rosen, 1993). Like IL-6 and most other immune-regulators, inhibition of IL-8 and RANTES expression can be influenced by steroid hormones. Glucocorticoids, acting via the glucocorticoid receptor (GR) are particularly potent inhibitors of IL-8 and RANTES expression (Kelly *et al.*, 1994; Chang *et al.*, 2001; Wingett *et al.*, 1996).

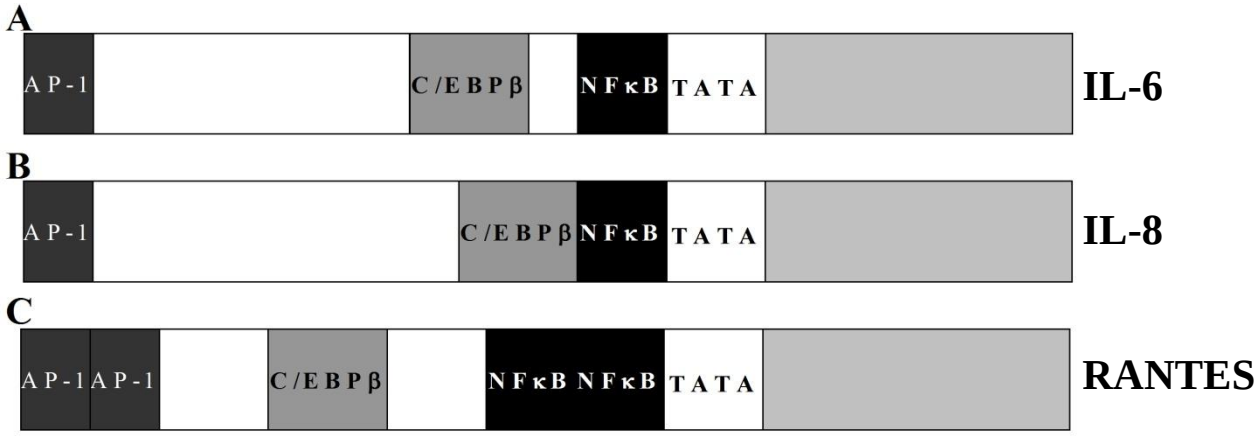


Figure 1.2. Schematic representation of (A) IL-6, (B) IL-8, and (C) RANTES promoters.
Abbreviations: AP-1; activating protein-1, C/EBPβ; CCAAT enhancer binding protein, NFκB; nuclear factor kappa B; IL-6; interleukin-6, IL-8; interleukin-8, RANTES; Regulated upon activation, normal T cell expressed and secreted.

RANTES and IL-8 have some similarity in their recruitment profiles of immune cells as both have been shown to recruit neutrophils (Larsen *et al.*, 1989; Pan *et al.*, 2000; Kunkel *et al.*, 1991) eosinophils (Kameyoshi *et al.*, 1992; Sehmi *et al.*, 1993), T lymphocytes (Kameyoshi *et al.*, 1992; Schall *et al.*, 1990b), basophils (Bischoff *et al.*, 1993; Krieger *et al.*, 1992), monocytes (Schall *et al.*, 1990b; Hayashida *et al.*, 2001), and macrophages (Holgate *et al.*, 1997; Mitsuyama *et al.*, 1994) to the site of infection. They are also activated by similar factors such as TNFα, IL-1β and various pathogens, and similar regulatory factors are subsequently recruited to their respective promoters (Nakamura *et al.*, 1991; Standiford *et al.*, 1990; Fichorova & Anderson, 1999; Miyamoto *et al.*, 2000; Andoh *et al.*, 2002; Song *et al.*, 2002; Matsukura *et al.*, 1996; Ammit *et al.*, 2002; Melchjorsen & Pedersen, 2002; Melchjorsen & Paludan, 2003).

IL-8 and RANTES are expressed in a variety of cell types, such as T-lymphocytes (Ortiz *et al.*, 1996; Song *et al.*, 2002) and epithelial cells, including those of the cervix (Nakamura *et al.*, 1991; Fichorova & Anderson, 1999; Barclay *et al.*, 1993; Narimatsu

et al., 2005). IL-8 expression, like IL-6 expression, is significantly increased during cervical dilation during parturition, resembling the effect of inflammation (Kemp *et al.,* 2002). IL-8 stimulates granulocyte attraction, activation, and leukocyte degranulation. The subsequent release of a variety of proteinases such as matrix metalloproteinase-8 and matrix metalloproteinase-9 from their granules can degrade extracellular matrix components leading to cervical ripening (Osmers *et al.,* 1995). Moreover, in cervical and colonic epithelial cells, both IL-8 and IL-6 mRNA expression are significantly upregulated during chlamydial infection (Rasmussen *et al.,* 1997). IL-8 has also been shown to increase HIV-1 infection in cervical explant tissue (Narimatsu *et al.,* 2005) while RANTES expression inhibits HIV transfection as both RANTES and HIV compete for binding to the same receptor (Alfano & Poli 2005). RANTES expression in tumour cells has been demonstrated to stimulate tumour regression (Mulé *et al.,* 1996; Kutubuddin *et al.,* 1999). However, RANTES has been implicated in breast cancer progression indicating that the effect of RANTES on tumour growth is cell-type specific (Niwa *et al.,* 2001; Azenshtein *et al.,* 2002). IL-8 has also been implicated in tumour progression in a wide variety of cell types, including lung, prostate, bladder, breast and cervix (Chopra *et al.,* 1998) with the exception of ovarian tissues where IL-8 has been demonstrated to attenuate tumour growth (Lee *et al.,* 2000). Therefore, like IL-6, both IL-8 and RANTES can affect cell growth and cancer progression, besides playing an important role in the immune response as chemoattactants.

1.2 Important signalling pathways involved in immunity

As discussed, cytokine gene promoters are predominately regulated by NFκB and AP-1 (Vanden Berghe *et al.,* 2000; Kassel & Herrlich, 2007; Blackwell & Christman 1997; Liberman *et al.,* 2007; Karin & Chang, 2001). TNFα, a potent early response pro-inflammatory cytokine, promotes the secretion of a large set of immune regulators by activating NFκB and, to a lesser extent, AP-1 (Barbara *et al.,* 1996; Vanden Berghe *et al.,* 2000). In the following sections, the signalling pathways induced by TNFα, NFκB and AP-1 will be discussed.

1.2.1 TNF alpha (TNFα) signalling

TNFα is a pro-inflammatory cytokine responsible for the expression of a wide variety of other immunological mediators such as IL-6 and IL-8 (Vlahopoulos *et al.,* 1999;

Vanden Berghe *et al.,* 2000; Brasier *et al.,* 1998) and is mainly expressed by activated macrophages, monocytes, lymphocytes, and keratinocytes (Baud & Karin, 2001). TNFα is a 17 kDa cytokine consisting of 157 amino acids, which functions as a homotrimer. It is involved in a wide variety of cellular responses such as lymphocyte and leukocyte activation and migration, cell proliferation, cell differentiation, apoptosis, cytotoxicity, and immune regulation (Jones *et al.,* 1989; Tracey & Cerami, 1993; Mohan *et al.,* 2001). TNFα belongs to family of trimeric cytokines, which include lymphotoxin-a, Fas ligand, receptor-activator of NFκB ligand, CD40 ligand, and TNFα related apoptosis-inducing ligand (Locksley et al., 2001). TNFα acts primarily via its cognate receptor, a 415 amino acid polypeptide with a single membrane-spanning region (Barbara *et al.,* 1996; Fessler *et al.,* 2004). Binding of the trimeric TNFα protein to its membrane-bound receptor, induces receptor trimerisation, which is required for receptor activation (Barbara *et al.,* 1996; Fessler *et al.,* 2004). TNFα activates a cascade of signalling pathways such as protein kinase C (PKC) and the MAPKs, which leads to the expression of various immune-regulators such as IL-6, IL-8 and RANTES by activating transcription factors such as NFκB and AP-1 in a wide variety of cell types (Fichorova & Anderson, 1999; Barnes & Karin, 1997; Karin *et al.,* 1997; Rothe *et al.,* 1995; Hsu *et al.,* 1996; Liu *et al.,* 1996; Natoli *et al.,* 1997). However, TNFα can also activate the caspase cascade, resulting in apoptosis (Chang & Yang, 2000). The human TNFα promoter itself contains both NFκB and AP-1 binding sites and is therefore subjected to positive auto-regulation thereby ensuring a sustained inflammatory response (Barbara *et al.,* 1996). TNFα, bound to its receptor, recruits TNFα receptor 1-associated death domain protein (TRADD), which serves as a platform recruiting other mediators including TNFα receptor associated factor 2 (TRAF2). TRAF2 recruitment leads to the activation of IκB kinase (IKK) and the activation MAPK pathways, including c-Jun N-terminal kinase (JNK) and p38 signalling pathways (Baud & Karin, 2001; Liu *et al.,* 1996; Song *et al.,* 1997; Natoli *et al.,* 1997). This subsequently leads to the activation of AP-1 and NFκB, respectively (Rothe *et al.,* 1995; Hsu *et al.,* 1996b; Liu *et al.,* 1996; Natoli *et al.,* 1997). It is noteworthy that NFκB is activated when the IκB kinase (IKK) complex is activated, whereas AP-1 activity is regulated by multiple mechanisms such as the phosphorylation of MAPKs, most importantly JNK.

TRAF2 has been shown to be essential for TNFα activation of AP-1. TRAF2 interacts with the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1), a member of the MAPK kinase kinase (MAPKKK) family. This interaction subsequently activates JNK, which is important for TNFα–induced AP-1 activation (see Figure 1.3) (Minden *et al*., 1994). Lymphocytes expressing dominant negative TRAF2 protein were unable to activate JNK on TNFα stimulation and subsequently AP-1, but not NFκB, activation was affected (Lee *et al*., 1997), highlighting the importance of TRAF2 in TNFα–mediated activation of AP-1.

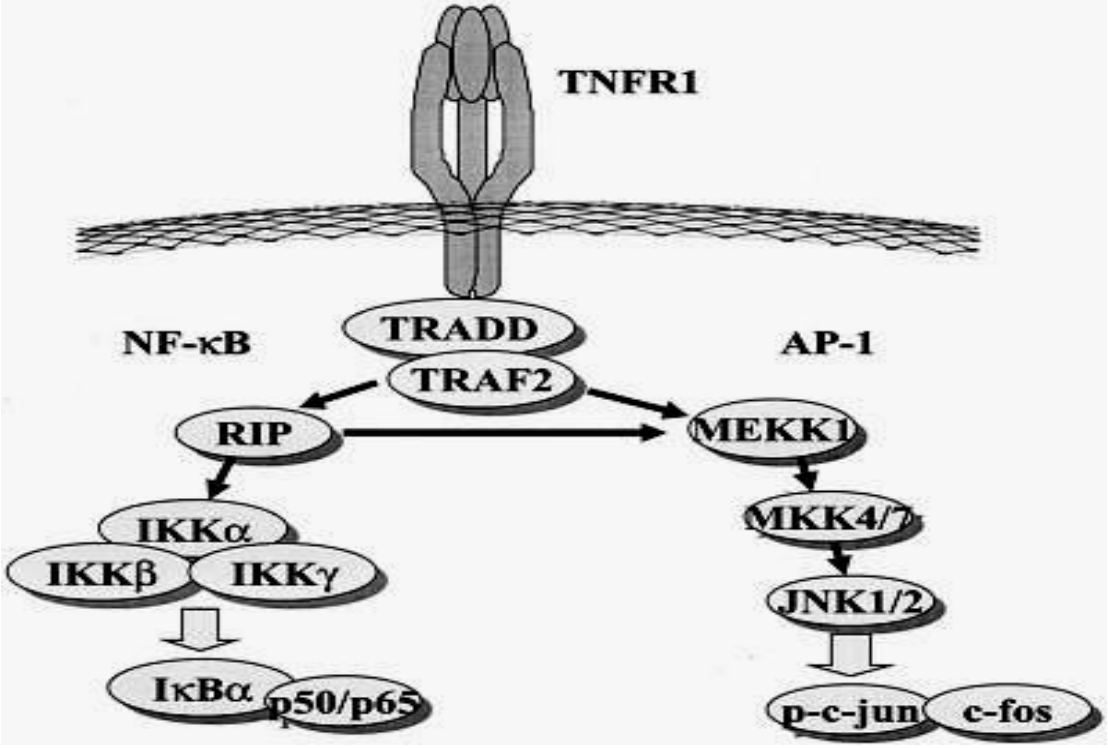


Figure 1.3: Simple schematic presentation of TNFα signalling via TNFR1, highlighting the two major activation pathways, IKK and MEKK1 which activate NFκB and AP-1, respectively. Trimeric TNFα binds to the TNFR1, which activates the association of TRADD, TRAF2, and RIP, which is required for the recruitment, and activation of IKK, which in turn phosphorylates IκBα leading to its ubiquitination and proteosomal degradation. NFκB is then free to translocate to the nucleus. Association of TRADD and TRAF2 leads to the recruitment and activation of MEKK1 which triggers a cascade of pathways which finally activate JNK. JNK phosphorylates and activates c-Jun of the c-Jun/Fos heterodimer (AP-1) (adapted from Fessler *et al.*, 2004).
Abbreviations: TNFR1, Tumour necrosis factor receptor1; TRADD, TNFα receptor 1- associated death domain protein; NFκB, nuclear factor-kappaB; RIP, Receptor-interacting protein; IKK, inhibitory kappa kinase; IκB, inhibitor kappaB; MEKK1; mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1; MKK, mitogen kinase kinase; JNK, c-JUN N-terminal kinase

However, TNFα treatment may also lead to the association of receptor-interacting protein 1 (RIP1) with TRAF2, which is involved in NFκB activation (Hsu *et al.*, 1996a; Fessler *et al.*, 2004). RIP1 has also been shown to directly interact with TRADD

independent from TRAF2 (Hsu *et al.,* 1996b; Devin *et al.,* 2000). Similarly, TRAF5 has been shown to be recruited by TRADD directly and is involved in NFκB activation only, distinct from AP-1 activation (Hsu *et al.,* 1996a; Marsters *et al., 1997;* Hsu *et al.,* 1996b). TNFα activation of NFκB is a complex process involving numerous proteins and pathways, many of which could be bypassed without affecting NFκB activation by TNFα (Leitges *et al.,* 2001; Ishida *et al.,* 1996; Hsu, *et al*. 1996a; Marsters *et al.,* 1997). This could also explain why in some cells TNFα activates NFκB but not AP-1 (Vanden Berghe *et al.,* 1999). These factors also highlight the very potent activation of NFκB by TNFα. To summarise, TNFα initiates a cascade of events resulting in the proteolytic degradation of IκB by the proteosome, thereby allowing NFκB nuclear translocation. TNFα also causes activation of MEKK1 resulting in the activation of AP-1 (Figure 1.3), which will be further discussed in the following section.

1.2.2 AP-1 signalling

AP-1 is a transcriptional activator composed of a heterogeneous collection of Jun (v-Jun, c-Jun, JunB, JunD), Fos (v-Fos, c-Fos, FosB, Fral, Fra2), and activating transcription factor (ATF2, ATF3/LRF1, B-ATF) families, all belonging to the basic-leuzine zipper (bZIP) group of DNA binding proteins (Angel & Karin, 1991). They form a variety of homo- or heterodimers. Fos proteins are unable to form homodimers (Turner & Tjian, 1989), while Jun proteins are most commonly found as homodimers (Chiu *et al.,* 1988; Ziff, 1990; Kouzarides & Ziff, 1989; Matsui *et al.,* 1990; Hai & Curran 1991; Hsu *et al.,* 1991; Dorsey *et al.,* 1995). The Jun-Jun homodimers or Fos-Jun heterodimers preferentially bind to the DNA binding site known as the TPA response element (TRE) (Lee *et al.,* 1987), whereas Jun-ATF heterodimers and ATF homodimers bind to the cAMP-responsive element (CRE) (Hai & Curran 1991). Both promoter sequences (TPA and CRE) are palindromic and contain an AP-1 half-site. Other bZIP proteins have also been shown to bind to the TRE and CRE forming dimers with Jun and Fos (Kerppola & Curran, 1994).

A wide variety of stimuli are able to activate AP-1, for example neurotransmitters, UV irradiation, growth factors, and cytokines, the latter including TNFα as previously discussed (Caelles *et al.,* 1997; De Bosscher *et al.,* 2003). These stimuli activate various protein kinases including the MAPKs, which have been demonstrated to increase AP-1

activity. The JNK pathway phophorylates c-Jun at serine 63 and 73, thereby increasing its stability and transcriptional activity (Musti *et al.,* 1997; Smeal *et al.,* 1994; Davis, 2000; Caelles *et al.,* 1997). Phosphorylation of c-Jun results in the recruitment of cAMP responsive element binding protein (CBP) that aids in AP-1 transcriptional activity in fibroblast cells (Arias *et al*., 1994). Fos protein is extensively modulated by phosphorylation on several serine and threonine residues. Cyclic AMP-dependent protein kinase A (PKA) phosphorylates Fos at serine residue 362 (Tratner *et al.,* 1992) thereby increasing its inhibitory effect on transcription. Similarly, protein kinase C (PKC) and 4α-phorbol 12-myristate 13-acetate (PMA) have been shown to induce phosphorylation of Fos (Abate *et al.,* 1991; Barber & Verma, 1987). Interestingly, phosphorylation of Fos proteins obstructs dimerisation with Jun proteins. Thus Fos negatively regulates its own expression when phosphorylated, as a Fos binding site is present in the *c-fos* gene promoter (Ofir *et al.,* 1990; Sassone-Corsi *et al.,* 1988).

1.2.3 NF-κB signalling

Ubiquitously expressed NF-κB regulates the expression of numerous genes involved in inflammation, immunity, apoptosis, differentiation, cell growth and cell adhesion, including Il-6 and IL-8 (Vanden Berghe *et al.,* 1998; De Bosscher *et al.,* 2006; Rasmussen *et al.,* 2008; Galien *et al.,* 1996; Vanden Berghe *et al.,* 1999; Brasier *et al.,* 1998). NFκB is a dimeric transcription factor, which in its inactive state is bound to the inhibitory IκB proteins, IκBα and IκBβ in the cytoplasm, thereby preventing it from entering the nucleus (Vermeulen *et al.,* 2002; Malek *et al.,* 2001; Tam *et al.,* 2001). A wide variety of stimuli, such as stress and cytokines, like TNFα, activate NF-κB by the phosphorylation of IκB, which is subsequently ubiquitinated and rapidly degraded. This results in the rapid nuclear translocation of the NF-κB proteins (Vermeulen *et al.,* 2002), that are then further activated by several kinases including MAPK and PKC (Vermeulen *et al.,* 2003; Anrather *et al.,* 1999).

The NF-κB proteins are structurally related and are able to interact with each other and bind to DNA as dimers. Five different NF-κB proteins have been described, NF-κB1 (p50/p105) (Bours *et al.,* 1990), NF-κB2 (p52/p100) (Neri *et al.,* 1991), RelA(p65) (Nolan *et al.,* 1991; Ruben *et al.,* 1992), RelB (Ruben *et al.,* 1992; Ryseck *et al.,* 1992), and c-Rel (Galien & Garcia, 1997). Generally, the transcriptionally active NF-κB

heterodimer consists of the p65 and p50 subunits (Barnes & Karin, 1997; Ahn & Aggarwal, 2005), which bind to NFκB binding sites, positively regulating transcription. Similar to AP-1, NFκB also interacts with co-factors thereby increasing transcription. The co-factors CBP and p300 have been shown to interact with p65 and augment its transactivational ability (Gerritsen *et al.,* 1997; Perkins *et al.,* 1997). Transfection studies showed that overexpression of CBP and p300 augmented p65 trancriptional activity on E-selectin and VCAM-1 promoter-reporter constructs. In addition, direct physical interactions of CBP and p300 with p65 were demonstrated by glutathione S-transferase fusion protein binding, and co-immunoprecipitation studies (Gerritsen *et al*., 1997). Interference with NFκB signalling to diminish NFκB regulated transcription, either through an increase in IκB concentrations or direct interaction of other transcription factors with NFκB, results in a reduced inflammatory response *(*Keller *et al.,* 1996). Steroid-mediated repression of NFκB-dependent transactivation is widely associated with the anti-inflammatory properties of steroids and their receptors. Most sex and adrenal steroid have been implicated in the repression of NFκB gene activation (De Bosscher *et al.,* 2006), but glucocorticoids exhibit the most powerful anti-inflammatory activity. Similarly, androgens and progesterone have also been shown to suppress the immune response. In contrast, in some tissues, estrogen has both enhanced and suppressed immunity (reviewed by Grossman 1984 and Beagley & Gockel, 2003). Interestingly, synthetic progestin treatments have also been implicated in suppression of immune function (Wakatsuki *et al.,* 2002; Koubovec *et al.,* 2004; Bamberger *et al.,* 1999). In the next sections, these synthetic progestins become the focus of this chapter.

1.3 Progesterone and the synthetic progestins Medroxyprogesterone acetate & Norethisterone enanthate/acetate

1.3.1 The natural hormone-Progesterone

Progesterone (P4), a derivative of cholesterol, is synthesised primarily by the corpus luteum in the ovaries of females, and the testis and adrenal cortex in males (Graham & Clarke 1997). It serves as a precursor for the synthesis of estrogens and androgens (Graham & Clarke 1997; Gellersen *et al.,* 2009). P4 concentrations are generally higher in females than males, but during the follicular phase of the menstrual cycle P4 concentrations in females decrease to concentrations similar to that found in males

(Kirschbaum *et al.,* 1999). The functions of P4 include the regulation of the menstrual cycle, pregnancy support and antagonising the highly proliferative effect of estrogen (Gellersen *et al.,* 2009). Mood changes, anxiety, depression, infertility, and miscarriages, are only a few of the medical conditions associated with low P4 production in humans (Gellersen *et al.,* 2009). As P4 has a very short half-life and limited bio-availability synthetic progestins were developed to mimic the physiological effects of P4 (Whitehead *et al.,* 1980). These progestins are extensively used in hormone replacement therapy (HRT) to inhibit the proliferative effect of estrogen and as contraceptives to inhibit ovulation.

1.3.2 Medroxyprogesterone acetate & Norethisterone enanthate/acetate: Synthetic progestins

Synthetic progestins were primarily developed as contraceptive agents and have been used extensively for decades. The introduction of synthetic progestins as contraceptives in the 1960's radically revolutionised birth control, making it safer and far more convenient for women (Sitruk-ware, 2006). Steroidal contraceptive methods fall into two categories, combined (synthetic progestins and estrogen) oral contraceptives or progestins-only contraceptives (Sitruk-ware, 2004). Progestin-only contraceptives were primarily developed due to side-effects associated with estrogen sensitivity in combined oral contraceptive users (Mansour, 2005). These side-effects include some associated health risks, such as hypertension, headaches, breast tenderness, and the risk of venous thromboembolism or arterial disease (Sitruk-ware, 2003; Mansour, 2005; Spencer *et al.,* 2009). Progestin-only contraceptives are administered as implants, intrauterine systems, oral preparations, or by injection. Moreover, the dosages of the different progestins also differ. Progestin-only injectable contraceptives offer a convenient alternative for those women who forget to take the daily contraceptive pill. Moreover, for women who want or are forced to keep contraception a secret from their partners, injectable contraception offers a safe alternative. Thus, injectable long-acting contraception offers women convenient, safe, and reversible birth control.

Generally, synthetic progestins are classified according to the steroid from which they are derived. This could be either from progesterone 17-hydroxy (OH) progesterone derivatives: pregnanes and norpregnanes or from testosterone 19-nortestosterone

derivatives: estranges and gonanes (Sitruk-ware, 2004). The two most commonly used progestin-only injectable contraceptives in sub-Saharan Africa are Depo-Provera® and Noristerate®, in which the active compounds are medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN), respectively. MPA, a 17-OH progesterone derivative and norethisterone (NET, the active metabolite of NET-EN) a 19-nortestosterone derivative, are both highly effective reversible contraceptive agents and are both available at no cost to users at public health facilities in South Africa. MPA is also commonly referred to as depo-medroxyprogesterone acetate (DMPA) (Mansour, 2005). Additionally, MPA and NET-A (the acetate form of NET-EN) are also extensively used in hormone replacement therapy (HRT) and cancer treatment (Stahlberg *et al.,* 2004). The structures of MPA and NET-EN, as well as P4 are depicted in Figure 1.4.

MPA is administered in an aqueous solution at a dosage of 150 mg every 12 weeks or 91 days while NET-EN (Noristerat®) is administered at 200 mg every two months in an oily solution. Although both MPA and NET-EN are effective in preventing pregnancy, there are some side-effects associated with their use. The side-effect profile of both these progestins includes irregular bleeding, breast tenderness, acne, amenorrhea, weight gain, headaches, and vaginal discharge (Kaunitz 2000; Greydanus *et al.,* 2001; Westhoff, 2003; Haider & Darney, 2007; Spencer *et al.,* 2009).

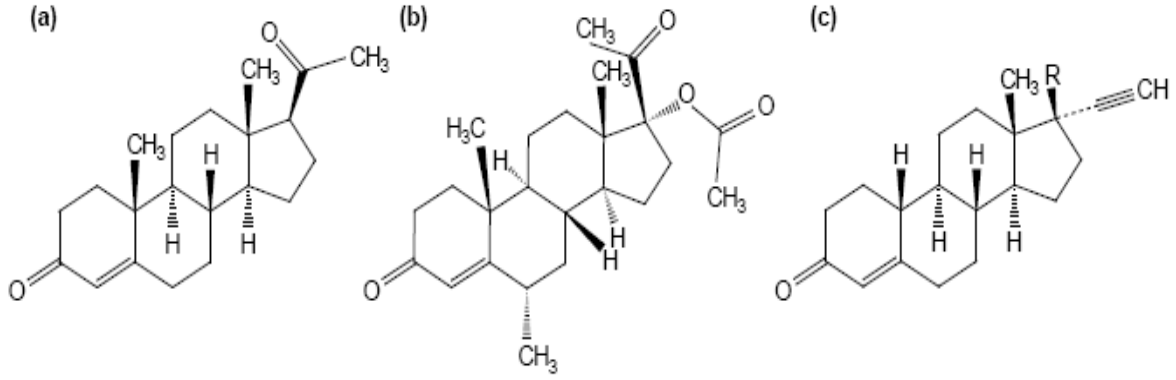


Figure 1.4: The chemical structures of (a) progesterone (P4), (b) medroxyprogesterone acetate (MPA),and (c) norethistherone (NET) (R=OH), norethisterone acetate (NET-A) (R=$OCOCH_3$) and norethisterone enathate (NET-EN) (R=$OCO(CH_2)_5CH_3$ (Hapgood *et al.,* 2004).

1.3.2.1 Mode of contraceptive action

DMPA and NET-EN inhibit ovulation, largely by inhibiting the release of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), by decreasing the pulse frequency of gonadotropin-releasing hormone (GnRH) release (Mishell, 1996; Kaunitz 2000; Greydanus *et al.,* 2001). This results in the prevention of follicular maturation and ovulation, which consequently prevents increased estradiol levels (Mishell, 1996; Kaunitz, 2000; Greydanus *et al.,* 2001). Secondary effects include preventing implantation of fertilised eggs, increasing the thickness of the cervical mucus thereby obstructing sperm mobility and atrophy, as well as the thinning of the endometrium (Mishell, 1996; Greydanus *et al.,* 2001).

1.3.2.2 HRT and other applications

Both MPA and NET are also commonly used in the treatment of a range of physiological conditions. MPA and NET-A are commonly used in HRT in combination with estrogen (Sitruk-ware, 2003; Nath & Sitruk-Ware, 2009; Warren 2004). Moreover, high dosages of MPA and NET are regularly used for the treatment of gynaecological cancers including breast cancer and endometriosis (Maltoni *et al.,* 2001; Cavalli *et al.,* 1984; Muss *et al.,* 1990; Telimaa *et al.,* 1987). These applications of synthetic progestins will be briefly discussed.

1.3.2.2.1 MPA and NET in HRT

Synthetic progestins are included in hormone replacement therapy (HRT) primarily to alleviate menopausal symptoms, such as hot flushes, urogenital atrophy, bone loss and vaginal dryness, which are side-effects associated with decreased estrogen levels (Hickey *et al.,* 2005; Greendale *et al.,* 1999). Two forms of HRT are prescribed, estrogen only or combined estrogen and progestin. The latter is given to women with an intact uterus, so as to counteract the stimulatory effect of estrogens on endometrial hyperplasia and carcinoma (Gambrell Jr *et al.,* 1980; Taitel & Kafrissen, 1995; Brunelli *et al.,* 1996). However, concern has been raised regarding the use of progestins in HRT. Two clinical studies, the Women's Health Initiative (WHI) study (Rossouw *et al.,* 2002; Anderson *et al.,* 2003) and the Women's International Study of Long Duration Oestrogen after the Menopause (WISDOM) (Vickers *et al.,* 2007), both which investigated post-menopausal women using combined HRT (MPA and conjugated equine estrogens (CEE)) were prematurely terminated due to adverse side-effects.

These side-effects included increased risk of breast cancer, coronary heart disease, venous thromboembolism, stroke and dementia (Rossouw *et al.,* 2002). In a follow-up study to the WHI study, an increased risk of ovarian cancer was also shown (Anderson *et al.,* 2003). Consistent with the observed side-effect of breast cancer, the 'Million Women Study' found that both MPA and NET substantially increased the risk of breast cancer in long-term HRT users (Beral *et al.,* 2003). A trial with estrogen alone, however, found no increase risk of breast cancer indicating that MPA is responsible for this adverse effect although this trial itself was also prematurely terminated due to the increase risk of strokes (Anderson *et al.,* 2004). Further investigation at the clinical and experimental level is thus needed to determine the exact role of MPA and NET in the side-effects experienced by HRT users. At this stage however, the current approach when using MPA and NET in therapy is that the benefits outweigh the risks.

1.3.2.2.2 High dosage progestin therapy

In addition to HRT and contraception, progestins at high dosages are commonly used for the treatment of metasized endometrial-, breast-, and ovarian cancer. MPA and megestrol acetate are two of the most commonly used progestins in the treatment of cancer (Muss *et al.,* 1990; Schindler *et al.,* 2003). MPA has also successfully been used in the treatment of endometriosis for decades, significantly relieving pelvic pain with only breakthrough bleeding reported as a side-effect (Telimaa *et al.,* 1987).

1.4 Effect of synthetic progestins on immunity

In addition to the side-effects already mentioned, various epidemiological studies have reported that synthetic progestins can modulate immunity (Majumder *et al.,* 1987; Brunelli *et al.,* 1996; Malarkey *et al.,* 1997). However, very little research has been conducted regarding the role of synthetic progestins in immunity at the molecular level. NET has been shown to modestly inhibit an immune response in mice (Hulka *et al.,* 1965) while the immunosuppressive effect of MPA in mice has been shown to be similar to that of cortisol, the endogenous glucocorticoid (Hulka *et al.,* 1965). Similar anti-inflammatory effects of MPA have been reported in other animal models (Turcotte *et al.,* 1968; Gomez *et al.,* 1998; Wakatsuki *et al.,* 2002). In post-menopausal women using combined CEE plus MPA for HRT, a decrease in pro-inflammatory mediators was observed (Wakatsuki *et al.,* 2002; Stopińska-Głuszak *et al.,* 2006). While CEE had

little if any effect on cell adhesion molecule expression, MPA significantly inhibited its expression in human endometrial biopsies (Wakatsuki *et al.,* 2002). Similarly, the very high doses of MPA used in the treatment of breast cancer caused a decrease in the number of immune regulatory cells such as T-helper cells and other T-lymphocyte subsets (Mallmann *et al.,* 1990; Scambia *et al.,* 1988; Kurebayashi *et al.,* 1999; Naglieri *et al.,* 2002). Furthermore, fewer T-lymphocyte subsets were observed in postmenopausal women using high-dose MPA in the treatment of breast cancer (Mallmann *et al.,* 1990; Scambia *et al., 1*988).

Numerous *in vitro* studies have also reported suppressive effects of MPA on immune regulators, which are summarised in Table 1.1 (Mantovani *et al.,* 1997; Lan *et al.,* 1999; Bamberger *et al.,* 1999; Kelly *et al.,* 1994). Briefly, MPA was reported to suppress phytohemaglutinin-induced IL-6, IL-1β, and TNFα expression in peripheral blood mononuclear cells (Mantovani *et al.,* 1997). Similarly, Lan and colleagues showed that IL-1α expression is significantly repressed by MPA in primary cultures of rat uterine smooth muscle cells (Lan *et al.,* 1999). Consistent with these studies, another study demonstrated that both IL-1 and IL-6 expression are significantly suppressed by MPA in normal human lymphocytes (Bamberger *et al.,* 1999). Dexamethasone (DEX), a strong GR agonist, and MPA significantly inhibited lipopolysaccharide (LPS)-induced IL-8 production in the endometrium and chorio-decidual primary cells, while P4 had no effect on IL-8 production (Kelly *et al.,* 1994). In one of the very few comparative studies of the two progestins, MPA and NET-A, MPA was shown to extensively repress TNFα induced IL-8 promoter activity in the human embryonic kidney HEK293 cell line, while NET-A only elicited a slight decrease in IL-8 promoter activity (Koubovec *et al.,* 2005). The effect of MPA on immunity has also been shown not only to be cellular but also systemic (Parr *et al.,* 1994; Kaushic *et al.,* 2003; Gillgrass *et al.,* 2003).

MPA is extensively used in mice as an immune compromising agent to investigate STIs (Parr *et al.,* 1994). Consistent with this, MPA treatment of mice was found to increase their susceptibility to genital herpes simplex virus type 2 (HSV-2) infection (Kaushic *et al.,* 2003). Interestingly, longer treatment (15 days) with MPA failed to protect mice from subsequent HSV-2 challenge due to a decrease in both innate and adaptive

immune responses (Gillgrass *et al.,* 2003). Vaginal thinning and subsequent simian immunodeficiency virus infection in macaque monkeys as a result of progesterone implants used as a contraceptive have also been reported (Marx *et al.,* 1996). Another study conducted with macaque monkeys demonstrated that MPA at injectable contraceptive dosage repressed antiviral immune responses and increased susceptibility to SIV infection (Trunova *et al.,* 2006) suggesting that MPA may have deleterious effect on sexually transmitted diseases (STDs) and also suggesting that MPA's effect on SIV infection is immune based rather than transmission based. Such an association has been made in a prospective study with 819 women. Morrison and colleagues reported that injectable MPA treatment increased the acquisition of cervical chlamydia and gonococcal infections (Morrison *et al.,* 2004). Moreover, an increase in both HIV and HSV cervical shedding has been shown in women using MPA as contraception (Mostad *et al.,* 1997; Mostad *et al.,* 2000; Wang *et al.,* 2004). In agreement with these studies, another study reported that HIV-positive women using MPA as a contraceptive have increased viral loads as well as a higher number of viral variants compared to non-users (Lavreys *et al.,* 2004). Similarly, increased HIV-1 infection rates have been reported amongst Kenyan sex workers using hormonal contraceptives, compared to non-users (Martin *et al.,* 1998). However, caution should be exercised in interpreting these findings, as women on contraceptives might be less likely to use condoms, which may be the reason for the increase in STIs, rather than a physiological effect of MPA. However, an alternative explanation proposed by Ildgruben *et al.* (2003) suggests that hyperplasia of vaginal epithelium and changes of immune cell milieu induced by MPA could account for the increased risk of STIs in MPA-users compared to non-users (Ildgruben *et al.,* 2003). In contrast to the studies showing an increased risk of HIV acquisition, a few studies have suggested that MPA has no effect on HIV acquisition (Myer *et al.,* 2007; Kleinschmidt *et al.,* 2007; Morrison *et al.,* 2007). Nonetheless, the use of MPA has also been associated with cervical ectopy (Mauck *et al.,* 1999; Morrison *et al.,* 2004), which in turn is linked to higher HIV-1 shedding in women (Moss *et al.,* 1991; Kreiss *et al.,* 1994; Royce *et al.,* 1997). A recent study also shows an association between MPA and an increased risk of cervical infection with chlamydia and gonorrhea (Pettifor *et al.,* 2009).

In summary, results from clinical studies are conflicting regarding the role of synthetic progestins, especially MPA, on immune function. However, *in vitro* evidence from

studies in mice and primates would strongly suggest that progestins do have an effect on the immune response. Synthetic progestins are used by millions of women worldwide, either as contraceptive or in HRT. It is therefore imperative that the molecular mechanism of action of these compounds be fully understood.

Table 1.1: Immune cells and regulators inhibited by MPA

Immune cells or-regulators	**Cell type**	**Reference**
cell-adhesion molecule	Endometrial biopsies	Wakatsuki *et al.*,, 2002
NK cells, IL-2, & IFNγ	PBMC	Stopinska-Głuszak *et al.*,, 2006
T-helper cells	PBMC	Mallmann *et al.*,, 1990
Monoclonal antibodies	PBMC	Scabia *et al.*,, 1988
IL-6	KPL-4 human breast cancer	Kurebayashi *et al.*,, 1999,
IL-2, IL-6, & IL-1	Normal human lymphocytes	Bamberger *et al.*,, 1999
IL-1α	myometrial smooth muscle cells	Lan *et al.*,, 1999
IL-6, IL-1β, & TNFα	PBMC	Mantovani *et al.*,, 1997
IL-8 promoter activity	human embryonic kidney HEK293	Koubovec *et al.*,, 2005
IL-8	endometrium and chorio-decidual cells	Kelly *et al.*,, 1994

1.5 Molecular mechanism of progestin signalling

Like the natural hormone P4, the synthetic progestins MPA and NET-A both have a very high binding affinity for the progesterone receptor (PR) (Bray *et al.*, 2005). In addition both progestins have been shown to interact with other steroid receptors such as the androgen receptor (AR), mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and although not yet well defined also the estrogen receptor (ER) (Philibert *et al.*, 1999; Bentel *et al.*, 1999; Koubovec *et al.*, 2005; Africander PhD thesis, 2010). The molecular mechanism of action of these two progestins via different steroid receptors will be discussed with particular emphasis on their effects on the immune response.

1.5.1 Steroid receptor signalling

The PR is a steroid receptor belonging to the steroid hormone receptor sub-family, which forms part of the nuclear receptor superfamily. Steroid receptors consist of distinct domains as illustrated in Figure 1.5. The amino-terminus primarily contains the highly variable transcriptional activation function 1 domain (AF1). Situated next to the AF1 domain is the highly conserved zinc finger DNA-binding domain (DBD). This domain plays an important role in receptor dimerization, DNA-binding specificity, and interaction with co-factors (Griekspoor *et al.*, 2007). The ligand-binding domain (LBD) is situated at the COOH-terminus and is a moderately conserved region, which is

involved in protein-protein interactions with chaperones or co-regulators (Beato *et al.*, 1995; Gronemeyer & Moras, 1995; McEwan *et al.*, 1997; Moras & Gronemeyer, 1998). Furthermore, the nuclear localisation signal is embedded in both the DBD and LBD (Tang *et al.*, 1998).

Figure 1.5: Functional domains of the steroid receptor. The highly variable amino terminus contains the transcriptional activation funtion 1 (AF-1), while the ligand binding domain (LBD) and ligand-dependent activation function 2 (AF-2) are present in the carboxy-terminus. The highly conserved DNA-binding domain (DBD) is situated in the centre of the protein. The domains concerned with receptor dimerization, co-factor binding, and nuclear localisation are mainly present in the carboxy-terminal end of the steroid receptor protein. H refers to the hinge region. (Diagram adapted from Zhou & Cidlowski, 2005).

In the absence of ligand, the AR, GR and MR are predominantly present in the cytoplasm whereas the ER and PR are predominately present in the nucleus (Griekspoor *et al.*, 2007) in an inactive state in a protein complex with heat shock proteins (hsp)-90 and hsp70, immunophillins, and other factors (Pratt & Toft, 1997). Hsp90 acts as a molecular chaperone, and prevents unliganded steroid receptor from translocating to the nucleus. The liphophilic steroids passively diffuse across the plasma membrane where they bind to the ligand-binding domain of the steroid receptor (Hammes & Levin, 2007). On ligand binding, a conformational change of the steroid receptor occurs, followed by rapid nuclear translocation (Griekspoor *et al.*, 2007; Zhou & Cidlowski, 2005). Furthermore, steroid receptors are hyper-phosphorylated upon ligand binding, which is generally associated with a transcriptionally active steroid receptor. In the nucleus, the ligand-bound steroid receptor binds to specific palindromic DNA sequences known as steroid responsive elements (SREs), thereby positively regulating transcription (Griekspoor *et al.*, 2007). In contrast, steroid receptors can negatively regulate transcription by either directly or indirectly interfering with other

DNA-bound transcription factors such as NFκB, AP-1, and C/EBP (Kassel & Herrlich 2007; De Bosscher & Haegeman, 2009; Revollo & Cidlowski, 2009). The direct protein-protein interaction also referred to as the tethering mechanism, is often associated with the repression of immune-regulator genes such as IL-6 and IL-8 (Barnes & Adcock 1993; Scheinman *et al.,* 1995; van der Saag *et al.,* 1996; De Bosscher *et al.,* 1997; De Bosscher *et al.,* 2000; Adcock & Caramori, 2001; Reichardt *et al.,* 2001; De Bosscher *et al.,* 2006; Jonat *et al.,* 1990; Nissen & Yamamoto, 2000). Although steroid receptor signalling is essentially similar for all steroid receptors, they regulate the transcription of different genes in different tissues in response to their specific ligands (Griekspoor *et al*., 2007).

Steroid receptors can also regulate transcription in the absence of ligand when activated by various stimuli such as peptide growth factors, neurotransmitters, and cyclins (Bunone *et al.,* 1996; El-tanani & Green 1997; Pierson-Mullany & Lange 2004; Tanaka *et al.,* 1996; Eickelberg *et al.,* 1999; Kotitschke *et al.,* 2009; Cenni & Picard 1999). The ER, AR, PR, and GR have all been shown to be activated in the absence of ligand, with most studies identifying phosphorylation of the specific receptor as a marker of activation (Bunone *et al.,* 1996; El-tanani & Green, 1997; Power *et al.,* 1991; Trowbridge *et al.,* 1997; Zwijsen *et al.,* 1998; Chauchereau *et al.,* 1994; Nazareth & Weigel, 1996; Kotitschke *et al.,* 2009). Ser-118 of transiently transfected ER has shown to be hyper-phosphorylated at least 3-fold in response to epidermal growth factor (EGF), a similar level to that induced by 17-β-estradiol in SK-Br-3 cells (Bunone *et al.,* 1996). Similarly, Pierson-Mullany & Lange (2004) showed that in breast cancer T47D whole cell extracts, hyper-phosphorylation occurs at Ser-400 of the PR in response to EGF, PMA, and insulin-like growth factor (IGF) (Pierson-Mullany & Lange, 2004). PR hyper-phosphorylation induced by these mitogens was similar to the extent of hyper-phosphorylation induced by the PR-specific synthetic agonist R5020 (Pierson-Mullany & Lange, 2004). Consistent with that shown for the ER and PR, a recent study in the mouse pituitary cell line LβT2 showed that endogenous GR is selectively phosphorylated in response to Gonadotropin releasing hormone (GnRH) at Ser-234 and not Ser-220 (equivalent to the human Ser-211 and Ser-226, respectively) (Kotitschke *et al*., 2009). In addition, phosphorylation of unliganded ERβ has also shown to facilitate co-factor binding, thereby increasing ligand-independent ERβ induced transcription in

response to EGF (Tremblay *et al.*, 1999; Tremblay & Giguère, 2001; Dutertre & Smith, 2003).

1.5.2 Interaction of P4 and synthetic progestins with steroid receptors

Even though MPA and NET have similar binding affinities and elicit similar biological responses via the PR (Bray *et al.*, 2005; MacLaughlin & Richardson, 1979; Bergink *et al.*, 1983) they have been shown to have differential interactions with other steroid receptors. Both MPA and NET have been shown to bind to endogenous AR with equal affinity in human breast cancer MCF-7 cells (Bergink *et al.*, 1983). However, binding experiments with cytosolic fractions of the same cell line showed that MPA has a slightly higher binding affinity for the AR than NET (29% versus 17% of binding relative to the natural AR ligand, dihydrotestosterone (DHT) (Bergink *et al.*, 1983). Although determined in different cell systems, the K_d of MPA for the AR in MFM-223 human mammary cancer cells was found to be between 1.2 nM-3.6 nM *(*Hackenberg *et al.*, 1993), which is 20 times less than the K_d of DHT as determined in the rat hypothalamus and pituitary (Pérez-Palacios *et al.*, 1983). A recent study in transfected COS-1 monkey kidney fibroblast cells reported that the K_d of both MPA and NET-A for the AR were found to be similar, to each other and DHT (~20 nM) (Africander PhD thesis, 2010). Furthermore, using promoter reporter assays, both MPA and NET-A were shown to exhibit weak agonist activity for transactivation similar to that of DHT via the AR in transfected COS-1 cells on a promoter reporter construct, (Africander PhD thesis 2010). It has been suggested that the androgenic effects of MPA may counteract the protective effects of endogenous androgen signalling in the breast (Birell *et al.*, 2007), which might also apply to NET-A. Consistent with this idea, both MPA and NET-A were demonstrated to increase the risk of breast cancer in long term HRT (Beral, 2003).

P4 has been shown to exhibit weak binding affinity for the AR and GR. In contrast, it has a high binding affinity for the MR, in both transfected COS-1 cells (Africander PhD thesis, 2010) and cytosols of adrenalectomized rat kidneys (Wambach & Higgins, 1978) and exhibits potent antagonist and weak partial agonist activity for the MR (Wambach *et al.*, 1979; Rafestin-Oblin *et al.*, 1992; Quinkler *et al.*, 2002; Quinkler *et al.*, 2002; Africander PhD thesis, 2010). MPA and NET, however, both exhibit no agonist or antagonist activity via the MR (Winneker *et al.*, 2003; Africander PhD thesis,

2010). The inability of synthetic progestins to mimic the effects of P4 via the MR could have negative consequences for women on HRT, as antagonism of aldosterone (Ald) activity by P4 prevents health risks such as high blood pressure and hypertension is a cardiovascular disease risk factor. Thus the choice of progestin used in HRT is especially crucial to protect women from cardiovascular disease.

In terms of the interaction of MPA and NET with the ER, reports are contradictory. Both MPA and NET have been reported to weakly bind the ER both *in vitro* and *in vivo* (Di Carlo *et al.,* 1983). The weak binding of NET and its metabolites to the ER is also supported by other studies (Markiewicz & Gurpide, 1994; Mendoza-Rodríguez *et al.,* 1999; Sasagawa *et al.,* 2008). However, some reports have shown that NET does not bind to the ER (Schoonen *et al.,* 2000; Bergink *et al.,* 1983). These discrepancies could be due to the metabolism of NET in different cell systems, as it is the NET metabolites that appear to have estrogenic activity (Larrea *et al.,* 2001). In contrast to the early study by Di Carlo and co-workers (1983) showing that MPA binds to the ER, several subsequent studies have shown that MPA has no binding affinity for endogenous or transfected ER in numerous cell types (Teulings *et al.,* 1980; Bergink *et al.,* 1983; Markiewicz & Gurpide, 1994; Sasagawa *et al.,* 2008). Binding of synthetic progestins to the ER is therefore a controversial issue, and is most likely cell- and tissue type-specific.

Numerous studies have reported binding of MPA to the GR (Bojar *et al.,* 1979; Teulings *et al.,* 1980; Kontula *et al.,* 1983; Winneker & Parsons, 1981; Bamberger *et al.,* 1999; Simoncini *et al.,* 2004; Koubovec *et al.,* 2004; Koubovec *et al.,* 2005; Ronacher *et al.,* 2009). Kontula and colleagues (1983) demonstrated in leukocytes that although MPA did not interact with the GR as strongly as the potent synthetic glucocorticoid, DEX, it did have a higher binding affinity for the GR than cortisol, the endogenous hormone (Kontula *et al.,* 1983). Similar results were shown in human renal carcinoma cells (Bojar *et al.,* 1979) and human breast cancer cells (Teulings *et al.,* 1980). On the other hand, NET interacts only weakly with the GR (Koubovec *et al.,* 2005; Kontula *et al.,* 1983; Schoonen *et al.,* 1995; Ronacher *et al.,* 2009). In fact, NET exhibited a similar binding affinity for the GR to P4, both 25 times less than that of MPA in the human alveolar epithelial carcinoma (A549) cell line (Koubovec *et al.,* 2005). Furthermore, MPA has also been shown to have dissociative glucocorticoid-like

properties (Bamberger *et al.,* 1999). While MPA and DEX were equally capable of transrepressing an IL-2 promoter, transactivation of a glucocorticoid response element (GRE) in the same cell system was lower in the presence of MPA than DEX (Bamberger *et al.,* 1999; Ronacher *et al.,* 2009). The effects of MPA were reported to occur via the GR, as neither PR nor the AR are expressed in the lymphocyte cell line in which the experiments were conducted (Bamberger *et al.,* 1999). In a human embryonic kidney (HEK293) cell line, MPA induced both GR-mediated transactivation of a GRE containing promoter reporter (EC_{50}=7.2 nM) as well as GR-mediated transrepression of an IL-8 promoter (EC_{50}=2.7 nM) (Koubovec *et al.,* 2005). NET was unable to induce transactivation although it did elicit some transrepression (approximately 22% of that elicited by DEX) (Koubovec *et al*., 2005). Furthermore, in contrast to MPA, NET was shown to antagonise GR-mediated transactivation in transfected COS-1 cells (Africander PhD thesis 2010).

Taken together, MPA and NET may exert biological effects via interaction with the GR, AR, MR, or ER. Furthermore, most of the effects by MPA regarding immune regulation are believed to be via the GR (Bamberger *et al.,* 1999; Koubovec *et al.,* 2004) although anti-inflammatory effects could also be via the AR (Africander Phd thesis 2010; Wakatsuki *et al.,* 2002) The mechanisms by which progestins such as MPA and NET may elicit anti-inflammatory responses via the GR are discussed below.

1.6. Glucocorticoid receptor-mediated repression of immune-regulators

Inflammatory diseases such as asthma and rheumatoid arthritis are extensively treated with glucocorticoids, which inhibit the production of immune-regulators, promote apoptosis and inhibit cell proliferation of immune cells. Inflammation also triggers a negative feedback loop to the hypothalamic-pituitary-adrenal (HPA) axis, which stimulates the synthesis and secretion of glucocorticoids by the adrenal cortex. As previously stated, ligand-activated GR can either stimulate or inhibit gene transcription. Glucocorticoids are extensively used as anti-inflammatory agents and there are various mechanisms by which ligand-bound GR can inhibit expression of immune regulators. These mostly involve interference with transcription of pro-inflammatory genes, which have been extensively discussed by several groups (Kassel & Herrlich, 2007; De

Bosscher *et al.,* 2003; Newton & Haegeman, 2009; Rogatsky & Ivashkiv, 2006; Liberman *et al.,* 2007). The gene repression can occur in one of three ways. Firstly, the GR can directly interact with pro-inflammatory transcription factors such as NFκB and AP-1 (Barnes & Adcock, 1993; Scheinman *et al.,* 1995; van der Saag *et al.,* 1996; De Bosscher *et al.,* 1997; De Bosscher *et al.,* 2000; Adcock & Caramori, 2001; Reichardt *et al.,* 2001; Jonat *et al.,* 1990; Nissen & Yamamoto, 2000; De Bosscher *et al.,* 2006). Secondly, activated GR can compete with transcription factors for nuclear co-factors thereby limiting their transcriptional activity (Sheppard *et al.,* 1998; Kamei *et al.,* 1996). Thirdly, the GR can indirectly influence immune-regulators either by interfering with upstream signalling proteins, responsible for the activation of pro-inflammatory transcription factors, or by the transcriptional up-regulation of anti-inflammatory genes (Caelles *et al.,* 1997; Ayroldi *et al.,* 2002; Alexander & Hilton 2004; Auphan *et al.,* 1995). These mechanisms, which are schematically represented in Figure 1.6, will be discussed in more detail with a focus on the mechanism by which glucocorticoids, MPA, and to a lesser extent NET-A, may exert their anti-inflammatory effects via the GR.

1.6.1 Direct interaction of the GR with pro-inflammatory transcription factors

The inhibitory activity of ligand-bound GR on immune-regulatory genes can be mediated by direct protein-protein interaction with pro-inflammatory transcription factors (Kassel & Herrlich, 2007; De Bosscher *et al.,* 1997; Tuckermann *et al.,* 1999; Ray & Prefontaine, 1994; De Bosscher *et al.,* 2000; Kleinert *et al.,* 1996). The interacting complex of the GR and other transcription factors is well established. Numerous studies in different cell types have shown by co-immunoprecipitation and/or glutathione S-transferase fusion binding protein (GST) pull down experiments, that the GR directly interacts with pro-inflammatory transcription factors such as NFκB and AP-1 (Touray *et al.,* 1991; Barnes & Adcock 1993; Scheinman *et al.,* 1995; van der Saag *et al.,* 1996; De Bosscher *et al.,* 1997; De Bosscher *et al.,* 2000; Jonat *et al.,* 1990; Nissen & Yamamoto 2000). The interactions between the GR and the transcription factors NFκB and AP-1 are the best studied, mainly because they are commonly associated with increased expression of a wide variety of inflammatory cytokines (e.g. IL-6, IL-8, RANTES, TNFα, IL-1β etc) (Barnes, 1998). The interaction of the GR with NFκB and AP-1 requires the zinc finger region situated in the DNA-binding domain of

the GR as determined by deletion mutants and/or domain mapping in a wide variety of cell types (Stein & Yang, 1995; Liden *et al.,* 1997; Wissink *et al.,* 1997; Nissen & Yamamoto, 2000; Scheinman *et al.,* 1995; Jonat *et al.,* 1990; Yang-Yen *et al.,* 1990; Ray *et al.,* 1990). In addition, interactions between the GR and a number of other transcription factors have been demonstrated, such as signal transducer and activator of transcription (STAT)-5 (Stöcklin *et al.,* 1996) and CREB (Imai *et al.,* 1993).

Early studies proposed that the direct interaction of the GR with other transcription factors prevents DNA binding of transcription factors NFκB and AP-1 and consequently repression of target genes (Scheinman *et al.,* 1995a; Scheinman *et al.,* 1995b; Ray & Prefontaine 1994; Mukaida *et al.,* 1994; Schüle *et al.,* 1990; Yang-Yen *et al.,* 1990). These studies showed that DEX treatment diminished binding of AP-1 or NFκB to DNA in electrophoretic mobility shift assays (EMSAs). However, other studies also using EMSAs, reported no reduction in DNA binding of AP-1 (Jonat *et al.,* 1990) or NFκB (De Bosscher *et al.,* 1997). Similarly, *in vivo* footprinting demonstrated that AP-1 binding to DNA remained unaltered in response to glucocorticoids (König *et al.,* 1991). It could be argued that these differences are due to promoter- or cell specificity. However, the studies by Yang-Yen *et al.* (1990) and Jonat *et al.* (1990) both investigated the collagenase-1 promoter (Yang-Yen *et al.,* 1990; Jonat *et al.,* 1990) and a later study involving chromatin immunprecipitation (ChIP) confirmed that AP-1 subunit levels at the collagenase-1 promoter do not change upon glucocorticoid treatment (Kassel *et al.,* 2004). Similar findings were also reported for NFκB recruited to the IL-8 promoter, which remained unchanged in response to DEX (Nissen & Yamamoto, 2000; Luecke & Yamamoto, 2005; Kassel *et al.,* 2004; Garside *et al.,* 2004), in contrast to the earlier studies. Several other studies also show unaltered pro-inflammatory transcription factor recruitment to various promoters, with DEX-dependent GR recruitment (Rogatsky *et al.,* 2001; Ogawa *et al.,* 2005; Garside *et al.,* 2004). Taken together, these findings suggest a repression model in which the protein-protein interaction between GR and pro-inflammatory transcription factors occurs while the latter are bound to DNA, at the promoter.

Protein-protein interactions at the promoter DNA is also commonly referred to as tethering. Tethering of GR to pro-inflammatory transcription factors gives rise to

different molecular events that lead to transcriptional repression. These molecular events include the involvement of co-factors (McKay & Cidlowski, 2000; Rogatsky *et al.,* 2001; Rogatsky *et al.,* 2002; He & Simons, 2007; Ogawa *et al.,* 2005) and destabilising of basal transcription machinery recruited to the promoter (Nissen & Yamamoto, 2000; De Bosscher *et al.*, 2000; Luecke & Yamamoto, 2005).

1.6.1.1 Co-factors involved in transrepression

The involvement of co-factors in GR-mediated transcriptional repression via tethering is complex and several mechanisms have been proposed. Both CBP and GR interacting protein-1 (GRIP-1) are historically associated with transcriptional activation (Glass & Rosenfeld, 2000). However, these two co-activators have recently been shown to also be involved in GR-mediated repression. Overexpression of CBP, a histone acetyl transferase, has been shown to increase the physical interaction between NFκB and GR, and augment GR-mediated repression of an NFκB responsive reporter (McKay & Cidlowski, 2000). Similarly, overexpressed GRIP-1 augmented GR-mediated repression of the collagenase-3 promoter, which is AP-1 dependent (Rogatsky *et al.,* 2001). The authors demonstrated by ChIP analysis that GRIP-1 is recruited to the collagenase-3 promoter in response to DEX, and not in response to the pro-inflammatory agent PMA, suggesting it is in the role as co-repressor (Rogatsky *et al.,* 2001). In addition, it has been established that GRIP-1 also functions as a co-repressor of liganded-GR on a synthetic NFκB-responsive promoter reporter (Rogatsky *et al.,* 2002). The exact mechanism that determines how these two co-factors are involved in transcription inhibition needs to be elucidated. However one could postulate that they serve as bridging proteins that interact with other proteins with histone deacetylase activity needed for repression (Kassel & Herrlich, 2007; Ito *et al.,* 2000). In fact, GRIP-1 has been shown to interact with histone deacetylase 1 (HDAC1) by co-immunoprecipitation (Liu *et al.,* 2006).

The GR can also block the recruitment of co-activators to transcription factors associated with pro-inflammatory genes. Two studies have shown that the GR disrupts the formation of NFκB and interferon regulatory factor (IRF) complexes (Ogawa *et al.,* 2005; Reily *et al.,* 2006). The NFκB/IRF complex is required for TLR-4- or TLR-9-dependent transcriptional activation of inflammatory genes (Ogawa *et al.,* 2005) and

IRF3 is a co-activator for NFκB responsive genes such as IL-6 and TNFα (Ogawa *et al.,* 2005). Co-factor involvement in transrepression mediated by the tethered GR is complex and may be co-factor-, as well as promoter-specific.

1.6.1.2 The effect of tethered GR on basal transcription machinery

The GR has also been shown to negatively affect the basal transcription machinery of various promoters via tethering (Nissen & Yamamoto, 2000; De Bosscher *et al.,* 2000; Luecke & Yamamoto, 2005). Although the GR does not prevent the recruitment of RNA polymerase II (Pol II) to the NFκB responsive IL-8 promoter (Luecke & Yamamoto 2005; Nissen & Yamamoto 2000), it has been shown to inhibit serine-2 phosphorylation of the Pol II carboxy-terminal domain (CTD), which is essential for Pol II transcriptional activity (Nissen & Yamamoto, 2000). Prevention of Pol II phosphorylation is achieved by the GR competing for promoter binding with transcription elongation factor b (P-TEFb) (Luecke & Yamamoto, 2005), which is responsible for the phosphorylation of the CTD of Pol II (Barboric *et al.,* 2001). Ligand activated GR has also been demonstrated to inhibit phosphorylation of H3 at serine-10 (Hasegawa *et al.,* 2005). Since this histone modification is linked to transcription of pro-inflammatory genes (Martenset *et al.,* 2005; Karrasch *et al.,* 2006; Chan *et al.,* 2005), this represents yet another mechanism by which the GR can decrease transcriptional activity.

1.6.2 Co-factor competition model

Besides the models discussed above, early studies proposed that GR-mediated repression is influenced by limited amounts of co-factors present in the cell (Kamei *et al.,* 1996; Sheppard *et al.,* 1998; Lee *et al.,* 1998; Aarnisalo *et al.,* 1998). CBP and steroid receptor co-activator (SRC)-1 act as co-factors for AP-1, NFκB, and the GR (Arias *et al.,* 1994; Perkins *et al.,* 1997; Kamei *et al.,* 1996; Lee *et al.,* 1998; Na *et al.,* 1998) and it has been suggested that the GR and pro-inflammatory transcription factors compete for binding to these cofactors (Kamei *et al.,* 1996; Sheppard *et al.,* 1998; Aarnisalo *et al.,* 1998). Consistent with this hypothesis, overexpressed CBP was shown to potentiate NFκB and c-Jun activated gene transcription (Gerritsen *et al.,* 1997; Arias *et al*., 1994) and was also subsequently shown to decrease GR-mediated repression (Kamei *et al.,* 1996; Sheppard *et al*., 1998). A similar mechanism has been shown for

SRC-1 and AP-1, where overexpression of SRC-1 in CV1 cells induced AP-1-mediated promoter activity, while also attenuating DEX-mediated repression (Lee *et al*., 1998). However, overexpressing CBP in murine L929A fibrosarcoma cells showed that CBP did not affect DEX-mediated repression of an IL-6 promoter reporter construct (De Bosscher *et al*., 1997). DEX-mediated transactivation of a GRE-containing promoter construct was negatively affected by co-expression of CBP and p65, a result which does not support the hypothesis of transrepression through co-factor competition (De Bosscher *et al*., 1997). These authors also showed that increasing the co-factor protein does not affect GR repression of IL-6 promoter induced by staurosporine, a compound that selectively activates AP-1 but not NFκB. A similar finding was demonstrated on an AP-1-driven promoter construct, suggesting that the lack of co-factor competition is not promoter specific. In contrast, using a GR mutant unable to bind to co-factors *(*Kassel *et al.,* 2004), the GR was still able to mediate repression of an AP-1 driven promoter. Further studies are thus needed to address these disparities concerning the co-factor competition model of GR transrepression.

1.6.3 Indirect repression by GR

The above-mentioned mechanisms of GR-mediated repression (Section 1.6.1 & 1.6.2) all involve direct cross-talk between the GR and pro-inflammatory transcription factors. However, the GR can also interfere with upstream signal transduction involved in the activation of transcription factors, as well as increase of transcription of anti-inflammatory genes.

1.6.3.1 Cross-talk with signalling pathways

Signalling pathways like the MAPKs and PKA have been shown to activate the pro-inflammatory transcription factors AP-1 and NFκB, thereby influencing transcription (Gonzalez *et al.,* 2000; Stein *et al.,* 1989; Devary *et al.,* 1991; Radler-Pohl *et al.,* 1993; Caelles *et al.,* 1997; Zhong *et al.,* 1997; Doucas *et al.,* 2000). The GR has been demonstrated to negatively influence AP-1 and NFκB activity indirectly by inhibiting the activity of the MAPKs, ERK, JNK, and p38 (Kassel *et al.,* 2001; Imasato *et al.,* 2002; Lasa *et al.,* 2002; Caelles *et al.,* 1997; Gonzalez *et al.,* 2000; Rider *et al.,* 1996; Hulley *et al.,* 1998). The main protein subunit of AP-1, c-Jun, is phosphorylated at serine residues 63 and 73 by JNK, which is required for its activity (Stein *et al.,* 1989;

Devary *et al.,* 1991; Radler-Pohl *et al.,* 1993; Caelles *et al.*, 1997). By quantitation of tryptic phosphopeptide maps of *in vivo*-labeled c-Jun in HeLa cells, Caelles and colleagues demonstrated that UV stimulation increased phosphorylation on Ser-63 and Ser-73 at least 2-fold whereas DEX abolished this increase without affecting total JNK protein levels (Caelles *et al.,* 1997). Interestingly, although the GR antagonises AP-1 transcriptional activity, both the GR and JNK were shown to be recruited to the *c-jun* promoter by means of ChIP analysis in HeLa cells (Bruna *et al.,* 2003). Co-immunprecipitation experiments showed a direct interaction between JNK and GR (Bruna *et al.,* 2003).

The PKA catalytic subunit phophorylates and physically interacts with NFκB (Zhong *et al.,* 1997; Doucas *et al.,* 2000) thereby increasing NFκB transcriptional activity, and the GR has been shown to interfere with the interaction between the PKA catalytic subunit and NFκB (Doucas *et al.,* 2000), thereby reducing NFκB activity. The GR can also associate with the PKA catalytic subunit, thus inhibiting activation of NFκB. Doucas and colleagues showed by means of *in vitro* pull-down experiments that *in vitro* translated human GR interacts with GST-PKA. Subsequently, the same study demonstrated in human HEK293 cells, that transfected human GR co-immunoprecipitated with PKA (Doucas *et al.,* 2000). Overexpressed GR decreased p65/ PKA catalytic subunit interaction in a ligand-dependent manner as determined by co-immunoprecipitation (Doucas *et al.,* 2000). The authors concluded that the GR competes with p65 for binding to the PKA subunit. However it could also weaken the association between p65 and PKA (Doucas *et al.,* 2000). Ligand-activated GR has also been shown to decrease ERK and p38 activity (Kassel *et al.,* 2001; Imasato *et al.,* 2002; Lasa *et al.,* 2002). However, this requires *de novo* protein synthesis, unlike inhibition of PKA and JNK (Kassel *et al.,* 2001; Lasa *et al.,* 2002).

1.6.3.2 Increased transcription of anti-inflammatory genes

Ligand-activated GR can also indirectly downregulate anti-inflammatory gene expression by the induction of numerous genes (summarised in Table 1.2) that are directly involved in the repression of the immune response. Besides direct interaction with signalling pathways, the GR can also indirectly influence MAPKs by increasing the expression of MAPK phosphatase-1 (MKP-1) (Kassel *et al.,* 2001; Lasa *et al.,*

2002; Jang *et al.,* 2007; King *et al.,* 2009). MKP-1 is a member of the dual specific (Thr/Tyr) phosphatase family (DUSPs) and is known for its negative regulation of immune processes, by dephophorylation of MAPKs (Camps *et al.,* 2000). MKP-1 is induced by pro-inflammatory stimuli and mitogenic stress and therefore its attenuation of MAPK activation serves as a negative feedback mechanism (King *et al.,* 2009). Endogenous GR increases MKP-1 expression at the promoter level in both RBL-2H3 mouse bone marrow-derived mast cells and NIH-3T3 fibroblasts cells in a ligand-dependent manner (Kassel *et al*., 2001). MKP-1 mRNA expression in mast cells and fibroblasts derived from mouse bone was shown to increase in response to 0.1 μM DEX with maximal expression after one hour of treatment. GR-mediated activation of MKP-1 has shown to occur via a tethering mechanism involving C/EBP in HEK293 cells stably transfected with human GR on the DUSP1 promoter (Johansson-Haque *et al.,* 2008). MKP-1 expression induced by DEX was shown to inhibit NFκB-mediated transcription of IL-8 (King *et al.,* 2009). Additionally, when activated by the endogenous ligand cortisol, liganded GR antagonises MKP-1 degradation in RBL-2H3 mast cells compared to untreated cells (Kassel *et al.,* 2001). Therefore, GR-mediated effects of DEX on MKP-1 are two-pronged: its expression is increased, while proteosomal degradation is decreased. The DEX-induced increase of MKP-1 is yet another mechanism by which glucocorticoids may elicit an anti-inflammatory response.

Glucocorticoid-induced leucine zipper (GILZ) is another example of a GR-induced protein with a role in the anti-inflammatory response. Ligand-activated GR increases the expression of GILZ, a leuzine zipper protein that inhibits the expression of AP-1- and NFκB-responsive genes (Mittelstadt & Ashwell, 2001; Ayroldi *et al.,* 2001; Ayroldi *et al.,* 2002; Riccardi *et al.,* 2000; Riccardi & Cifone, 1999). Increased GILZ expression has been shown to be required for GR mediated repression of IL-2 mRNA in normal T-cells (Mittelstadt & Ashwell, 2001), as well as transrepression of AP-1- and NFκB-responsive reporters in transfected Jurkat and human kidney epithelial carcinoma HEK293 cell line respectively (Mittelstadt & Ashwell, 2001; Ayroldi *et al.,* 2001). In addition, GILZ was shown to interact with AP-1 and NFκB subunits by means of co-immunoprecipitation, and furthermore it prevents DNA binding of both transcription factors *in vitro* (Mittelstadt & Ashwell, 2001; Ayroldi *et al.,* 2001). Overexpressed GILZ has been shown to inhibit ERK, MEK, and Raf-1 phosphorylation

by interacting with upstream Raf-Extracellular Signal-Regulated Kinase, which consequently impairs AP-1 activation (Ayroldi *et al.,* 2002). Additionally, DEX-induced GILZ expression has shown to significantly increase the expression of the anti-inflammatory cytokine IL-10 in macrophages of non-lymphoid tissues from humans and mice (Berrebi *et al*., 2003). Suppressor of cytokine signalling-1 (SOCS-1) is another GR-induced protein that negatively influences the inflammatory response. SOCS inhibits the JAK/Stat pathway, through which cytokines such as IL-6 signal, by physically interacting with JAK and blocking STAT interaction, as well as increasing JAK degradation by recruiting E3 ubiquitin ligases (Alexander & Hilton, 2004). Ligand-mediated GR upregulates SOCS-1 mRNA expression in several cell types thereby increasing its inhibitory effect on the JAK/Stat pathway (Tonko *et al.,* 2001; Yoshida *et al.,* 2002).

Lastly, the ligand-activated GR has been shown to induce IκB expression, which directly inhibits NFκB activation (Auphan *et al.,* 1995; Crinelli *et al.,* 2000; Quan *et al.,* 2000; Thiele *et al.,* 1999; Scheinman *et al.,* 1995). Ligand-activated GR was demonstrated to increase both IκB mRNA and protein levels in Jurkat (Auphan *et al.,* 1995) and HeLa cells (Scheinman et al., 1995b). Additionally, NFκB remained in the cytoplasm in response to DEX as determined by immunofluorescent microscopic analysis (Auphan *et al.,* 1995) and subcellular biochemical fractionation (Scheinman *et al.,* 1995b). Newly synthesised IκB is proposed to sequester NFκB thereby inhibiting NFκB responsive genes (Auphan *et al.,* 1995; Scheinman *et al.,* 1995b). However, several studies have refuted the suggestion that glucocorticoids induce IκB synthesis (Kleinert *et al.,* 1996; Luecke & Yamamoto, 2005) and suggest that NFκB inhibition by glucocorticoids is independent of IκB synthesis (Heck *et al.,*, 1997; De Bosccher *et al.,*, 1997). Glucocorticoid treatment neither activated nor suppressed TNFα-induced IκB mRNA expression in A549 (Luecke & Yamamoto, 2005) and L929sA (De Bosscher *et al.,* 1997) cells, although the GR is recruited to the IκB promoter (Luecke & Yamamoto, 2005). Taken together, these studies suggest that the indirect GR-mediated repression of NFκB responsive genes by directly increasing IκB expression is cell-type specific.

Table 1.2: Proteins upregulated by glucocorticoids that play a role in the inhibition of inflammation

Protein	Role in inflammatory inhibition	Reference
MKP-1	Involved in the dephosphorylation of MAPK pathways including upstream kinases	Caelles *et al.*,, 1997; Kassel *et al.*,, 2001; Jang *et al.*,, 2007
GILZ	Inhibit phosphorylation of ERK, MEK and Raf-1 and the expression of immune-regulator genes such as IL-2	Ayroldi *et al.*,, 2002; D'Adamio *et al.*,, 1997; Riccardi *et al.*,, 1999;
SOCS	Inhibits Jak/Stat signalling pathway and promotes degradation of Jak	Alexander & Hilton, 2004
IκB	Inhibitor of NFκB, which keeps it sequestered in the cytoplasm	Auphan *et al.*,, 1995; Crinelli *et al.*,, 2000; Quan *et al.*,, 2000

The GR can therefore also indirectly influence the immune response as discussed above and summarised in Table 1.2 by inducing the expression of proteins i.e. *de novo* protein synthesis, responsible for the inhibition of signalling pathways involved in the activation of pro-inflammatory transcription factors.

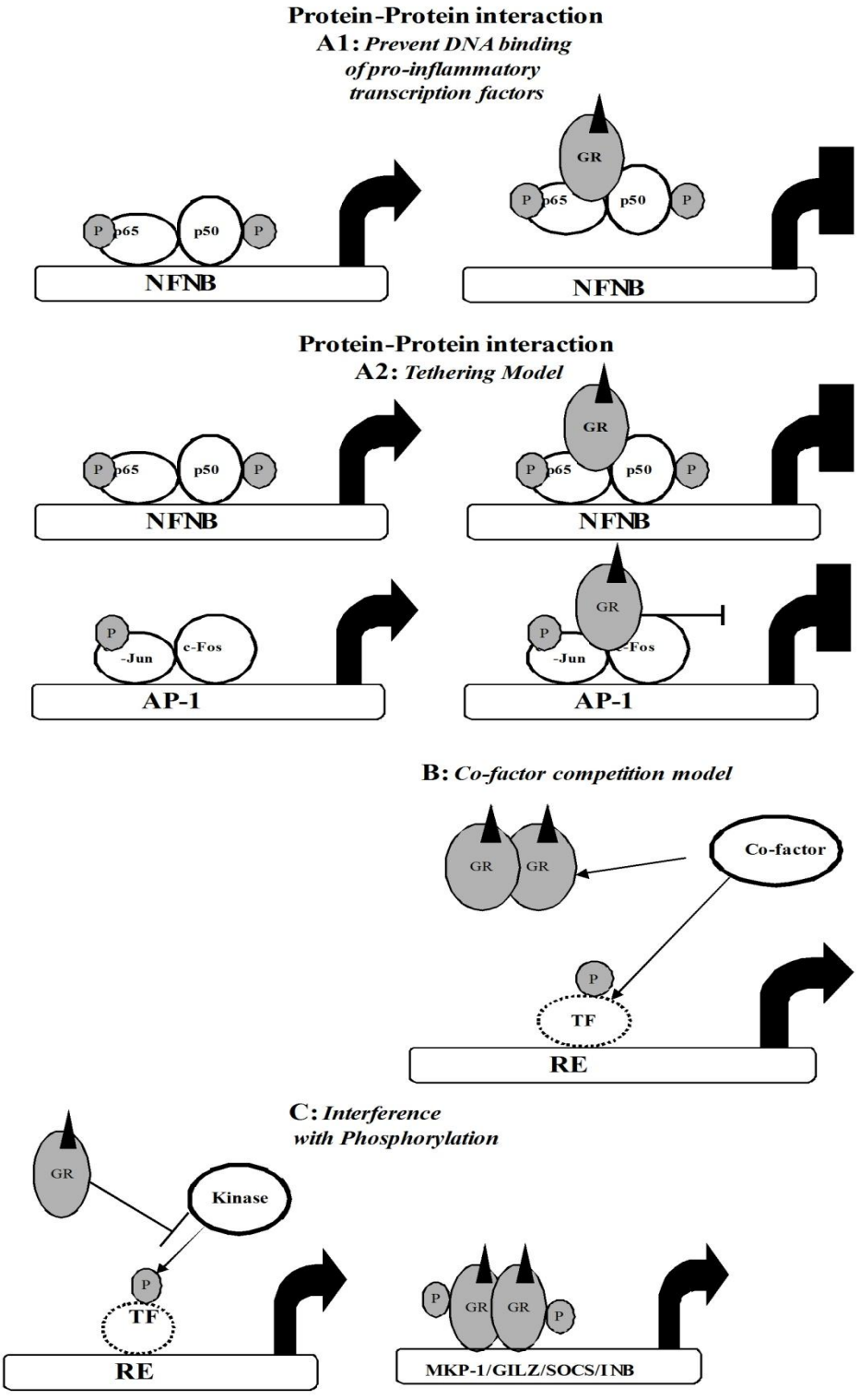


Figure 1.6: Illustration of GR repression models as described in text by (A1) inhibition of DNA binding , (A2) tethering, (B) competing for co-factors, and (C) either directly or indirectly interfering with signalling pathways responsible for the activation of pro-inflammatory transcription factors. Abbreviations: GR, glucocorticoid receptor; P, phosphorylation, IκB, inhibitory κB; NFκB, nuclear factor κB; AP-1, activation protein-1; c-Jun, cellular Jun; c-Fos, cellular Fos; TF, transcription factor; RE, response element; MKP-1, mitogen kinase phosphatase-1; GILZ, glucocorticoid inducible leuzine zipper; SOCS, suppressor of cytokine signalling

As summarised in Figure 1.5, there are numerous mechanisms by which the GR induces repression of immune regulators, and this is dependent on the promoter, local chromatin structure and the accessibility of interacting co-factors (Reily *et al.,* 2006). The most extensively studied and discussed method of GR cross talk with immune signalling is that of tethering. However, GR induced repression may encompass more than one of the mechanisms mentioned. This may explain why glucocorticoids are so effective in anti-inflammatory treatment and it is a challenge to elucidate a single mechanism.

1.7 Conclusion

The FRT is a complex anatomical structure and the epithelial cells lining the FRT play an important role in the local immune response induced by pathogens or stress (Wira *et al.,* 2005b). The epithelial cells of the FRT express a wide variety of both pro- and anti-inflammatory cytokines and chemokines including IL-6, IL-8 and RANTES (Fichorova & Anderson, 1999; Fahey *et al.,* 2005). Cytokines are under tight transcriptional regulation and hyper-expression is induced under conditions of stress or inflammation. The release of pro-inflammatory cytokines such as TNFα and IL-1β triggers a cascade of signalling pathways leading to the activation of pro-inflammatory transcription factors such as AP-1 and NFκB.

STIs commonly occur at the FRT and numerous studies have implicated the use of contraceptives in the increased risk of various STIs as well as viral shedding in both humans and primates (Lavreys *et al.,* 2004a; Lavreys *et al.,* 2004b; Morrison *et al.,* 2004; MacLean 2005; Marx *et al.,* 1996). MPA has also been shown induce thinning of the vaginal mucosa (Ildgruben *et al.,* 2003), which is associated with increased HIV infection (Guimarães *et al.,* 2007). Contraceptive use could directly influence susceptibility to various infections and diseases of the FRT. However, many unanswered questions remain regarding the molecular mechanism(s) of action of the synthetic progestins in the FRT. In addition, because MPA and NET-EN are used interchangeably as injectable contraceptives, and they are assumed to act similarly to P4, more comparative studies are needed to directly compare these compounds.

MPA and NET-A were synthesised to mimic the actions of P4 but have been shown to differ from P4 and to each other in their actions (Koubovec *et al.,* 2005; Stanczyk, 2003; Hapgood *et al.,* 2004). These differences are attributed to their different affinities for different steroid receptors and MPA's anti-inflammatory actions have been proposed to be mediated via the GR (Bamberger *et al.,* 1999; Koubovec *et al.,* 2004).

Various mechanisms are responsible for the anti-inflammatory effects mediated via ligand activated GR as summarised in Figure 1.5. This and could explain why the immuno-compromising effects of the progestins on the FRT are not well understood and certainly need to be examined further.

1.8 Aims of thesis

Little is known about the molecular mechanism of action of MPA and NET-A with even less information on the molecular effects in the cervical mucosae. Synthetic progestins are generally considered anti-inflammatory and the effect of MPA and NET-A on local endocervical epithelial immune function warrants investigation. Thus, in the present study the regulation of cytokines RANTES, IL-8, and IL-6 by synthetic progestins MPA, NET-A, and P4 was investigated in a cell model of the endocervix (End1/E6E7) (Chapter 3). It was hypothesised that cytokine gene regulation would occur and that, as suggested by the literature, MPA especially would be anti-inflammatory. In addition it was hypothesized that MPA and NET-A would differentially regulate the expression of the cytokine genes. Possible differential gene regulation by the progestins is hypothesised to be due to different steroid receptors and MAPK pathways and was investigated. In studying the role of different steroid receptors in IL-6 gene regulation by progestins an interesting observation was made which lead to the second part of this thesis (Chapter 4). A novel mechanism of GR repression in response to TNFα in endocervical epithelial (End1/E6E7) cells, was investigated.

CHAPTER TWO

MATERIAL AND METHODS

2.1 Cell culture

End1/E6E7 (endocervical cells immortalized with the human papillomavirus 16/E6E7) were purchased from the American Type Culture Collection (ATCC), United States of America. Cells were grown 175 cm^2 culture flasks (Greiner Bio-One International, Austria) in keratinocyte serum free (KSF) medium (Sigma-Aldrich, South-Africa) supplemented with $CaCl_2$ (final concentration 0.4 mM), 100 IU/mL penicillin, 100 μg/mL streptomycin, the provided bovine pituitary extract (BPE) and 0.1 ng/mL recombinant epidermal growth factor (EGF).

COS-1 (African green monkey kidney fibroblast) cells (ATCC, United States of America) and HeLa (human cervical carcinoma) cells were cultured in 175 cm^2 culture flasks (Greiner Bio-One International, Austria) in Dulbecco's modified Eagle's medium (DMEM) from Sigma-Aldrich, South-Africa, supplemented with 10% (v/v) fetal calf serum (FCS) from Delta Bioproducts, South-Africa and a penicillin (100 IU/ml) and streptomycin (100 μl/ml) mixture (penicillin-streptomycin) from Gibco-BRL Life Technologies, United Kingdom. All cells were maintained at 37°C in a 5% CO_2 humidified incubator.

Cell cultures were passaged twice weekly, except End1/E6E7 cells, which were sub-cultured at 60% confluency. Cells were passaged with pre-warmed 0.25% trypsin / 0.1% EDTA in calcium- and magnesium-free PBS (Highveld Biologicals, South Africa). For End1/E6E7 only, trypsinisation was terminated by adding 10 mL neutralisation medium (DMEM/F12 (1:1), 1% penicillin-streptomycin and 10% (v/v) fetal calf serum (Delta Bioproducts, South-Africa)). Cell were pelleted by centrifugation and resuspended in culture medium as described above.

All cultures were regularly tested for mycoplasma infection by means of Hoechst staining (Freshney, 1987), and only mycoplasma-negative cell lines were used in experiments.

2.2 Test compounds and antibodies

4-pregnene-3, 20-dione (progesterone; P4), 6α-methyl-17α-hydroxy-progesterone acetate (medroxyprogesterone acetate; MPA), 17α-ethynyl-19-nortestosterone 17β-acetate (norethindrone 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), 17-β-estradiol (1, 3, 5(10)-estratriene), and recombinant mouse 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 (Stanczyk & Roy, 1990). 17β-hydroxy-7α,17α-dimethylestr-4-en-3-one (mibolerone; MIB), 17β-17-hydroxy-17-methyl-estra-4,9,11-trien-3-one (methyltrienolone; R1881) and promegestone (R5020) were purchased from (PerkinElmer Life and Analytical Science, South Africa).

The JNK (SP600125)-, ERK (PD98059)- and p38 (SB230580) inhibitors were purchased from Merck Chemicals, South Africa. Hydroxyflutamide (OHFL) was obtained from Dr C. Tendler (Schering Plough Research Institute, USA). Tumour necrosis factor alpha (TNFα) was kept at -80°C in aliquots at a stock concentration of 0.1 μg/mL in filter sterilized water. Working stock (20 mg/mL) was diluted in End1/E6E7 culture medium and kept at –20°C.

Antibodies to glucocorticoid receptor (GR) (H-300, sc-8992), progesterone receptor (PR) (B-30, sc-811), androgen receptor (AR) (441, sc-7305), mineralocorticoid receptor (MR) (H-300, sc 11412), estrogen receptor alpha (ERα) (MC-20, sc-542) and GR interacting protein 1 (GRIP-1) (M-343, sc-8996) were obtained from Santa Cruz Biotechnology, USA. Antibodies to β-actin (#4967) and GAPDH (#14C10) were purchased from Cell Signaling, South Africa. The anti-histone H3 antibody (ab1791) and anti-rabbit HRP conjugate (NA934VS) were purchased from Abcam, UK and AEC Amersham, South Africa respectively.

2.3 Plasmids

The human MR expression vector, pRShMR, was a kind gift from Ronald Evans (Howard Hughes Medical Institute, La Jolla, USA) and previously described (Arriza *et al*., 1987) while the human AR expression vector, pSVARo (Brinkmann *et al*., 1999) was obtained from Frank Claessens (University of Leuven, Leuven, Belgium). The expression vector for the human PR isoform B, pSG5hPR-B (Kastner *et al*., 1990), was obtained from Stoney Simons Jr (NIH, Bethesda, USA) and the wild type HA-tagged human GR (pCMV-HA-human GR) was a kind gift from Prof. M. J. Garabedian. The expression vector for the human ERα, pSG5-hERα (Denger, 2001) was a gift from F. Gannon (European Molecular Biology Laboratory, Germany) and the estrogen response element (ERE)-containing promoter reporter construct derived from the vitellogenin promoter, ERE.vit2-luc (Curtis & Korach, 1991) was a kind gift from K. Korach (National Institute of Environmental Health Science, USA). The reporter plasmid pTAT-GRE-E1b-luc (Sui *et al*.,, 1999), driven by the E1b promoter that contains two copies of the rat tyrosine aminotransferase containing glucocorticoid response element (TAT-GRE) was obtained from Dr G. Jenster at Erasmus University of Rotterdam, Netherlands. Reporter constructs for selective androgen response elements (AREs), 4xSC-ARE1.2 and 4xSLP-HRE2 (Schauwaers *et al*., 2007) were obtained from Prof. Frank Claessens (University of Leuven, Leuven, Belgium). The pGL2-basic empty vector and cytomegalovirus (CMV)-driven-β-galactosidase expression vector (pCMV-β-gal) were purchased from Promega, Madison, USA and Statagene, USA, respectively. HA-GRIP was a gift from M. R. Stallcup (University of Southern California, USA).

2.4 Transient transfections

End1/E6E7 cells were transiently transfected using the DEAE-Dextran transfection method (Sambrook *et al.*, 1989). For reporter promoter reporter studies, End1/E6E7 cell were plated in 24-well plates at a density of 1 x 10^5 cells per well. Twenty-four hrs after plating cells were transiently transfected with 300 ng pTAT-GRE-E1b-luc plasmid or pGL2-basic empty vector (Promega Corp., USA) and 10 ng pCMV-β-galactosidase (Stratagene, USA) and incubated for 24 hrs prior to treatment. After the 24-hour induction period the medium was aspirated, 50 µl of lysis buffer (Tropic Inc, USA) was added and cells were frozen at -20°C overnight.

For GRIP-1 co-factor over-expression studies, 12-well plates were used. End1/E6E7 cell were plated at a density of 2 x 10^5 cells per well and 24 hrs after seeding cells were transfected with 500 ng pHA-GRIP-1 or pGL2-Basic, empty vector. Briefly, the transfection mixture consisted of 500 ng plasmid DNA, 0.1 mM chloroquine, 0.1 mg/ml DEAE-Dextran and KSF without supplements to a final volume of 500 μL. Transfection medium was added to each well and cells were incubated at 37°C in a humidified incubator for 1 hr, followed by washing of cells with pre-warmed 10% DMSO/PBS. Finally transfected cells were incubated overnight in culture medium at 37°C in a humidified incubator. Cells were treated 24 hrs after transfection and incubated for 24 hrs with various compounds, as described in figure legends. RNA was isolated as described in 2.7

2.5 Promoter reporter luciferase assays

Luciferase assay reagent (Promega Corp., USA) was used to quantify luciferase activity in accordance with the manufacturer's instructions. Briefly, 10 μl cell lysate was allowed to react with 50 μl luciferase assay reagent. The relative light units (RLU's) were measured using the Veritas luminometer (Turner Biosystems, Unites States of America). A further 5 μl cell lysate for each sample was used to measure β-galactosidase activity with the β-galactosidase chemiluminescent Galacto-Star™ reporter gene assay system for mammalian cells (Tropix Inc., USA). Luciferase RLU's were normalized to β-galactosidase readings to control for differences in transfection efficiency between wells, and results were expressed as fold induction compared to vehicle EtOH control (0.1% EtOH) set as 1.

2.6 Small interference RNA (siRNA) transfections

End1/E6E7 cells plated at a density of 1 x 10^5 cells/well in a 12-well culture plate were transfected with 10 nM validated GR HS_NR3C1_5 (cat# SI02654757) siRNA directed against the human GR, 10 nM validated AR Hs_AR_5 HP (cat# SI02757258 siRNA directed against the human AR or validated non-silencing scrambled sequence control (NSC) siRNA (cat#1027310) (Qiagen, Germanstown, MD) using HiPerfect transfection reagent (Qiagen, Germanstown, MD) as per the manufacturers instructions. Briefly, specific or NSC siRNA was diluted in pre-warmed Optimem

medium with GlutaMAX ™ (Gibco-BRL Life Technologies, UK) to which 3.5μL transfection reagent was added. The transfection mixture was incubated at room temperature for 10 min and then added drop-wise to the cells to a final concentration of 10 nM. Cells were incubated for 24 hrs before being treated for 24 hrs with compounds. RNA was then harvested and IL-6 gene expression was analysed by quantitative real time PCR, as described below. Cells which were transfected in parallel were analysed by Western blotting as described below to verify the protein knockdown.

2.7 RNA isolation and cDNA synthesis

RNA was isolated from End1/E6E7 cells using TRI Reagent® (Sigma-Aldrich, South Africa). Briefly, End1/E6E7 cells were plated in 12-well culture plates (Nunc, Denmark) at a density of 1 x 10^5 cells per well. At 70-80% confluency cells were treated with test compounds and incubated as described in figure legends. Induction medium was removed and 400 μL TRI Reagent® was added to cells and incubated for 5 min at room temperature to allow lysis. Lysates were transferred to clean 1.5mL microcentrifuge tubes and 80 μL chloroform was then added. The mixture was then vortexed vigorously for 1 min followed by incubation at room temperature for 15 min. The mixture was centrifuged at 12 200 g (4°C) for 15 min. Equal amounts of the top aqueous phase were transferred to clean microcentrifuge tubes and 200 μL isopropanol was added. Samples were then vortexed for 1 min and incubated for 10 min at room temperature, followed by centrifugation at 12 200 g (4°C) for 10 min to pellet the RNA. The RNA precipitate was sequentially washed twice with 75% EtOH diluted in DEPC (Sigma-Aldrich, South Africa) treated H_2O and centrifuged for 5 min at 12 200g (4°C). The pellet was allowed to air dry for no more than 10 min. RNA was resuspended in 15μL DEPC-treated water and incubated for 5 min at 55°C to allow RNA to dissolve. RNA samples were stored at -20°C.

RNA was quantified 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 denaturing formaldehyde agarose gels (Figure A1.1); (Sambrook *et al.*, 1989). Briefly, 5μL of RNA sample buffer (12% DEPC-H2O, 5% bromophenol blue solution; 6.67% glycerol, 10% 10 x MOPS, 2 M formaldehyde, and 49% formamide)

was added to 0.5 ug (independent of volume) of each RNA sample and incubated at 65°C for 10 min to allow for denaturation of secondary structures, followed by cooling on ice. Samples were then analysed on a 1% denaturing formaldehyde agarose gel in 1 x MOPS buffer (40 mM MOPS; 10 mM sodium acetate; 1 mM EDTA, pH 8.00) at 65V. A representative gel picture is shown in addendum A (Figure A1.1).

RNA (0.5 μg) was reverse transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche, South Africa), primed with anchored 2.5 μM oligo-dT(18) as per manufacturer's instructions in a total reaction volume of 10 μL. Briefly, 0.5 ug RNA and 0.5 μL, oligo-dT (50 μM) was added to DEPC-treated water to a final volume of 6.5 μL. This mixture was incubated at 65°C for 10 min after which samples were placed immediately on ice. Next, 2 μL Transcriptor Reverse Transcriptase reaction buffer, 0.25 μL Protector RNase Inhibitor (40 U/μL), 1 μL dNTP (10 mM) mix (final concentration of 1 mM of each dNTP) and 0.25 μL Transcriptor Reverse Transcriptase (20 U/μl) were added to each sample, mixed carefully and incubated at 50°C for one hour. The reaction was terminated by placing the samples at 85°C for 5 min. Synthesised cDNA samples were kept at –20°C.

2.8 Real time PCR

Equal volumes of synthesised cDNA were used for semi-quantitative real time PCR using the SensiMix dT Kit (Quantace Ltd, UK) and the Corbett real-time PCR machine. Interleukin-6 (IL-6), interleukin-8 (IL-8), and RANTES gene expression were measured using the mRNA specific primer sets (Table 2.1) at a final concentration of 0.3 μM. GAPDH, which served as an internal control, was used at a final concentration of 0.2 μM (Table 2.1). In short, 12.5 μL SensiMix dT, 0.5 μL 50 x SYBR® Green I solution (Quantace Ltd, UK), 1 μL cDNA, sense and anti-sense primers and PCR grade water, to a final reaction volume of 25 μL, made up the real-time PCR reaction mix. PCR conditions for all primer sets were as follows; 95°C for 10 min followed by 40 cycles of 95°C for 10 sec, 60°C for 10 sec and, 72°C for 10 sec. Melting curve analysis was performed to confirm amplification of a single product in each sample (See Addendum A, Figure A1.4.). Relative transcript levels were calculated using the method described by Pfaffl *et al.,* 2001 (Pfaffl, 2001) and were normalised to the relative GAPDH transcript levels.

Table 2.1: Primer sequences of cytokine/chemokines genes investigated

Gene	Primer Sequence (5' to 3')	Strand	Product size (bp)	Annealing Temp (°C)	Primer efficiency*	Reference
IL-6	TCTCCACAAGCGCCTTCG	**Forward**	**193**	**60**	2.22	Wolf *et al.*, (2002)
	CTCAGGGCTGAGATGCCG	**Reverse**				
IL-8	TGCCAAGGAGTGCTAAAG	**Forward**	**197**	**60**	2.02	Wolf *et al.*, (2002)
	CTCCACAACCCTCTGCAC	**Reverse**				
RANTES	TACCATGAAGGTCTCCGC	**Forward**	**199**	**60**	1.99	Wolf *et al.*, (2002)
	GACAAAGACGACTGCTGG	**Reverse**				
GAPDH	TGAACGGGAAGCTCACTGG	**Forward**	**307**	**55**	1.97	Ishibashi *et al.*, (2003)
	TCCACCACCCTGTTGCTGTA	**Reverse**				

*Primer efficiency was determined as described in Addendum A

2.9 Chromatin immunoprecipitation assay

2.9.1 Treatment of End1/E6E7 cells

To evaluate the association of the GR and the co-factor, GRIP-1, with the IL-6 promoter, the protocol described by Ma and co-workers (2003) was followed (Ma, *et al.*, 2003) with a few modifications. Briefly, End1/E6E7 cells were plated at a density of 5 x 10^6 cells per 15 cm^2 culture dish and allowed to reach 80% confluency after which culture medium was replaced with keratinocyte serum free medium not supplemented with BPE, EGF, $CaCl_2$ and PenStrep, followed by incubation for 24 hrs. Cells were treated with steroid for 1 hr prior to the addition of 20 ng/μL TNFα, and then incubated at 37°C for a further 2 hrs.

2.9.2 Formaldehyde crosslinking

Formaldehyde (37%) was added directly to treatment medium to a final concentration of 1%, followed by incubation at 37°C for 10 min to allow for cross-linking of proteins and the chromatin. Thereafter, glycine (1.25 M) was added to a final concentration of 0.125 M and the mixture was incubated for 5 min at room temperature, while shaking on an orbital shaker. Cells were washed twice with ice-cold 1 x PBS. Thereafter 4 ml PBS containing protease inhibitors tablet (1 x Complete Mini Protease Inhibitor Cocktail 1 tablet per 10 mL (Roche Applied Science, South Africa)) was then added to each dish and cells were harvested using a cell scraper, followed by centrifugation for 10 min at 1200 g. The pelleted cells were resuspended in 500 μL nuclear lysis buffer (1% (w/v) SDS, 50 mM Tris-HCL, pH 8.0, 10 mM

EDTA plus 1 tablet 1 x Complete Mini Protease Inhibitor Cocktail per 10 mL). Cell lysates were placed on ice and sonicated to allow for fragmentation of DNA to fragments of between 150 and 500 bp's (see Addendum A, Figure A2.1). Cells were sonicated on Power 3, for 10 cycles at 20 sec per cycle, with 40 sec intervals between pulses, using the Misonix Sonicator® 3000 sonicator. Sonicated chromatin was centrifuged for 10 min at 15 000 g at 4°C to pellet cell debris and the supernatant was transferred to a clean microcentrifuge tube followed by spectrophotometry of the sonicated lysate to measure the amount of A_{260} units/µl. Measuring the absorption at 260 nm allows for normalisation of the amount of sonicated chromatin DNA for the immunoprecipitation step. Nuclear lysis buffer was used to dilute samples to equal chromatin concentration. Sonicated chromatin was either stored at –80°C or prepared immediately for immunoprecipitation. 30 µg of diluted sonicated chromatin was made up to a volume of 30 µL with nuclear lysis buffer, followed by dilution with 90 µL IP dilution buffer (plus 1 x Complete Mini Protease Inhibitor Cocktail 1 tablet per 10 mL). This mixture served as the input sample.

2.9.3 Preparation of the sonicated cell lysate for immunoprecipitation

A total of 100 µg sonicated chromatin in 100 uL nuclear lysis buffer was diluted with 900 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 plus 1 x Complete Mini Protease Inhibitor Cocktail 1 tablet per 10 mL). Chromatin was pre-cleared with 20 µl 50:50 (v/v) pre-blocked Protein A/G PLUS beads (sc-2003, Santa Cruz Biotechnology, USA) for 1 hr on a rotating wheel at 4ºC to reduce non-specific binding. Pre-blocked Protein A/G PLUS beads were prepared by incubating 500 µl of pure beads with 2 ml IP dilution buffer, salmon sperm DNA (final concentration 0.2 mg/ml) and BSA (final concentration 1 mg/ml) for 1 h on a rotating wheel at 4ºC. After centrifugation the beads were re-suspended as 50% slurry in IP dilution buffer. Pre-cleared chromatin was centrifuged at 15 000g for 10 min at 4ºC to pellet beads and the supernatant was transferred to a clean microcentrifuge tube followed by the addition of 2 µg primary-antibody (anti-GR, anti-GRIP-1 or anti-IgG antibody). This mixture was incubated overnight at 4ºC 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 hrs at 4 ºC on a rotating wheel to allow the capture of the immuno-sample complexes. The samples were then

centrifuged at 5000 g for 1 min at 4ºC and the pellet was washed sequentially with 1 mL each of wash buffers I, II, and III (wash buffer I: (0.1 % (w/v) 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% (w/v) 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 pH 8.0) to remove DNA and proteins non-specifically associated with the protein A/G PLUS beads. This was followed by three washes with 1 ml TE (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 of elution buffer (1% (w/v) SDS and 100 mM $NaHCO_3$) for 15 min on a rotating wheel. The mixture was then centrifuged at 5000 g for 1 min and the eluted complexes were transferred to a clean microcentrifuge tube. The eluates were pooled and DNA was purified as described below.

2.9.4 Isolation and purification of DNA associated with the immunoprecipitated protein

To reverse the cross-linking, the eluted DNA-protein complexes, as well as input samples, were incubated at 65°C overnight after the addition of 5M NaCl to a final concentration of 300 nM. This was followed by a further incubation at 45°C for 1 hr in the presence of 15 nM EDTA, 125 nM Tris-HCL and 60 ng/mL proteinase K (Roche Applied Science, South Africa) to digest the protein. Both immunoprecipitated- and input DNA were purified using the QIAquick® PCR Purification Kit (Qiagen, USA) according to the manufacturer's instructions.

2.9.5 Analysis of DNA coupled with the immunoprecipitated protein

Purified immunoprecipitated and input DNA were analysed by means of real-time PCR using primers specific for the IL-6 promoter (hIL-6 sense, 5'-GCGCTAGCCTCAATGACGACCTAAG-3' and hIL-6 antisense, 5'-GAGCCTCAGACATCTCCAGTCCTAT-3') (VanDen Berghe *et al.*, 2006). Conditions for the real-time PCR reactions were as follows; 95°C for 10 min followed by 40 cycles of 95°C for 10 sec, 50°C for 10 sec and, 72°C for 10 sec. The volume of template DNA used was 2 μL for input- or 4 μL immunoprecipitated samples and 0.4 μM sense and antisense IL-6 primers. Both melting curve analysis and agarose gel

electrophoresis were performed to confirm specific product amplification in each sample. Relative protein recruitment was determined using real-time PCR and calculated by the method described by Pfaffl (2001) with slight modifications (Pfaffl 2001) as the primer efficiency was assumed to be 2 and normalised relative to input, which was set as one.

2.10 Subcellular nuclear fractionation assay

End1/E6E7 cells, plated at a density of 2 x 10^5 cells/well in a 6-well culture dish (Nunc, Denmark), were grown to 80% confluency after which culture medium was aspirated and replaced with KSF medium not supplemented with BPE, EGF, and $CaCl_2$, followed by incubation for 24 hrs. Cells were pre-treated with steroid or vehicle (EtOH) for 1 hour before TNFα stimulation (20 ng/μL) and further incubated for 2 hrs, after which they were washed with ice-cold 1 x PBS. Buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$ 10 mM KCl, 0.5 mM DTT, 0.05% (v/v) NP40, 1 proteinase minitablet (Roche, South-Africa)) was added to cells (100 μL per well) and cell lysates were harvested by scraping. Lysates were placed in 1.5 mL micocentrifuge tubes and tubes were placed on ice for 10 min. Lysates were centrifuged for 5 min at 3000 rpm at 4°C (Eppendorf 5417R). The supernatant was transferred to a clean tube and represented the cytoplasmic fraction. The nuclear pellet was washed with 1 mL 1 x PBS and centrifuged at 3000 rpm for 5 min. The pellet was re-suspended in 80 μL DNAse I buffer (40 mM Tris pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$) and 5 μL DNAse I enzyme was added, followed by incubation for 10 min at 37°C. To 80 μL of both cytoplasmic and nuclear fractions, 20 μL 5 x SDS-PAGE loading buffer (100 mM Tris-HCL pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% ß-mercaptoethanol and 0.1% (w/v) bromophenol blue) (Sambrook *et al.*, 1989) was added. For SDS-PAGE and Western blot analysis, samples were incubated at 100°C for 10 min followed by SDS-PAGE as described below (see 2.11). For Western blotting, hybridisation was performed with antibodies raised against GR, GAPDH, and H3. GAPDH and H3 served, as controls for cytoplasmic and nuclear fractions, respectively as well as loading controls (see Table 2.2).

2.11 Western blot analysis

For all Western blot analysis protein samples were separated by SDS polyacrylamide gel electrophoresis (PAGE) at 200 V in running buffer (25 mM Tris-HCl pH 6.8, 250 mM glycine and, 0.1% SDS (Sambrook *et al.*, 1989)) using a BioRad Mini Protean® II electrophoresis cell. Proteins were electroblotted onto Hybond™ ECL™ nitrocellulose membrane (AEC Amersham Biosciences, South Africa) for 90 min at 180 mA with BioRad Mini Trans-blot® cell in ice-cold transfer buffer (25 mM Tris, 200 mM glycine, 10% (v/v) methanol). Membranes were blocked in 10% (w/v) fat-free milk powder in Tris buffered saline (50 mM Tris, 150 mM NaCl) (TBS) containing 0.1% (v/v) Tween (TBS-T), unless otherwise stated, for one hr at room temperature, followed by incubation with primary antibody overnight at 4°C. After incubation with primary antibody the membranes were washed for 15 min and 2 x 5 min in TBS-T at room temperature and incubated with the appropriate secondary HRP conjugated antibody at room temperature for one hr. The membranes were subsequently washed as before. For antibodies dilutions see Table 2.2 below.

Table 2.1: Antibody concentrations used in Western blot analysis

Antibody	Dilution	Dilutant
GR-H300	1:4000	5% non-fat milk powder in 0.1% TBST*
AR (441)	1:4000	5% non-fat milk powder in 0.1% TBST
MCR (C-19)	1:1500	0.1% TBST
PR (B-30)	1:500	0.1% TBST
ER (MC-20)	1:1000	5% non-fat milk powder in 0.1% TBST
β-actin	1:1000	5% non-fat milk powder in 0.1% TBST
phospho-GR Ser-221	1:10 000	5% non-fat milk powder in 0.1% TBST
phospho-GR Ser- 226	1:10 000	5% non-fat milk powder in 0.1% TBST
GAPDH	1:1000	5% non-fat milk powder in 0.1% TBST
H3	1:1000	5% non-fat milk powder in 0.1% TBST

*TBST: Tris buffered saline (50 mM Tris, 150 mM NaCl) (TBS) containing 0.1% (v/v) Tween

Autoradiography and ECL visualisation were performed with AEC Amersham Hyperfilm™ MP high performance autoradiography film (AEC Amersham, South Africa). Autoradiograms were scanned and densometric analysis was performed using AlphaEaseFC™ Software (AlphaInnotech, USA).

2.12 Data and statistical analysis

GraphPad Prism® version 5.00 for Windows (GraphPad Software, USA) was used for graphical representations and statistical analysis. One-way ANOVA was performed with Dunnett's multiple comparison's test as post-test (when comparing treatment conditions to control (EtOH) only) or Tukey's post-test (when comparing all values to each other). For grouped analysis, i.e. how the response is affected by two factors, two-way ANOVA was used for statistical analysis with Bonferroni as post-test. P values for comparison of two samples were obtained by using the paired t-test. P-values are represented as follows: $p < 0.05$ by *, $p < 0.01$ by **, and $p < 0.001$ by ***. Where all values were compared to each other, different lower-case letters indicate statistically significant difference; therefore conditions with the same letter are not statistically significantly different from each other ($p > 0.05$), while those having different letters are statistically significantly different from each other ($p < 0.05$). For all experiments, unless otherwise indicated, the error bars represent the standard error of the mean (SEM) of three independent experiments.

CHAPTER THREE

RESULTS & DISCUSSION

Differential cytokine gene regulation by the synthetic progestins, medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A) in a human endocervical epithelial cell line

Abstract

Norethisterone (NET) and its derivatives, and medroxyprogesterone acetate (MPA) are widely used as injectable contraceptives and in hormone replacement therapy, although little is known about their molecular mechanisms of action. While MPA has been shown to increase HIV (Mostad *et al.,* 1997) and HSV cervical shedding in HIV-infected women (Mostad *et al.,* 2000), the effects of MPA and NET-A on gene expression in the female genital tract are not well understood. In this study, regulation of TNFα-stimulated cytokine/chemokine genes for interleukin (IL)-6, IL-8, and regulated upon activation, normal T cell expressed and secreted (RANTES) by MPA and NET-A, as compared to the endogenous hormone P4, was investigated by real time PCR in an *in vitro* cell culture model for the female endocervical mucosa, namely the immortalised human endocervical epithelial cell line End1/E6E7. Interestingly, the synthetic progestins were shown to have pro-inflammatory effects, increasing expression of the cytokine/chemokine genes investigated. Both MPA and NET-A significantly upregulated IL-8 and RANTES gene expression, unlike P4, which did not elicit a significant response. IL-6 mRNA levels were differentially upregulated by P4, MPA, and NET-A, showing progestin-specific effects on the same gene. For the first time it was shown that proteins for the glucocorticoid receptor (GR), estrogen receptor (ERα), androgen receptor (AR), and mineralocorticoid receptor (MR) are expressed in the End1/E6E7 cell line, although only the GR and ERα are transcriptionally active. Using both GR siRNA and a GR antagonist, it was shown that P4, MPA, and NET-A induce IL-6 gene expression predominantly via the GR, in contrast to DEX, a potent GR agonist, which repressed IL-6 gene expression via the GR. The involvement of the PR-A and the ERα could not be excluded. Different MAPK signalling pathways appear to be responsible for the differential IL-6

gene regulation by the compounds. Using MAPK inhibitors, both ERK1/2 and p38 signalling pathways were shown to be involved in NET-A-mediated IL-6 gene regulation whereas only p38 is required for P4-induced IL-6 mRNA expression. In contrast ERK1/2 and JNK appear to inhibit MPA-induced IL-6 gene expression, although significance could not be established. These different kinase pathways may play a role in the differential effects of the progestins in promoter-specific gene expression. Differential gene regulation by the synthetic progestins compared to P4 via the GR may have important implications for women's risk of susceptibility to infections.

Introduction

Epithelial cells lining the female reproductive tract (FRT) not only serve as a physical barrier against microbial infection but they also express a wide variety of immune mediators such as cytokines and chemokines, aiding in both innate and adaptive immunity (Wira *et al.,* 2005a; Wira *et al.,* 2005b). Interleukin (IL)-6 and the chemotactic cytokines or chemokines, IL-8 and Regulated-upon-activation-normal-T-cell-expressed-and-secreted (RANTES), are expressed in vaginal and cervical epithelial cells, both primary and immortalised (Fichorova & Anderson 1999; Woodworth & Simpson, 1993; Barclay *et al.,* 1993). IL-6 gene expression can be induced by viral infection (Malejczyk *et al.,* 1991; Partridge *et al.,* 1991) and exposure to the pro-inflammatory cytokines IL-1β and tumour necrosis factor-alpha (TNFα) (Yoshizaki *et al.,* 1990; Zhang *et al.,* 1990). IL-6 activates T-cells, causing them to differentiate, regulates biosynthesis of acute phase proteins in hepatocytes and plays a role in cervical dilation and tumour angiogenesis (Hirano *et al.,* 1990; Jones *et al.,* 2005). IL-8 recruits neutrophils, eosinophils and macrophages (Huber *et al.,* 1991; Kelly *et al.,* 1994; Hoffmann *et al.,* 2002) and RANTES attracts T lymphocytes, eosinophils and monocytes (Schall *et al.,* 1990) to the site of infection.

Numerous pathogens such as herpes simplex virus (HSV), human papillomavirus **(**HPV), and human immunodeficiency virus (HIV) have been shown to infect epithelial cells of the lower FRT, and the process is affected by treatment with hormones such as progesterone (P4) (Brabin, 2002). The effects of steroidal hormones are variable and include increased susceptibility to infection, disease progression, as well as increased

risk of re-infection. These effects are also subject to variability with varying hormone levels (Sonnex, 1998; Forrest, 1991).

Progestin treatment of animals (mice and non-human primates) and humans has been reported to increase susceptibility to viral and bacterial infection (Kaushic *et al.*, 2003; Gillgrass *et al.*, 2003; MacLean 2005; Marx *et al.*, 1996; Trunova *et al.*, 2006; Morrison *et al.*, 2004). Rhesus macaques treated with injectable medroxyprogesterone acetate (MPA) five weeks prior to exposure to simian HIV were more susceptible to infection than MPA-naïve macaques irrespective of inoculum dose (Trunova *et al.*, 2006). The immunosuppressive effect of MPA was also reported to be associated with an increase in viral diversity in rhesus macaques due to acceleration in viral replication (Trunova *et al.*, 2006). This study also identified the effects of MPA on immunity to be immune based (i.e. involving immune regulators), rather than transmission based. Additionally, MPA treatment in mice increases susceptibility to HSV infection by decreasing immune responses (Kaushic *et al.*, 2003; Gillgrass *et al.*, 2003). A prospective cohort study, which included 819 women, reported that injectable contraceptive users were more susceptible to both chlamydia and gonococcal infections than oral contraceptive users (Morrison *et al.*, 2004). Additionally, increases in both HIV and HSV shedding have been reported in women using injectable contraceptives (Mostad *et al.*, 1997; Mostad *et al.*, 2000; Wang *et al.*, 2004) as well as the presence of more viral variants and higher viral loads in MPA users infected with HIV than non-users (Lavreys *et al.*, 2004). In contrast there are studies that have reported no correlation between hormonal contraception and an increase HIV infection or viral shedding (Myer *et al.*, 2007; Kleinschmidt *et al.*, 2007; Morrison *et al.*, 2007; McClelland *et al.*, 2002). A South African prospective study with 4200 women participants, showed no correlation between HIV acquisition and usage of injectable contraceptives norethisterone-enathate (NET-EN) or MPA (Myer *et al.*, 2007). In similar studies no evidence of an association between HIV infection and injectable contraceptives (MPA & NET-EN) was shown (Kleinschmidt *et al.*, 2007; Morrison *et al.*, 2007). Another prospective cross-sectional study reported that hormonal contraceptive usage did not detectably increase cervical HSV shedding (McClelland *et al.*, 2002). Nevertheless, the majority of evidence would suggest some adverse effects associated with synthetic progestins including cervical ectopy (Mauck *et al.*, 1999; Morrison *et al.*, 2004; Critchlow *et al.*, 1995), which in turn has been

shown to be linked to higher HIV-1 shedding in women (Morrison *et al.,* 2004; Moss *et al.,* 1991; Kreiss *et al.,* 1994; Royce *et al.,* 1997). MPA has also been reported to induce hyperplasia of vaginal epithelium in women (Ildgruben *et al.,* 2003) and it is regularly used as an immuno-compromising agent to induce viral infectability in mice (Parr *et al*., 1994). *In vitro* studies have reported that MPA has anti-inflammatory properties as it decreased IL-6 and IL-8 protein and gene expression in mouse fibroblast cells (Koubovec *et al.,* 2004) and IL-2, IL-1, and IL-6 protein expression in normal human lymphocytes, via the glucocorticoid receptor (GR) (Bamberger *et al.,* 1999). These studies suggest that progestins have an effect on the immune response and play an important role in female reproductive health.

The above studies have prompted a more detailed investigation into the role of synthetic progestins in cytokine gene regulation in the endocervix, a key physiological component of the lower FRT, which is vulnerable to infections. MPA and NET-EN are extensively used as progestin-only injectable contraceptives especially in sub-Saharan Africa, where infections such as HIV are rife. In addition MPA and norethisterone acetate (NET-A) are used in hormone replacement therapy (HRT) and in the treatment of endometriosis and cancer (Sitruk-ware 2003; Cavalli *et al.,* 1984; Schindler *et al.,* 2003). Both NET-EN and NET-A are metabolised to the active molecule NET, as well as other metabolites, unlike MPA, which is itself the active compound (Stanczyk & Roy, 1990). The synthetic progestins MPA and NET are steroidal synthetic hormones designed to mimic the biological effects of the endogenous hormone P4 (Bray *et al.,* 2005). The progestational effects of P4 are mainly attributed to acting via its cognate receptor, the progesterone receptor (PR) (Sitruk-ware, 2004). Indeed both MPA and NET bind with similar affinities as P4 to the PR (Bray *et al.,* 2005). However, studies have shown that both MPA and NET can bind to other steroid receptors such as the GR, androgen receptor (AR), mineralocorticoid receptor (MR) and estrogen receptor (ER) (Philibert *et al.,* 1999; Bentel *et al.,* 1999; Hapgood *et al.,* 2004; Koubovec *et al.,* 2005; Africander PhD thesis, 2010).

While MPA, NET, and P4 bind with a high affinity to the AR, only MPA and NET, but not P4, are strong agonists of the AR (Africander PhD thesis 2010; Bentel *et al.,* 1999; Hoppen & Hammann, 1987). Furthermore, P4 binds with high affinity to the MR, unlike MPA and NET (Winneker *et al.,* 2003; Palacios *et al.,* 2006; Africander PhD

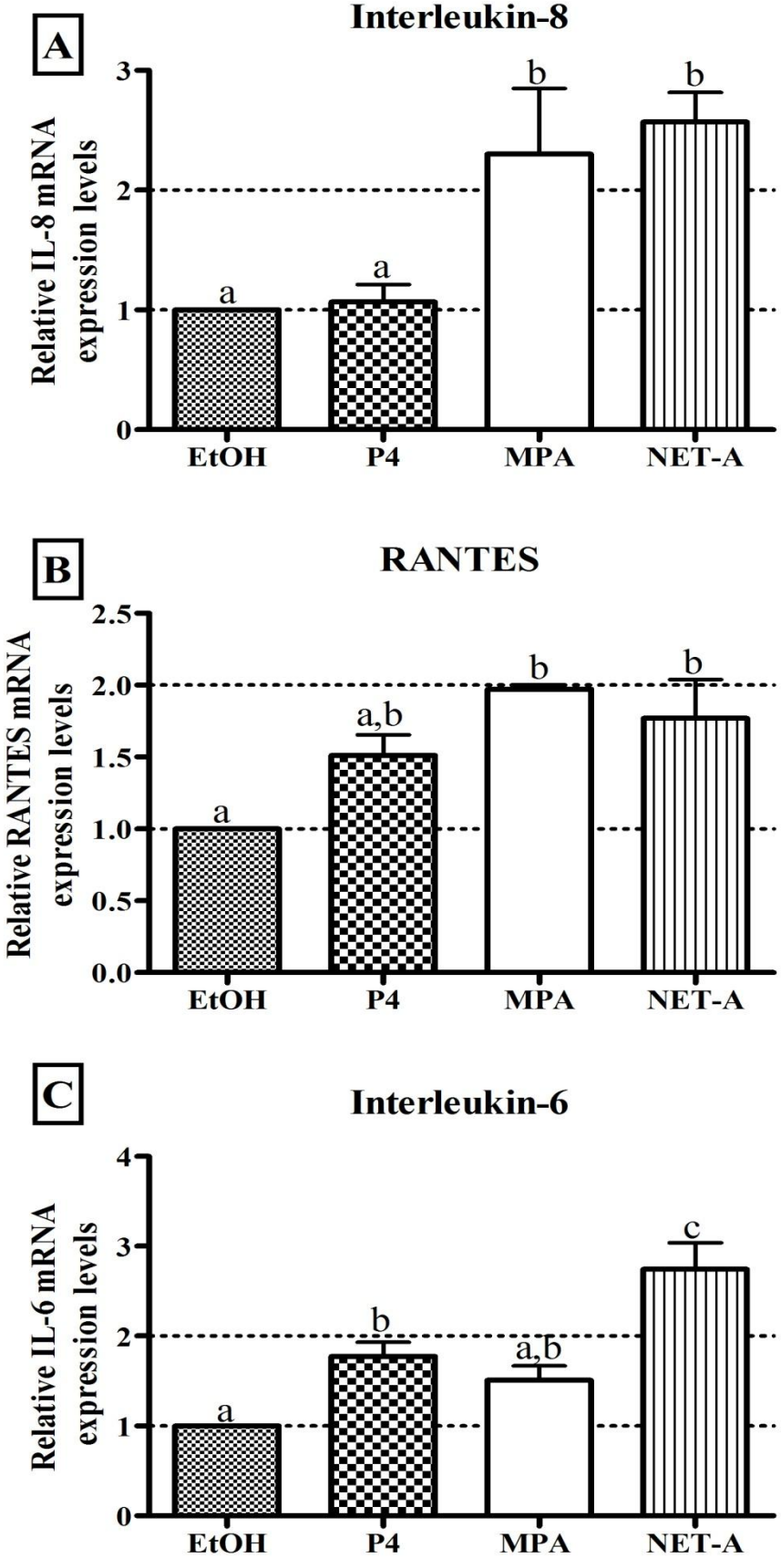
thesis 2010). MPA has a relatively strong binding affinity for the GR as compared to NET (Kontula *et al.*, 1983; Koubovec *et al.*, 2005). In addition, while MPA is an agonist of the GR, NET is an antagonist (Africander PhD thesis 2010). Moreover, NET-EN metabolites were shown to bind to the ER, albeit very weakly (Markiewicz & Gurpide 1994; Mendoza-Rodríguez *et al.*, 1999). Although MPA and NET elicit similar progestational effects to P4 (Sitruk-ware 2004; Bray *et al.*, 2005), differences in biological effects mediated via steroid receptors others than the PR could be expected (Hapgood *et al.*, 2004). Women using progestins either in HRT or as contraception may become more susceptible to viral and bacterial reproductive tract infections as these hormonal contraceptives could modulate the immune function of the cervical mucosae (Morrison *et al*., 2004b). Unfortunately, very few studies have directly compared the molecular mechanism of action of MPA and NET and no study has ever investigated the effect of these progestins on cytokine gene regulation in endocervical epithelial cells. The effect of these compounds on immune responses in the endocervix, a site vulnerable to infection, has not been determined. Using a human immortalised endocervical (End1/E6E7) epithelial cell line (Fichorova *et al.*, 1997) as a model for the mucosal surface of the endocervix, the molecular mechanisms involved in the regulation of endogenous IL-6, IL-8, and RANTES gene expression in response to MPA, NET-A, and P4, were investigated.

Results

MPA and NET-A regulate the expression of pro-inflammatory cytokine genes

Before experimental treatments with the synthetic progestins and P4 commenced, optimisation of cDNA and quantitative real-time PCR (qPCR), including primer efficiency determination, was performed as discussed in Addendum A. The human End1/E6E7 cell line was treated with 20 ng/μL TNFα and 1 μM P4, MPA, or NET-A for 24 hrs. Treatment in the presence of TNFα mimics infection, which upregulates the expression of pro-inflammatory cytokine genes. Total RNA was isolated and expression of IL-6, IL-8, and RANTES was measured by quantitative real-time PCR (qPCR) using specific primers for the relevant genes. The target gene expression was normalised to the housekeeping GAPDH gene and results are depicted in Figure 3.1. Both MPA and NET-A induced IL-8 gene expression (greater than 2-fold), which was significantly different to that of P4 ($p < 0.05$), which had no effect on IL-8 gene

expression (Figure 3.1A). Similarly, MPA and NET-A, but not P4, significantly ($p < 0.05$) increased RANTES mRNA expression by about 2-fold (Figure 3.1B). Interestingly, IL-6 gene expression was differentially regulated by MPA and NET-A ($p < 0.05$). NET-A induced an approximately 2.5-fold increase in IL-6 expression, while MPA showed a 1.5-fold increase, ($p=0.08$) when compared to vehicle control (Figure 3.1C). The finding that the synthetic progestins differentially regulate IL-6 mRNA expression in the presence of TNFα suggests that these two progestins act via different mechanisms. The effects of these progestins on IL-6 gene expression were thus further investigated, including their effects in the absence of TNFα.



(legend to follow on next page)

Figure 3.1: **Effects of P4, MPA and NET-A on the TNFα-induced expression of (A) IL-8, (B) RANTES, and (C) IL-6 in a human endocervical cell line.** End1/E6E7 cells were treated for 24 hrs with 20 ng/μL TNFα in the presence of 0.1% ethanol (EtOH, vehicle), 1 μM P4, 1 μM MPA or 1 μM NET-A. Total RNA was isolated and 500 ng RNA was reverse-transcribed. Relative IL-6, IL-8 and RANTES gene expression was measured by qPCR and normalised to GAPDH mRNA expression, which served as internal control. Relative cytokine/chemokine gene expression of treated samples was calculated relative to vehicle control, which was set as 1. Graphs represent pooled results of at least three independent experiments. For statistical analysis one-way ANOVA was used and Tukey's multiple comparison post-test. Different letters indicate statistically significant differences ($p < 0.05$); while the same letter indicates no statistically significant difference ($p > 0.05$). Using Dunnett's multiple comparisons post-test MPA is significantly different from vehicle control ($p < 0.05$) in C.

TNFα is a potent activator of IL-6 as it increased IL-6 mRNA expression approximately 22-fold (Figure 3.2A). Interestingly, it was found that P4, MPA, and NET-A also upregulated IL-6 expression in the absence of TNFα in the endocervical cell line (Figure 3.2B). NET-A and MPA induced IL-6 mRNA expression in the absence of TNFα approximately 4.5- and 2.5-fold respectively, while P4 increased IL-6 gene expression approximately 3.5-fold (Figure 3.2B). Induction of IL-6 by these progestins shows a similar trend in the absence (Figure 3.2B) and presence (Figure 3.1C) of TNFα i.e. NET-A ≥ P4 > MPA. Statistical differences could not be established due to variability between individual experiments for P4, and MPA, however, NET-A does significantly upregulate IL-6 mRNA expression in the absence of TNFα, $p = 0.043$, compared to vehicle. The results suggest a similar mechanism of action in the absence and presence of TNFα.

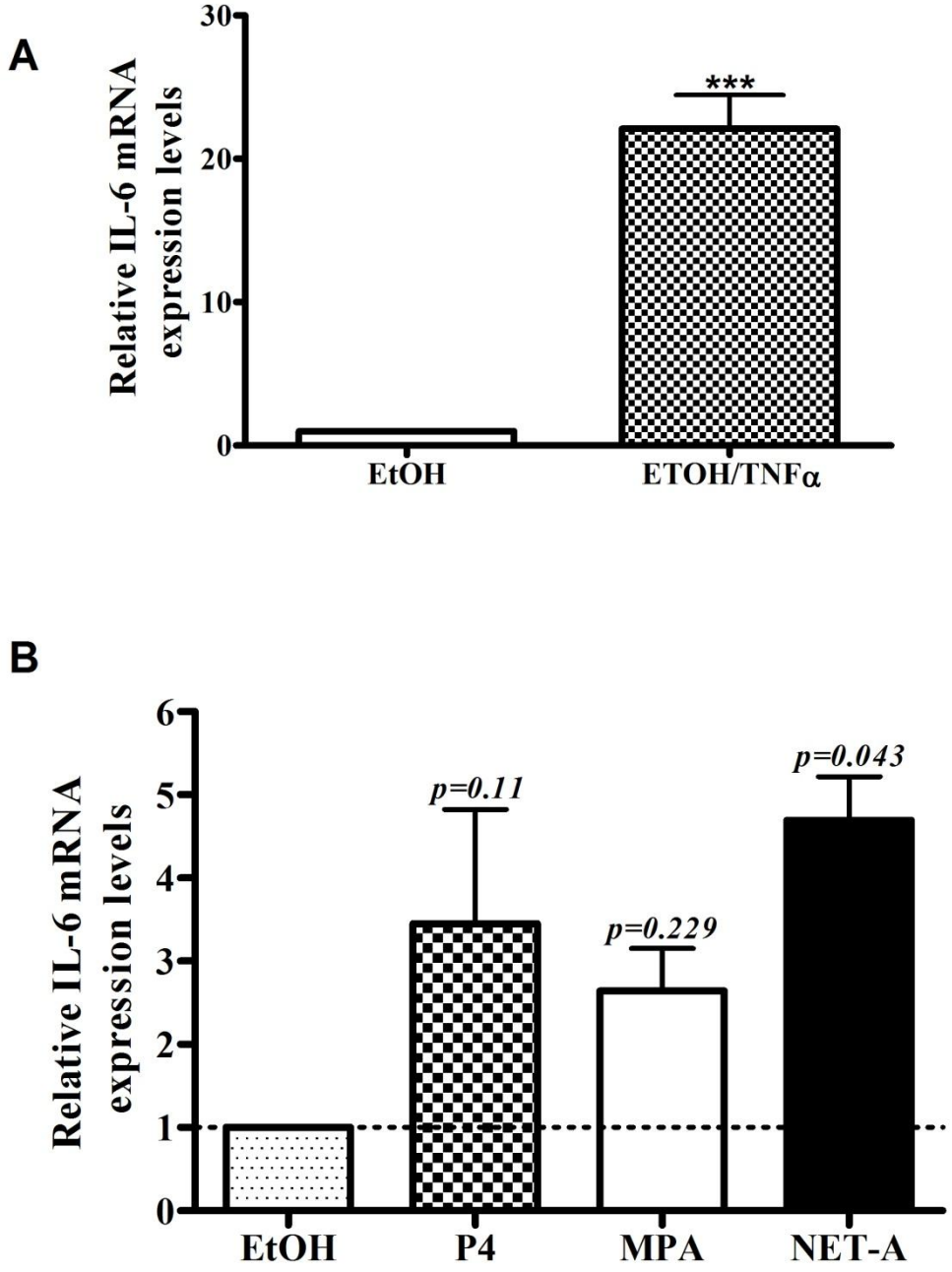


Figure 3.2: (A) TNFα induces IL-6 expression in the End1/E6E7 cell line and (B) P4, MPA and NET-A increase IL-6 expression in the absence of TNFα stimulation in a human endocervical cell line. End1/E6E7 cells were treated with (A) vehicle or 20 ng/μL TNFα or (B) with vehicle (0.1% EtOH) or 1 μM P4, MPA or NET-A for 24 hrs. Total RNA was isolated, cDNA synthesized and expression levels of IL-6 mRNA relative to GAPDH mRNA were determined by qPCR. Relative target gene expression of treated samples was calculated relative to vehicle control, which was set as 1. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least three independent experiments. For statistical analysis paired *t*-test (***; $p < 0.001$) in A was used and one-way ANOVA was used and Tukey's multiple comparison post-test in B.

The AR, MR, ERα, and GR are expressed in the End1/E6E7 cell line

As both MPA and NET-A have been shown to bind to and elicit a biological response via the GR, AR and MR (Koubovec *et al.,* 2005; Africander PhD thesis 2010; Philibert *et al.,* 1999) it was hypothesized that the differential regulation of the IL-6 gene by P4, MPA and NET-A may be due to their actions via different steroid receptors. To this end, the steroid receptor content of the End1/E6E7 cells was investigated. Whole cell lysates of untreated End1/E6E7 cell lines were prepared and subjected to Western blot analysis and protein expression of the PR-B, GR, AR, MR, and ERα (Figure 3.3A-E) was determined. Positive controls consisted either of COS-1 cells transiently transfected with the receptor of interest (GR, PR-B, MR and AR), or whole cell lysate of the HeLa cervical carcinoma cell line for the ER. ERα was shown to be expressed in End1/E6E7, corresponding in size to the positive control, as detected by Western blot analysis (Figure 3.3A). Similarly, the GR and MR are expressed in the End1/E6E7 cells as shown in figures 3.3B and 3.3D, corresponding in size to the positive control (Figures 3.3B and 3.3D). Although a strong band was detected in COS-1 cell lysates transiently transfected with hPR-B, no detectable PR-B protein was observed in End1/E6E7 cells (Figure 3.3C). Since immunohistochemical investigations have previously reported the expression of both PR isoforms (PR-A and PR-B) in primary cervical tissue (Nair *et al.,* 2005; Remoue *et al.,* 2003), it was surprising that Western blotting did not detect PR-B in the End1/E6E7 cells. However, several studies have reported the expression of the PR to be dependent on the menstrual cycle (Stjernholm-Vladic *et al.,* 2004; Konishi *et al.,* 1991). The present result cannot be ascribed to an inefficient antibody, as the positive control was clearly detected. As there are two functional isoforms of the PR, PR-A and PR-B (Kastner *et al.,* 1990; Kraus & Katzenellenbogen 1993), it may be that the PR-A isoform, which is less transcriptionally active than PR-B (Vegeto *et al.,* 1993), is expressed in this cell line. Optimization of the commercial antibody detecting the PR-A isoform was unsuccessful and thus the expression of the PR-A isoform in the End1/E6E7 cell line could not be confirmed. Finally, Western blot analysis also detected the MR expressed in End1/E6E7 cells corresponding in size to the overexpressed MR in COS-1 cells (Figure 3.3D).

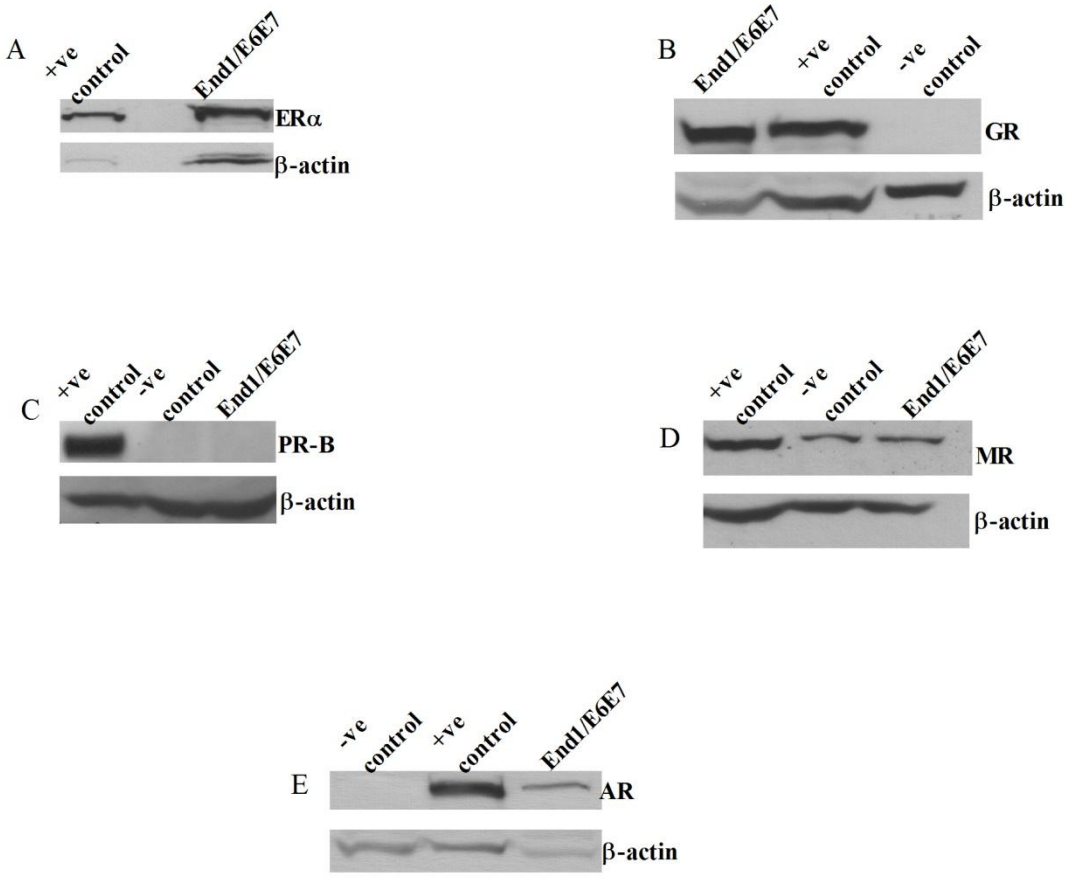


Figure 3.3: GR, AR, MR, and ERα, but not PR-B are expressed in End1/E6E7 cells. Whole cell lysates were prepared from the human End1/E6E7 cell line. Untransfected COS-1 cells served as negative control (-ve). COS-1 cells transiently transfected with steroid receptor expression vectors (pSG5hPR-B, pCMV-HA-hGR, pSVhARo, and pRShMR) served as positive controls (+ve). For ERα, the HeLa carcinoma cervical cell line endogenously expressing ERα, served as positive control (+ve). Equal volumes of lysate were analysed by Western blotting with antibodies against (A) hPR-B, (B) hGR, (C) hAR, (D) hMR, (E) hERα, and β–actin was used as a loading control. It should be noted that proteins on the SDS-gel of (B) migrated slightly skew in the last lane resulting in the β-actin band to be slightly above the position of the previous lane. It should be noted that proteins on the SDS-gel in (B) migrated slightly skew in the last lane resulting in the β-actin band migrating slightly above the position in the previous lane.

Only the GR and ERα are transcriptionally active in the End1/E6E7 cell line

To determine whether the endogenously expressed receptors are transcriptionally active, promoter-reporter assays were performed to measure transactivation activity in the presence of receptor-specific agonists. The results show that only the GR and ERα are transcriptionally active in the End1/E6E7 cell line (Figure 3.4A and 3.4B). For the GR, an ~8-fold induction was observed in the presence of the GR-specific agonist, dexamethasone (DEX), while for ERα, an ~4.5-fold increase was observed in the presence of the ER-specific ligand, 17-β-estradiol (E2). Consistent with the absence

of PR expression observed by Western blotting (Figure 3.3A), no transcriptional response was observed in the presence of the potent PR-specific ligand, R5020, in the End1/E6E7 cells (Figure 3.4A). Although Western blotting showed expression of the MR in the End1/E6E7 cell line (Figure 3.3D), the endogenously expressed MR was transcriptionally inactive, as aldosterone (Ald) the MR agonist, was unable to induce transactivation. Similarly, although Western blot analysis also showed the expression of the AR in the endocervical cells (Figure 3.3C), the AR-specific ligand, mibolerone (MIB), did not increase transcription from either of two AR-responsive promoters (Figure 3.4E). This lack of AR transcriptional activity could not be attributed to an inactive ligand as 1 μM MIB induced transactivation of a GRE-containing reporter plasmid, as well as the 4xSC-ARE1.2 promoter reporter in the presence of transiently overexpressed hAR. The 4xSC-ARE1.2 promoter reporter contains two AR-specific AREs. These resemble a direct rather than a palindromic repeat of the 5'-TGTTCT-3' hexamer. These AREs were previously shown to be AR-specific, as the AR but not GR is able to transactivate via these DNA motifs (Schauwaers *et al.,* 2007).

In summary, transactivation assays revealed that only the endogenous ERα (Figure 3.4A) and GR (Figure 3.4B) are transcriptionally competent. Although Western blot analysis did determine that endogenous MR and AR are expressed in the End1/E6E7 cells, both receptors were transcriptionally inactive on promoter reporter assays. In fact the AR was unable to induce transcription of both GRE- and the AR specific ARE-containing promoters (Figure 3.4E).

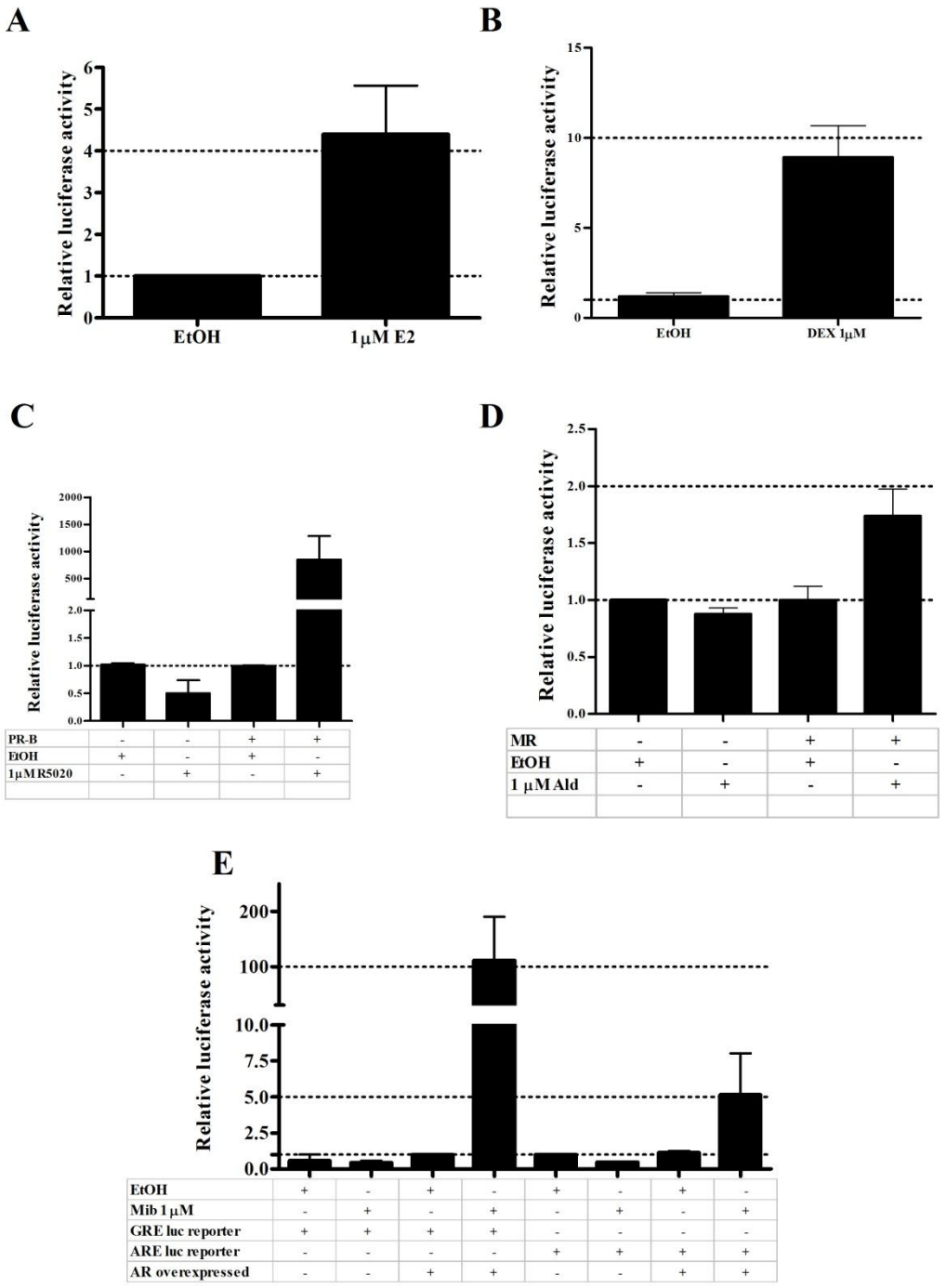


Figure 3.4: Transactivation of transiently transfected promoter-reporter constructs via endogenous or overexpressed steroid receptors. End1/E6E7 cells were transiently transfected with (A) 300 ng pSG5-ERE.vit2.luc promoter reporter construct, 30 ng pGL2-basic empty vector control, and 10 ng β-galactosidase expression plasmid or (B) 300 ng pTAT-GRE-E1b-luc reporter construct, 30 ng pGL2-basic empty vector control, and 10 ng β-galactosidase expression plasmid or (C) 300 ng pTAT-GRE-E1b-luc reporter construct, 30 ng pSG5hPR-B or pGL2-basic empty vector control, and 10 ng β-galactosidase expression plasmid (D) 300 ng pTAT-GRE-E1b-luc reporter construct, 30 ng pRShMR or pGL2-basic empty vector control, and 10 ng β-galactosidase expression plasmid or (E) 300 ng pTAT-GRE-E1b-luc - or 4xSC-ARE1.2-luc reporter construct, 30 ng pSVARo or pGL2-basic empty vector control, and 10 ng β-galactosidase expression plasmid. Twenty-four hrs after

transfection, cells were treated with vehicle control (0.1 % EtOH) or (A) 1 μM of the ER agonist, 17β-estradiol (E2), (B) 1 μM of the GR agonist, dexamethasone (DEX), (C) 1 μM of the synthetic PR agonist, R5020, (D) 1 μM of the MR agonist, aldosterone (Ald), or (E) 1 μM of the AR agonist, mibolerone (MIB), for 24 hrs. Cells were lysed and both luciferase and β-galactosidase activities were measured. Luciferase activity in relative light units (RLU's) was normalised to β-galactosidase activity in RLU's with EtOH in the absence of transfected receptor set as one. Results shown are pooled results of two independent experiments performed in triplicate.

The role of the GR in the regulation of IL-6 by MPA and NET-A in the endocervical cell line

As MPA and NET-A exhibited striking differences in IL-6 regulation, in-depth investigations into the molecular mechanism of differential IL-6 mRNA expression in End1/E6E7 cells by P4, MPA, and NET-A were performed.

Having established that the GR protein is expressed and functional in the End1/E6E7 cells (Figure 3.3B & 3.4B), it was next investigated whether the GR plays a role in IL-6 gene expression in response to MPA and NET-A. MPA is known for its anti-inflammatory effects, which are mainly attributed to its glucocorticoid activity (Koubovec *et al.*, 2004; Bamberger *et al.*, 1999), while NET-A has been reported to be an antagonist of the GR (Africander PhD thesis, 2010). Western blotting was used to determine whether P4, MPA, and NET, in the presence of TNFα, have an effect on GR protein levels. End1/E6E7 cells treated with the progestins in the presence of TNFα for 24 hrs were analysed by means of Western blotting using a GR specific antibody and β-actin as loading control. None of the progestins had any significant effect on GR expression following 24 hr treatment (Figure 3.5A & Figure 3.5B).

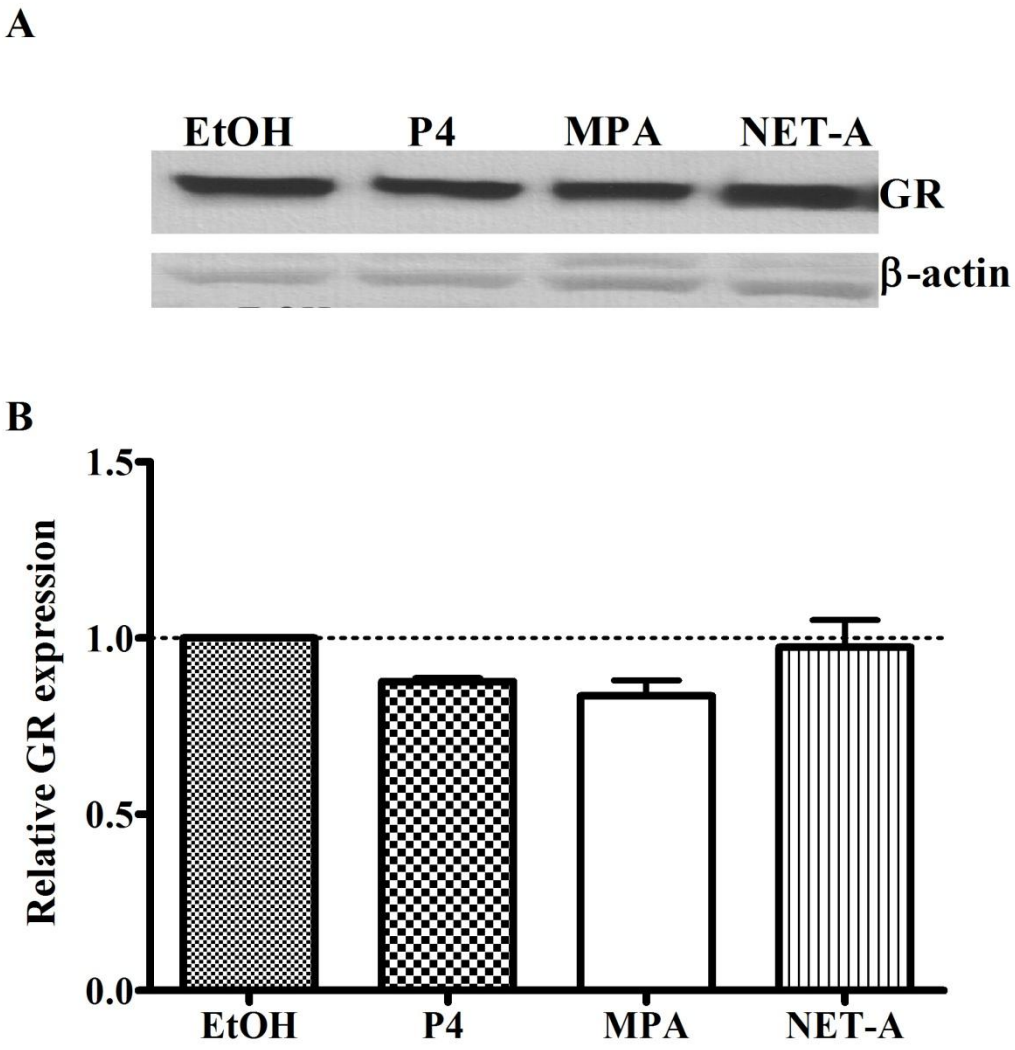


Figure 3.5: **P4, MPA and NET-A do not affect GR protein levels after 24 hrs**. End1/E6E7 cells were treated with 1 μM of each progestin, P4, MPA, or NET-A, in the presence of 20 ng/μL TNFα for 24 hrs. (A) Cells were harvested and whole cell lysates were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane. A GR specific antibody was used for Western blotting analysis and a β-actin specific antibody as loading control. (B) GR protein levels on Western blot were quantified and normalised to β-actin levels. Graphs are representative of pooled results of two independent experiments with vehicle control (EtOH) set as one.

To investigate the possible involvement of the GR in the IL-6 response to progestin treatment, the endocervical cells were treated with 1 μM MPA or NET-A in the presence of TNFα for 24 hrs in the absence or presence of 1 μM of the GR antagonist, RU486. Total RNA was isolated, cDNA synthesised and IL-6 gene expression determined by qPCR. Interestingly, RU486 alone showed a significant upregulation of IL-6 gene expression ($p < 0.001$) (Figure 3.6) of approximately 3-fold. This result was unexpected, as it had previously been shown that 1 μM RU486 in murine fibrosarcoma

L929 cells has no effect on IL-6 protein levels (Vanden Berghe *et al.*, 1999). A slight decrease in of DEX-mediated repression by RU486 was observed, although this was not significant. RU486 had no effect on the response to either NET-A or P4, although a slight increase in the response was observed for MPA (Figure 3.6). When normalising for the induction seen with RU486 alone however, RU486 significantly antagonises P4, MPA, and NET-A- mediated IL-6 induction, suggesting the involvement of the GR (Addendum B, Figure B1).

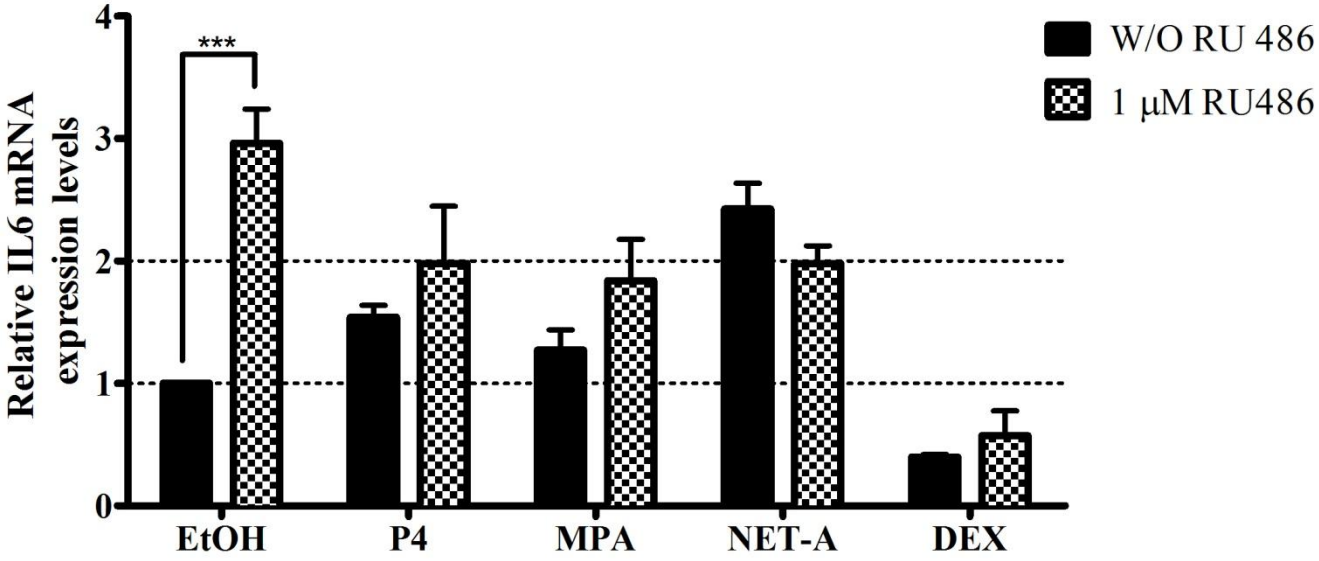


Figure 3.6: The role of the GR in IL-6 gene expression in response to P4 and the synthetic progestins MPA and NET-A in the endocervical cell line End1/E6E7. End1/E6E7 cells were treated with 20 ng/μL TNFα and 1 μM P4, MPA, NET-A, or DEX in the absence or presence of 1 μM RU486. Total RNA was isolated after 24 hrs and 500 ng mRNA was reverse-transcribed. Relative IL-6 mRNA expression was measured by quantitative real-time PCR and normalised to relative GAPDH gene expression, which served as internal control. Relative IL-6 gene expression of treated samples was calculated relative to vehicle control, which was set at 1. Graphs represent pooled results of at least three independent experiments. For statistical analysis two-way ANOVA was used with Bonferroni as post-test (***; $p < 0.001$).

To confirm the results obtained with the GR antagonist, the GR protein expression was reduced, by transfecting End1/E6E7 cells with GR specific siRNA oligonucleotides (oligos). As a control, cells were transfected in parallel with validated non-silencing RNA oligos (NSC). Transfected cells were analysed by Western blotting to confirm GR knockdown (Figure 3.7). Approximately 42% knockdown of GR protein was detected (Figure 3.7).

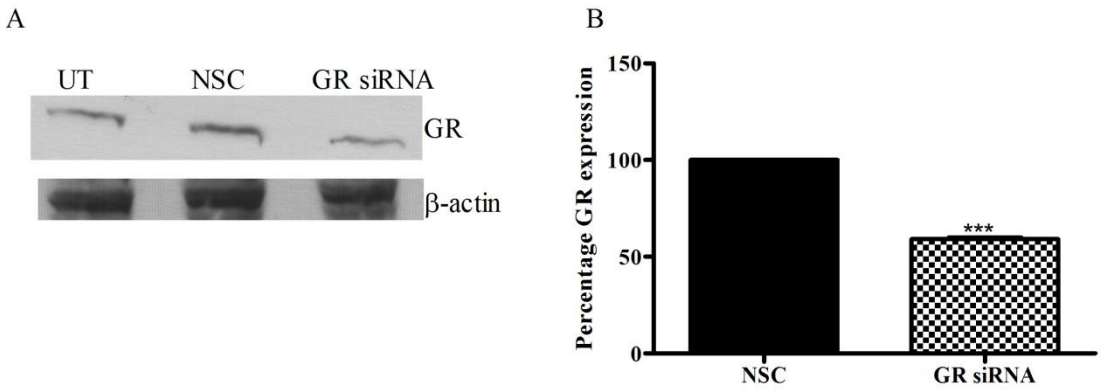


Figure 3.7: Reduction of GR protein by GR siRNA (A) For verification of GR knockdown, End1/E6E7 cells were transfected with 10 nM non-silencing control (NSC) or GR siRNA oligos. Forty-eight hrs after transfection, cells were harvested and whole cell lysates were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane. A GR-specific antibody was used for Western blotting analysis and β-actin-specific antibody as loading control was used. A representative blot is shown. (B) Western blots of at least four independent experiments were quantified to determine the percentage GR protein knockdown. For statistical analysis student *t*-test was used. p value represents $p < 0.001$; *****.**

As expected, DEX-induced repression of IL-6 expression was significantly reduced by the decrease in GR expression (Figure 3.8). NET-A-induced IL-6 mRNA expression was potentiated by the decrease in GR expression, suggesting a role for the GR in NET-A mediated induction of IL-6 mRNA transcripts (Figure 3.8A). For both P4 and MPA a slight increase in IL-6 gene expression was observed ($p = 0.226$ and $p = 0.062$, respectively). Interestingly, the response to RU486 was unaffected by the decrease in GR protein expression (Figure 3.8A). To normalise for the induction seen with GR siRNA alone, the results were also normalised to the vehicle control of each transfection condition (NSC vs GR siRNA) as shown in Figure 3.8B. As expected, DEX-mediated repression of IL-6 was abrogated by GR knockdown ($p = 0.08$) (Figure 3.8B). Furthermore, reduction in GR protein levels significantly ($p < 0.05$) reduced P4, NET-A and RU486 induced IL-6 mRNA expression (Figure 3.8B). In addition, a decrease in MPA-induced IL-6 mRNA expression ($p = 0.062$) was also observed in the presence of GR siRNA.

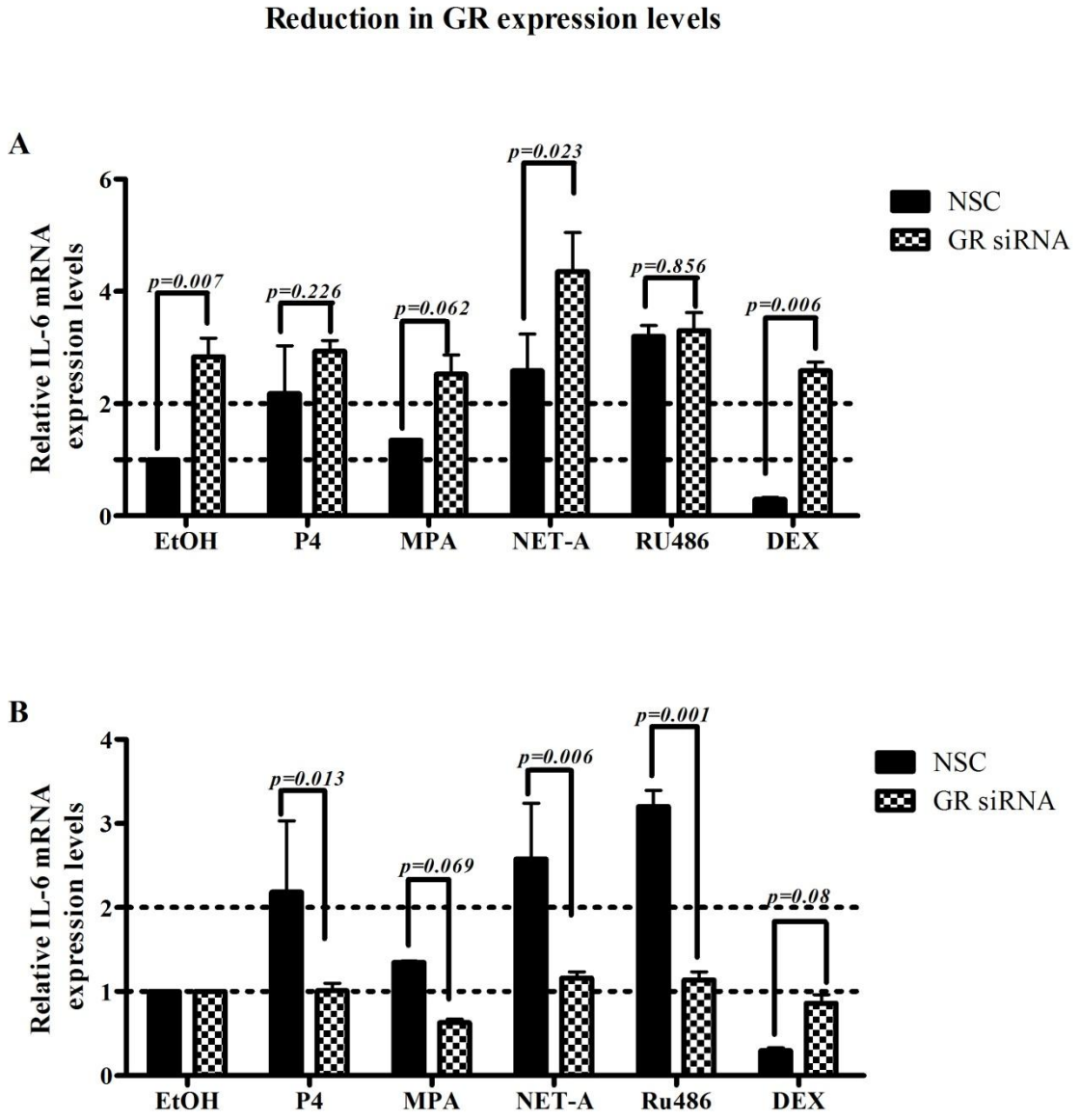


Figure 3.8: Effect of reduction of GR levels by siRNA on IL-6 gene expression in response to P4 and the synthetic progestins MPA and NET-A in the endocervical cell line End1/E6E7. End1/E6E7 cells were transfected with 10 nM NSC or GR siRNA and then treated with 1 μM P4, MPA, NET-A, RU486, or DEX for 24 hrs in the presence of 20 ng/μL TNFα. Total RNA was isolated after 24 hrs and 500 ng RNA was reverse-transcribed. Relative IL-6 mRNA expression was measured by quantitative real-time PCR and normalised to relative GAPDH gene expression, which served as an internal control. Relative IL-6 gene expression of treated samples was calculated relative to vehicle control. Graphs presented are differently normalised. Results in (A) were normalised to EtOH of NSC transfection, which was set as 1 and in (B) each different transfection condition was normalised to its own EtOH, which was set as 1 (i.e. all the black bars were normalized to NSC EtOH and all the checked bars normalized to GR siRNA EtOH). Graphs represent pooled results of at least three independent experiments. For statistical analysis two-way ANOVA was used with Bonferroni as post-test. P-values are presented on the graph.

Taken together, the results suggest that the GR is required for the upregulation of IL-6 gene expression by RU486, P4, and the synthetic progestins MPA and NET-A and for the repression by DEX (Figure 3.8B).

Different MAPK pathways are involved in P4-, MPA-, and NET-A-induced IL-6 gene expression

Both P4 and MPA are reported to activate ERK1/2 in hippocampal neurons, although only P4 induced ERK1/2 nuclear translocation in these cells (Nilsen & Brinton 2003). To the best of the author's knowledge no studies have investigated whether NET-A can activate the mitogen activated pathway kinases (MAPKs). Thus the involvement of members of the MAPK family of kinases i.e. extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK1/2), and p38 kinase pathway was next investigated, to determine whether they play a role in TNFα-induced IL-6 gene expression in response to P4, MPA, and NET-A (Figure 3.9).

MAPK inhibitors were used to determine the involvement of MAPKs in IL-6 gene expression in response to the progestins. Inhibitors of both the ERK1/2 and p38 pathways were found to abolish NET-A induced IL-6 mRNA expression ($p < 0.05$) (Figures 3.9A & 3.9B), while only the p38 MAPK inhibitor significantly repressed P4-induced IL-6 mRNA expression (Figure 3.9B). Neither the ERK nor the JNK pathway inhibitor showed any effect on P4-induced IL-6 expression (Figures 3.9A & 3.9C). Minor, non-significant differences were seen in the presence of all the MAPK inhibitors in co-treatment with MPA (Figure 3.9A-C). A slight reduction in IL-6 mRNA expression was observed in response to MPA in the presence of the ERK pathway- and JNK inhibitors (Figures 3.9A & 3.9C), opposite to the effect seen with NET-A. As observed for NET-A, the p38 inhibitor decreased the small induction of IL-6 mRNA expression in response to MPA treatment (Figure 3.9B). In summary, ERK1/2, p38, and JNK1/2 pathways appear to differentially regulate P4-, MPA- and NET-A-induced IL-6 gene regulation (Figure 3.9A-C) in the endocervical epithelial cell line End1/E6E7.

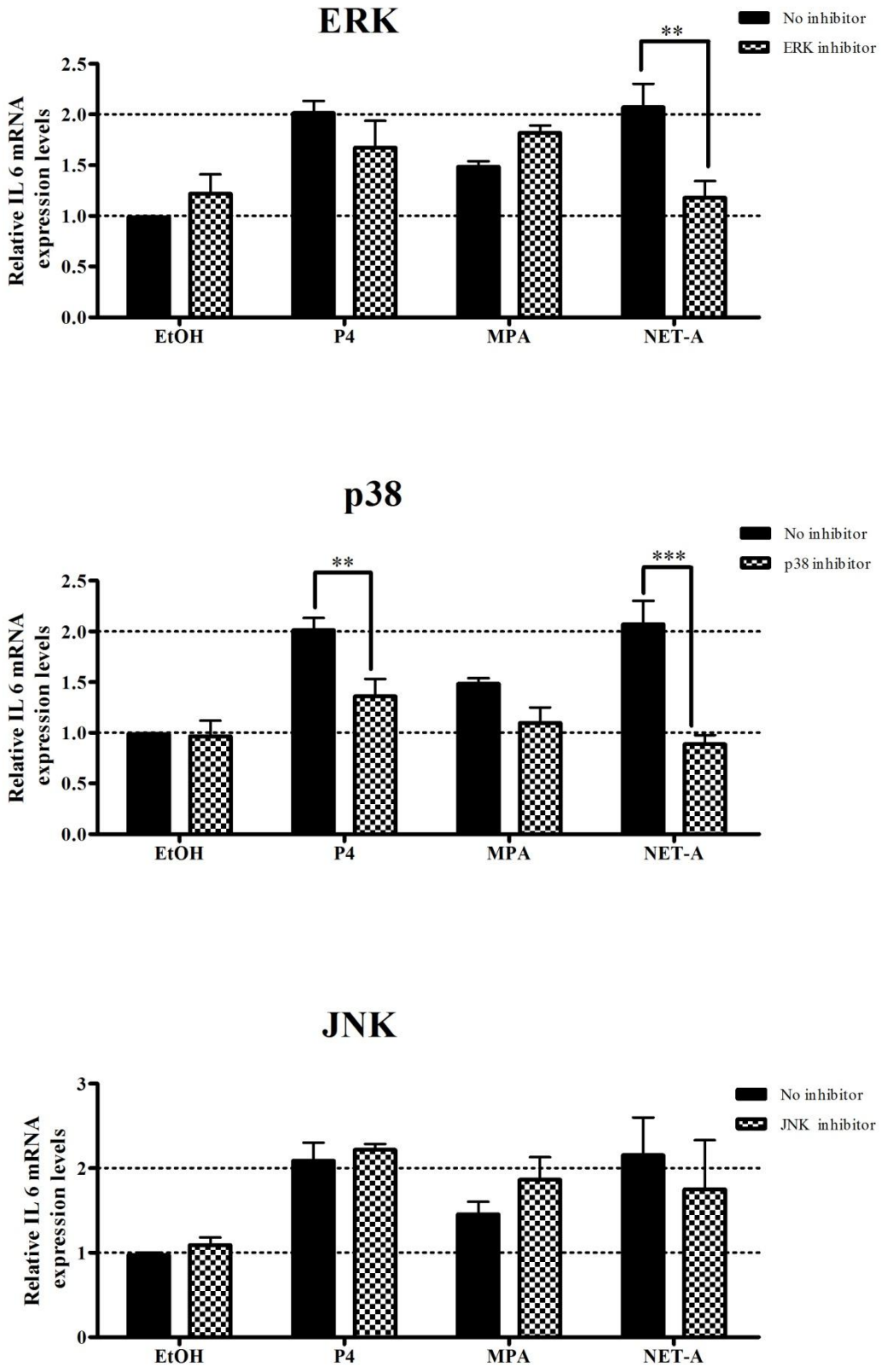


Figure 3.9: Effects of MAPK inhibitors on IL-6 gene expression in response to the synthetic progestins, MPA and NET-A. End1/E6E7 cells were treated with 20 ng/μL TNFα and 1 μM P4, MPA, or NET-A in the absence (no inhibitor) or presence of 10 μM MAPK inhibitors, (A) ERK1/2 inhibitor (PD 98059), (B) p38 inhibitor (SB 203580), or (C) JNK inhibitor (SP 600125). Total RNA was isolated after 24 hrs of treatment and 500 ng

RNA was reverse-transcribed. Relative IL-6 mRNA expression was measured by quantitative real-time PCR and normalised to relative GAPDH gene expression, which served as internal control. IL-6 gene expression of treated samples was calculated relative to vehicle control without inhibitor, which was set as 1. The graphs represent pooled results of at least three (A) & (B) or two (C) independent experiments. For statistical analysis Two-way ANOVA was used with Bonferroni's post-test. P-values are represented as follows: $p < 0.01$ by ** and $p < 0.001$ by ***.

Discussion

Differential cytokine gene regulation by P4, MPA, and NET-A

Epithelial cells at mucosal surfaces such as at the female reproductive tract are important role players in the early immune response induced by pathogens or stress (Wira *et al.,* 2005b). The simple columnar epithelial cells of the endocervix express a wide variety of both pro- and anti-inflammatory cytokines and chemokines (Fichorova & Anderson, 1999; Fahey *et al.,* 2005) including IL-6, IL-8, and RANTES. These cytokines are constitutively expressed at low levels and upregulated in response to TNFα (Fichorova & Anderson, 1999). The endocervix is a site of infection by HIV and other sexually transmitted diseases. Susceptibility may be influenced by factors affecting local immunity such as hormonal contraception. Little is known about the effects of MPA and NET-A, as compared to P4, in the endocervical mucosa, and whether or not they interfere with the local epithelial immune function.

This novel study investigated the effect of P4 and the commonly used progestins, MPA and NET-A on pro-inflammatory cytokine/chemokine expression in the endocervical environment. In the present study, End1/E6E7 cells were used as a model cell system for the endocervical epithelium. These cells are immortalised by expression of E6 and E7 genes of the human papillomavirus type 16 and closely resemble primary cultures both morphologically and in their immunocytochemical expression profile (Fichorova *et al.,* 1997; Fichorova & Anderson 1999; Fichorova *et al.,* 2001).

The effects of P4, MPA, and NET-A on TNFα-induced expression of endogenous IL-6, IL-8, and RANTES genes were investigated. These specific cytokines/chemokines were chosen because they have been shown to be repressed by MPA in other cell types (Koubovec *et al.,* 2004; Zhao *et al.,* 2002). These immune-regulators have also

been reported to play a role in HIV pathogenesis (Fichorova *et al*., 2004). Surprisingly, none of the compounds repressed the cytokine/chemokine genes investigated, in the presence or absence of TNFα (Figures 3.1 & 3.2). MPA slightly increased TNFα-induced IL-6 gene expression but statistical significance could not be established ($p > 0.05$) (Figure 3.1C). In contrast NET-A ($p < 0.001$) and P4 ($p < 0.05$) significantly induced IL-6 mRNA expression in the presence of TNFα. Furthermore, all progestins increased IL-6 expression in the absence of TNFα, even though statistical significance could not be established for P4 and MPA (Figure 3.2B). This may be attributed to the fact that IL-6 is expressed at very low levels in the absence of TNFα and variability between experiments was thus high relative to the response. A higher fold induction was also observed by the progestins in the absence of TNFα (Figure 3.2B). This is probably due to the very high fold increase induced by TNFα alone (Figure 3.2A), which could mask any further IL-6 gene induction by the progestins. Most studies in other cell types have reported that MPA and NET-A decrease IL-6 expression, for example in peripheral blood mononuclear cells (Mantovani *et al.,* 1997), mouse L929 fibroblast cells (Vanden Berghe *et al.,* 1999), and a thyroid cancer cell line, KTC-2 (Kurebayashi *et al*., 2003). To the best of the author's knowledge, there are no other reports of an increase in IL-6 expression in response to MPA except a recent study in an ectocervical cell line, which showed a slight increase of IL-6 mRNA expression (Africander PhD thesis, 2010). Taken together, these results for MPA suggest that the upregulation observed in the End1/E6E7 cells and the ectocervical cell line is specific to the cervicovaginal milieu. NET-A has been shown to both up- and downregulate IL-6 expression (Zitzmann *et al.,* 2005; Kriek MSc thesis 2005). IL-6 protein expression was shown to be repressed in activated monocytes from combined gender groups (Kriek MSc thesis 2005), while males administered NET-EN showed a significant increase in IL-6 levels (Zitzmann *et al.,* 2005). In the present study, P4 upregulated TNFα-induced IL-6 expression in the End1/E6E7 cell line (Figure 3.1). The level of endogenous P4 in women reaches its highest levels during pregnancy. These high concentrations inhibit the expression of IL-6 in trophoblast cells from placentae obtained from women with uncomplicated pregnancies (Das *et al.,* 2002) and have also been shown to reduce IL-6 expression in rat corpus luteum tissue (Telleria *et al.,* 1998). In contrast, P4 has been shown not to change IL-6 mRNA expression in normal human osteoblasts and human bone marrow

stromal cells (Rifas *et al*., 1995). Taken together, the regulation of IL-6 by P4, MPA, and NET-A appears to be cell specific. Moreover, the effects of MPA and NET-A on IL-6 gene regulation are different, indicating progestin-specific effects.

Both MPA and NET-A increased TNFα-induced IL-8 mRNA expression to the same extent (Figure 3.1A), while P4 had no effect (Figure 1A). Consistent with this result, 1 μM MPA was also reported to increase IL-8 mRNA expression in primary human endometrial stromal cells (Arici *et al.*, 1996). In contrast to this result, IL-8 inhibition has been reported to occur in response to MPA in human embryonic kidney HEK293, as well as endometrial and chorio-decidual cells (Kelly *et al.*, 1994; Koubovec *et al.*, 2005). In contrast, NET-A has been shown not to repress IL-8 promoter activity in HEK293 cells (Koubovec et al., 2005). To the best of the present author's knowledge, no other reports exist showing an increase in IL-8 expression in response to NET-A. Furthermore, the results showing a significant increase in IL-8 expression in response to P4 is contrary to other reports in the literature. In rabbit uterine fibroblasts cells, P4 significantly repressed IL-1β induced IL-8 mRNA expression (Ito *et al.*, 1994), whereas an increase in IL-8 expression was reported in human endometrial stromal cells in response to P4 (Arici *et al.*, 1996). Taken together, IL-8 regulation by progestins, like that of IL-6, appears to be cell specific. Moreover, in the End1/E6E7 cells, the effect of MPA and NET-A on IL-8 gene regulation are similar to each other, but dissimilar to the endogenous hormone, P4, indicating progestin-specific effects.

Similar to the effects of MPA and NET-A on IL-8 expression, both progestins significantly enhanced TNFα–induced RANTES expression (Figure 3.1B) while P4 elicited only a small, non-significant induction of the RANTES gene. Consistent with these results, MPA has previously been shown to significantly enhance RANTES mRNA expression in human endometrial tissue obtained from women following short-term treatment with MPA for endometriosis (Deng *et al*., 2007). In contrast, another study reported a decrease in RANTES protein expression in response to MPA in human endometrial stromal cells from women following long-term MPA treatment (Zhao *et al.*, 2002) suggesting that time of exposure to progestins influences their effect on RANTES expression. Previous studies have also shown contradictory results for P4 on RANTES gene expression. P4 significantly inhibited RANTES expression

in human CD8+ T lymphocyte cells, while RANTES expression was unaffected by P4 treatment in CD4+ T lymphocyte cells (Vassiliadou *et al.,* 1999). As observed for IL-6 and IL-8, RANTES gene regulation thus appears to be cell and tissue specific. However, unlike the progestin-specific regulation of IL-6 and IL-8 genes, all the progestins tested elicited a similar response on the RANTES gene (Figure 3.1B).

Due to the low bioavailability of P4 and short half-life, synthetic progestins were synthesised to act similarly to each other as well as to the natural hormone P4. This study clearly shows that the synthetic progestins MPA and NET-A are not always similar to each other, or the natural hormone P4 in their effects on gene expression of some cytokine genes in endocervical epithelial cells.

This is the first study to show that both MPA and NET-A elicit pro-inflammatory responses in the End1/E6E7 cells. Both synthetic progestins significantly induced the mRNA expression of IL-8 and RANTES (Figure 3.1) and induction of IL-8 gene expression by MPA and NET-A is significantly different from that elicited by the natural progestin, P4. The physiological implications of the pro-inflammatory effects of the synthetic progestins could be two-pronged. Firstly, one could postulate that these progestins may increase the local immune response in the endocervical epithelial cells to protect against bacterial and viral infections, thereby decreasing the infection rate. Secondly, however, increased levels of these immuno-regulators could lead to chronic inflammation of the cervix, which could increase cervical cancer progression and HIV and HSV pathogenesis. IL-8 and IL-6 are implicated in cervical cancer progression and all three immuno-mediators are involved in the increase of HIV and HSV shedding and transmission (Tjiong *et al.,* 1999; Das *et al.,* 2000; Brabin, 2002; Wira *et al.,* 2005a; Wei *et al.,* 2001; Wei *et al.,* 2003; Tartour *et al.,* 1994). Long-term usage of oral contraceptives has for example been correlated to cervical cancer progression (Brinton *et al.,* 1986; Moreno *et al.,* 2002; Brabin, 2002; Ursin *et al.,* 1994; La Vecchia *et al.,* 1986). Furthermore, both MPA and NET-EN used as long-term injectable contraceptives have been implicated in increasing the risk of invasive cervical cancer (Herrero *et al.,* 1990). Thus the results of the present study suggest that the progestins could both positively and negatively influence infections of the endocervical mucosa by altering the local immune response. In addition, progestins could lead to chronic inflammation of the endocervix. This certainly needs to be investigated further to

ascertain the exact effects that synthetic progestins have on the lower FRT and how they affect the local epithelial immune response. It would be of interest to investigate the expression of these immune-regulators in endocervical swabs from healthy-, and HIV-1 infected- women using MPA and NET as injectable contraception, as compared to women not using these contraceptives. In the present study the molecular mechanisms of progestin regulation of the IL-6 gene were further investigated, since this gene exhibits differential regulation by the synthetic progestins MPA and NET (Figure 3.1C).

Steroid receptors expressed in the End1/E6E7 cell line

As progestins primarily act via steroid receptors, the steroid receptor content of the End1/E6E7 cell line was characterised using Western blotting and receptor-specific promoter-reporter analysis. Results showed that the GR, AR, MR and ERα are expressed in the End1/E6E7 cells (Figure 3.3). Unexpectedly, the PR-B was found not to be expressed at detectable levels in these cells. Immunohistochemical analysis, Western blotting and RT-PCR of primary human endocervical cells have identified the expression of both PR isoforms (PR-A and PR-B) in the endocervix (Al-Hendy, *et al.,* 2006) although PR expression is dependent on the menstrual cycle. Decreased P4 concentrations are associated with decreased PR-B levels (Stjernholm-Vladic *et al.,* 2004; Konishi *et al.,* 1991). The End1/E6E7 cells might have been established during the follicular phase of the menstrual cycle, which could explain the lack of PR-B protein present, given that these cells were not maintained in the presence of P4. Similarly, the AR was shown to be present in the human ectocervix (Ruizeveld de Winter *et al.,* 1991; van der Kwast *et al.,* 1994) but not detectable in the endocervix (Ruizeveld de Winter *et al.,* 1991), in contrast to the findings of the present study. Consistent with the current results however, immunohistochemical analyses of primary human endocervical cells have also shown the ERα to be expressed in the endocervix (Hodgins *et al.,* 1998, Snijders *et al.,* 1992; Remoue *et al.,* 2003; Al-Hendy *et al.,* 2006). Also consistent with the present study, the GR has been reported to be expressed in the HeLa cervical carcinoma cell line (Burnstein *et al.,* 1991; Wallace & Cidlowski 2001) and immunohistochemical analysis of cervical biopsies also showed GR expression (Vladic-Stjernholm *et al.,* 2009). To summarise, this is the first study to show the expression of the ERα, GR, AR, and MR in the End1/E6E7 cell line. P4,

MPA, and NET-A are able to bind to the GR, AR, and MR (Philibert *et al.,* 1999; Koubovec *et al.,* 2005; Africander Phd thesis, 2010) and the potential exists for the progestins to mediate a biological response via any or all of these steroid receptor in the End1/E6E7 cell line. However, on further investigation only the ERα and GR were shown to be transcriptionally competent on an exogenous promoter reporter (Figure 3.4). Contradictory, to the results of the Western blotting showing the presence of the AR, in the End1/E6E7 cells, MIB only induced a strong transcriptional response in the presence of transiently overexpressed AR, but showed no activity in mock-transfected End1/E6E7 cells. This result would suggest that the AR found to be expressed in the End1/E6E7 cell lysate is transcriptionally inactive or that the levels of endogenously expressed AR are too low to elicit a transcriptional response. Interestingly, similar findings were reported in U2OS cells (Hadley PhD thesis 2010) and in ectocervical (Ect1/E6E7) and vaginal (Vk2/E6E7) epithelial cells (Africander PhD thesis 2010) for the AR. Likewise, a band similar in size to the positive control was detected with the anti-MR antibody (Figure 3.3D) although the MR also was transcriptionally incompetent in upregulation via the GRE reporter (Figure 3.4D). This could be attributed to a faulty MR-antibody as the MR is detected in the negative control and it has previously been shown not to be expressed in the COS-1 cells (Han *et al.,* 2005). In addition, because the loading of the positive control is slightly higher the possibility exists that the MR is not overexpressed in the End1/E6E7 cells. This hypothesis is supported by the low fold-induction induced by Ald in transiently overexpressed MR in End1/E6E7 cells. It is possible that this low induction could also be via the GR because Ald also acts via the GR (Ronacher *et al.,* 2009).

The role of the GR in IL-6 gene regulation by the progestins

Having established that the GR is expressed and transcriptionally competent in the End1/E6E7 cells, the role of the GR in the regulation of IL-6 mRNA expression in response to P4, MPA, and NET-A was further investigated. To investigate the role of the GR in IL-6 gene regulation by the compounds, two approaches were used: GR antagonism by RU486, and GR knockdown using siRNA. Unexpectedly, treatment with RU486 and reduction of GR expression both augmented TNFα–induced IL-6 gene expression in the absence of DEX (Figures 3.6 & 3.8A), suggesting that the unliganded

GR inhibits TNFα–induced IL-6 gene expression. In the next chapter (Chapter 4) this response was further investigated.

RU486 was unable to significantly ($p > 0.05$) antagonise DEX-mediated IL-6 mRNA repression in End1/E6E7 cells as only a slight lift in DEX-mediated repression was observed with DEX in the presence of RU486 (Figure 3.6). In the present study equimolar concentrations of DEX and RU486 were used and this might suggest that the concentration of RU486 is insufficient to significantly antagonise the DEX-mediated repression. However, the binding affinity of RU486 is approximately 10 times greater than that of DEX with K_d values equal to 0.68 nM (Wagner *et al.,* 1999) and 9.4 nM (Charmandan *et al.,* 2005) respectively. The fractional occupancy of the GR for RU486 as calculated by the equation [ligand]/[ligand]+K_i would therefore suggest that approximately 90% of the total GR should be bound by RU486. An alternative explanation for the lack of significant antagonism by RU486 might be the steroid receptor density in the End1/E6E7 cells. Zhao and colleagues suggested that GR density plays a role in RU486 antagonism of a DEX response (Zhao *et al.,* 2003). These authors showed that at low GR density, RU486 could antagonise DEX- mediated repression of a NFκB promoter in COS-7 cells, whereas RU486 was unable to inhibit DEX-mediated repression at high GR levels (Zhao *et al.,* 2003). Thus high endogenous GR levels in the End1/E6E7 cells may be responsible for the inability of RU486 to significantly antagonise DEX-mediated IL-6 mRNA repression. It would be interesting to compare GR expression levels of the End1/E6E7 cells to a cell line such as L929 where RU486 has been shown to successfully antagonise DEX mediated repression, to test this theory (Vanden Berghe *et al.,* 1999). Nonetheless, the results are difficult to interpret due to the observed induction of IL-6 mRNA expression with RU486 alone. Furthermore, although Western blotting analysis showed that the PR-B isoform is not expressed, the expression of endogenous PR-A has not been excluded and RU486 is also a well-known PR antagonist. Therefore GR siRNA was also used to determine GR involvement in IL-6 gene regulation and corroborate the RU486 results.

Unlike RU486, GR siRNA significantly lifted DEX-mediated repression of IL-6 (Figure 3.8A) and normalising to each treatment condition's vehicle control, DEX-mediated repression is abrogated by GR knockdown ($p = 0.09$) (Figure 3.8B). The GR

knockdown results confirm the involvement of the GR in the DEX-mediated IL-6 response and that it is an effective experimental approach to establish the involvement of the GR in the effects of P4, MPA, NET-A on IL-6 gene regulation.

Interestingly, although P4, MPA and NET-A differentially regulate IL-6 gene expression (Figure 3.1C), several lines of evidence would suggest they all act via the GR (Figure 3.8B & Addendum B, Figure B1) as depicted in Figure 3.10. Decrease in GR protein levels attenuated IL-6 gene expression in response to P4, MPA, and NET-A (Figure 3.8B) and further support is provided by the observation that RU486 significantly antagonised P4, MPA, and NET-A-induced IL-6 expression (Addendum B, Figure B1) when normalised for the induction of RU486 alone. A similar trend, although less pronounced, is also seen with the GR antagonist (RU486) experiments when compared to GR knockdown results for P4, MPA, and DEX, although a slight decrease is observed for NET-A in the presence of RU486, which is difficult to interpret (Figure 3.6 vs Figure 3.8A).

All three compounds have previously shown to bind to overexpressed GR in COS-1 cells (Koubovec *et al.,* 2005; Ronacher *et al.,* 2009) with the rank order for binding affinity being MPA > P4 > NET-A. MPA was shown to display much greater glucocorticoid agonist potency than P4 for both transactivation and transrepression on a (GRE)-driven reporter- and an IL-8 promoter-reporter constructs, respectively, in the human embryonic kidney (HEK293) cell line stably transfected with a rat GR expression vector (Koubovec *et al.,* 2005). In contrast to MPA, NET-A did not transactivate the GRE-driven promoter, even at 10 μM, and only marginally (22%) transrepressed the IL-8 reporter at this concentration (Koubovec *et al.,* 2005). Similar results in a COS-1 cell system, transiently transfected with hGR and a GRE-driven reporter construct (for transactivation) or an AP-1 or NFκB promoter–reporter construct (for transrepression) were also reported (Ronacher *et al.,* 2009). However, in End1/E6E7 cells, despite showing that P4, MPA, and NET-A appear to act predominantly via the GR in IL-6 regulation, these responses were not agonist responses as obtained for the potent synthetic GR agonist, DEX (Figure 3.8B). This is very interesting and should be examined further. It is plausible that these differences are a result of differences in the type or relative amount of co-factors recruited by the GR

when bound to P4, MPA, and NET-A versus DEX. Different signalling pathways could also be activated by P4 and the synthetic progestins MPA and NET-A, which could modulate different downstream transcription factors compared to DEX. Unfortunately when examining the role of the MAPK signalling pathways DEX was not included. It would be interesting to examine what role these signalling pathways play on the DEX-mediated response compared to that observed for the progestins. Another possibility, although not shown in the model presented in Figure 3.10, is that the GR bound to the progestins is unable to tether to any of the transcription factors associated with IL-6 promoter activity. It would thus be interesting to investigate in future the GR recruitment to the IL-6 promoter in response to P4, MPA, and NET-A. In addition, another explanation might be that the opposite effects seen with the progestins compared to DEX might be due to differential non-genomic effects by the progestins as compared to DEX. The observation that MPA mediates a biological response opposite to DEX is difficult to explain, especially as MPA has previously been shown to be a partial agonist (Koubovec et al., 2004). However, NET-A has been reported to be a GR antagonist under certain conditions (Africander PhD thesis, 2010), and hence may induce a different conformation of the GR as compared to DEX.

Role of other steroid receptors

Steroid receptors other than the GR might also play a role in progestin-mediated IL-6 regulation. The ER might mediate some of the effects of NET-A on IL-6 gene expression, since NET-A and its metabolites have been shown to have estrogenic activity (Markiewicz & Gurpide, 1994; Mendoza-Rodríguez *et al.,* 1999), although the evidence of the estrogenicity of NET is controversial (Schoonen *et al.,* 2000; Bergink *et al.,* 1983). However, preliminary results indicate that the endogenous ERα is not responsible for the NET-A-mediated induction of IL-6 gene expression. Overexpression of ERα resulted in the transrepression of IL-6 mRNA expression in response to NET-A, in contrast to the increase in NET-A mediated IL-6 gene expression when the GR is overexpressed (Addendum B, Figure B2).

Although PR-B protein was not detected by Western blot analysis and no transactivationally competent PR was identified, this does not exclude the possibility that the trancriptionally less active PR-A is expressed in the End1/E6E7 cells. This

could affect both MPA and NET-A induced IL-6 gene expression, although GR knockdown studies (Figure 3.8B) suggest that the GR is the main steroid receptor involved in P4,- MPA- and NET-A-induced IL-6 expression. Interestingly, overexpression of PR-B resulted in NET-A induced IL-6 transrepression (Addendum B, Figure B2). The potent PR agonist R5020 strongly repressed IL-6 gene expression (Figure B2) in contrast to P4, MPA, and NET-A, and overexpression of PR-B lifted the repression induced by R5020 (Figure 3.1B). Because Western-blotting showed that the PR-B is not expressed in these cells it is very likely that the PR-A isoform is expressed in the End1/E6E7 cells and that this isoform is responsible for the R5020-induced repression of IL-6 mRNA. The PR-A isoform is a potent activator of transrepression and not transactivation (Kalkhoven *et al.,* 1996) further supporting this hypothesis. The involvement of PR-A in the responses induced by MPA and NET-A certainly needs to be investigated further.

Both MPA and NET-A are reported to have androgenic activity (Bentel *et al.,* 1999; Teulings *et al.,* 1980; Bamberger *et al.,* 1999; Kemppainen *et al.,* 1999; Pérez-Palacios *et al.,* 1981; Pérez-Palacios *et al.,* 1983; Bergink *et al.,* 1983) and are able to bind to the MR (Philibert *et al.,* 1999; Africander PhD thesis, 2010). However, both the MR and AR were shown to be biologically inactive in a transactivation assay on a GRE (Figure 3.4).

Collectively the results of the present study suggest that the GR is the primary steroid receptor involved in the increase in IL-6 gene expression mediated by P4 and the synthetic progestins MPA and NET-A. The only steroid receptor that caused an increase in NET-induced IL-6 mRNA expression when overexpresssed, was the GR (Addendum B, Figure B2). This is in agreement with the findings of the GR knockdown studies (Figure 3.8B) and RU486 antagonism studies (Addendum B, Figure B1).

MAPK involvement in the differential IL-6 regulation by the progestins

The initial hypothesis for this study was that the differential effects of MPA and NET-A may be due to activation of different steroid receptors. However, this was shown not to be the case, as both MPA and NET-A were shown by protein knockdown to act via the GR. Another mechanism that might explain the differential regulation of IL-6

by the progestins was thus investigated. It is known that TNFα as well as hormonal steroids are capable of activating various signalling pathways including the MAPKs (Aronica *et al.,* 1994; Muslin *et al.,* 1993; Di Domenico *et al.,* 1996; Migliaccio *et al.,* 1998; Ballaré *et al.,* 2003; Lange, 2004; Skildum *et al.,* 2005; Boonyaratanakornkit *et al.,* 2007) and the possible role of the MAPKs in the differential IL-6 gene regulation by MPA and NET-A was thus investigated.

The results of the present study show that different MAPK pathways are involved in IL-6 mRNA expression induced by P4, MPA, and NET-A (Figure 3.8) as summarised in Figure 3.9. Both ERK1/2 and p38 pathways play a role in the induction of IL-6 by NET-A in the End1/E6E7 cell line. In contrast, only p38 is involved in P4-induced activity. Although no significance was established, both ERK1/2 and JNK pathways, but not p38, appear to inhibit MPA-induced IL-6 mRNA expression. This is the first study comparing P4, MPA, and NET-A showing a differential requirement of MAPK pathways in progestin-induced IL-6 gene expression. Both ERK and p38 were shown to be required for the activation of nuclear factor-κB (NFκB) by TNFα and maximal IL-6 induction in the mouse fibrosarcoma L929 cell line (Vanden Berghe *et al.,* 1998), suggesting that the effects of NET-A on IL-6 gene expression may be due to activation of NFκB by the GR when bound to NET-A or possibly by non-genomic effects. However, the results obtained in the present study in the presence of the p38 MAPK inhibitor should be interpreted with caution as the specificity of this inhibitor has been shown to be questionable (Kotitschke PhD thesis, 2009).

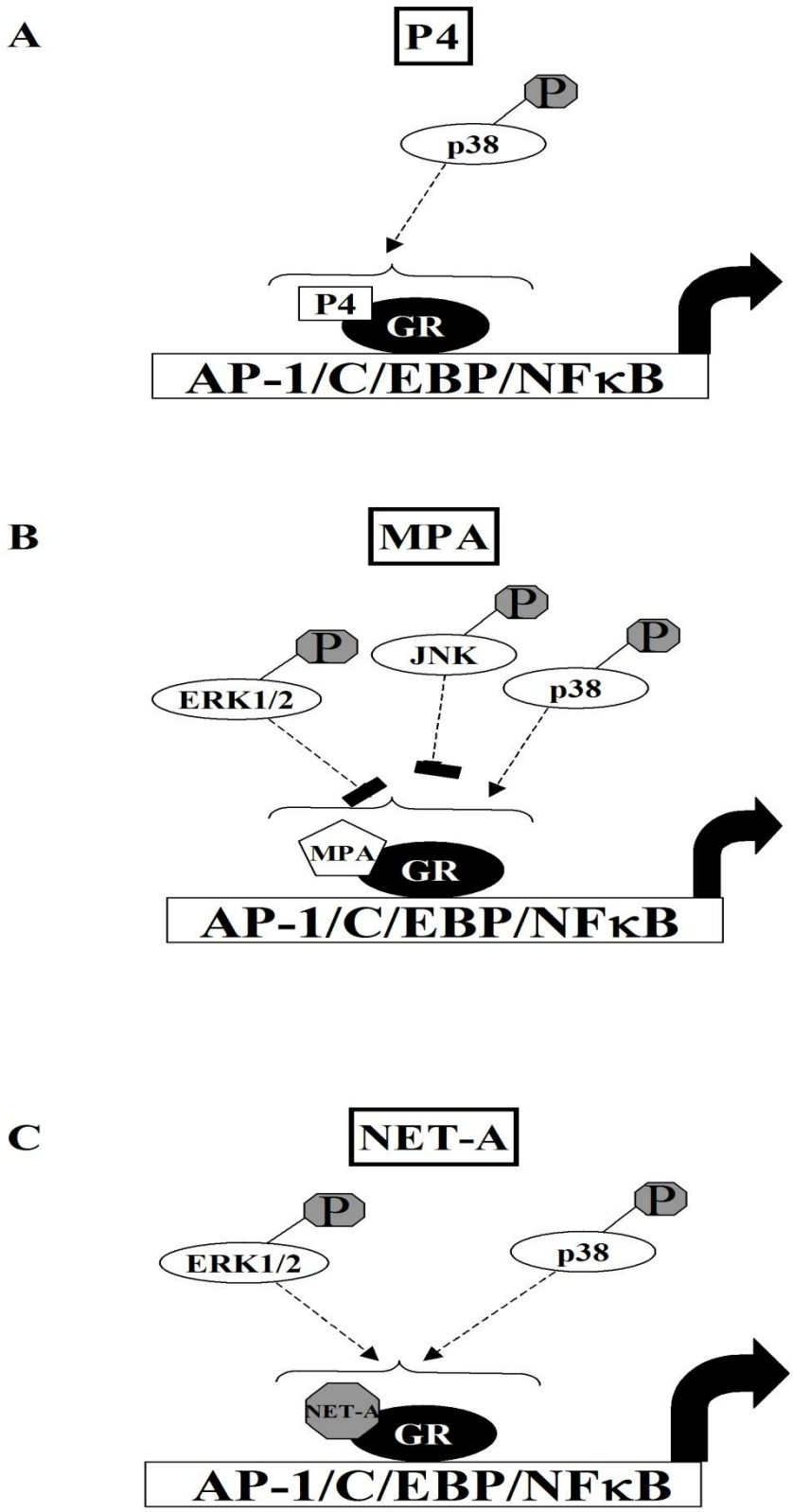


Figure 3.10: Schematic model for the role of the GR and MAPK pathways in regulation of the IL-6 gene by (A) P4, (B) MPA, and (C) NET-A .

Abbreviations: P4: progesterone, MPA: medroxyprogesterone acetate, NET-A: norethisterone acetate, IL-6: interleukin-6, ERK1/2: extracellular signal-regulated kinase, JNK: c-Jun N-terminal kinase, GR: glucocorticoid receptor, NFκB: nuclear factor kappa B, AP-1: activating protein 1, C/EBP: CAAT enhancer binding protein.

To summarise, although IL-6 is differentially regulated by P4, MPA, and NET-A (Figure 3.1C), both MPA and NET-A appear to act via the GR. In addition, both MPA and NET-A also increase expression of IL-8 and RANTES, thus exhibiting different effects to the endogenous hormone P4. Interestingly, the responses induced by MPA and NET-A are opposite to those of the potent GR agonist, DEX. This fact is especially surprising for MPA, as it has been reported to have anti-inflammatory properties similar to DEX in various cell systems (Koubovec *et al.*, 2004; Koubovec *et al.*, 2005; Bamberger *et al.*, 1999). Although both MPA and NET-A act via the GR, as schematically represented in Figure 3.10, different MAPK pathways are involved in the divergent extents of IL-6 gene induction by the progestins (Figure 3.10). Future studies could investigate the role of other transcription factors, which might be modulated by progestin activated GR such as AP-1, C/EBP, and NFκB, all of which have been shown to tether to the GR (De Bosscher & Haegeman 2009) as presented in Figure 3.10.

The results of the present study highlight the importance of further research into the molecular mechanisms of action of progestins, as well as the signalling pathways involved. This will allow physicians to better evaluate the risk and benefit ratio of particular progestins, and may even ultimately lead to the design of improved progestin therapies without these possible negative effects on immune function. Knowing the molecular mechanisms by which MPA and NET-A influence gene expression of pro-inflammatory regulators in the endocervix compared to P4, is important for women's health and will give further insight at the molecular level into inflammatory responses during infection. Understanding the extent of gene regulation by MPA and NET compared to P4 is an important issue for women's health, since these progestins influence the levels of pro-inflammatory regulators in the endocervix. Furthermore, a better understanding of the molecular mechanisms of action of these compounds will provide insight into the immune responses of mucosal surfaces within the FRT, which form a crucial, yet vulnerable, barrier to infection.

CHAPTER FOUR

RESULTS & DISCUSSION

Ligand-independent GR-mediated repression of IL-6 in response to tumour necrosis factor-alpha (TNFα) in an endocervical epithelial cell line

Abstract

Tumour necrosis factor-alpha (TNFα), an early response pro-inflammatory cytokine, is a potent inducer of interleukin-6 (IL-6) gene expression, while ligand-activated glucocorticoid receptor (GR) has strong anti-inflammatory properties and is able to significantly antagonise TNFα-induced IL-6 expression. In this study, the role of liganded and unliganded GR in IL-6 gene regulation was examined in response to TNFα in an immortalised human endocervical epithelial cell line (End1/E6E7). Both decreased GR protein expression by means of GR siRNA, as well as incubation with the GR antagonist RU486, resulted in potentiation of the TNFα-induced IL-6 mRNA expression, suggesting a role for the unliganded GR in modulation of TNFα-induced IL-6 expression. TNFα was subsequently shown to induce phosphorylation of the unliganded GR at Ser-226 but not Ser-211, unlike dexamethasone (DEX), which induced hyperphosphorylation at both serine residues. The GR was also shown to partially translocate to the nucleus in response to TNFα. Furthermore, TNFα induced recruitment of the unliganded GR to the IL-6 promoter to a similar extent as DEX, providing further evidence of TNFα-induced activation of the GR. Unliganded GR appears to actively attenuate IL-6 gene expression in response to TNFα. In addition, TNFα also induced the recruitment of GRIP-1 to the IL-6 promoter and overexpression studies indicate that in this context GRIP-1 functions as a co-repressor. These results identify a novel mechanism of ligand-independent GR activation by TNFα that modulates IL-6 gene expression. These findings suggest the presence of an auto-regulatory mechanism that prevents overproduction of IL-6 in endocervical epithelial cells and thus protects against negative effects of excessive inflammation.

Introduction

TNFα is a pleiotropic regulatory pro-inflammatory cytokine, which mediates its inflammatory effects via binding to the membrane-bound TNFα receptor. It is responsible for the induction of various cytokines including IL-6 (Vanden Berghe *et al.*, 1999; Cromwell *et al.,* 1992). IL-6 is a multifunctional cytokine with potent pro-inflammatory properties. It is responsible for T-cell and lymphocyte activation, B-cell differentiation, leukocytosis and acute phase protein synthesis (Kishimoto 1989). IL-6 plays an important role in the host defence against pathogen infections as well as in cell proliferation and cell differentiation (Iglesias *et al.,* 1995). IL-6 has also been associated with various autoimmune diseases and the progression of cervical cancer in the cervix in response to pathogen infections (Rasmussen *et al.,* 1997). The IL-6 promoter contains numerous regulatory elements within the region 300 bp upstream of the transcriptional start site. These include a nuclear factor κB (NFκB) binding element between positions –73 and –63, CCAAT enhancer binding protein (C/EBPβ) binding sites at positions –173 and –145 and an activator protein-1 (AP-1) binding site located between –283 and –277 relative to the transcriptional start site (Vanden Berghe *et al.,* 1998; Vanden Berghe *et al.,* 1999). Bacterial and viral infections and other stress situations increase TNFα expression and binding to its receptor, triggering a cascade of signalling events that leads to the activation of the transcription factors NFκB and AP-1 (Rothe *et al.,* 1995; Hsu *et al.,* 1996; Liu *et al.,* 1996; Natoli *et al.,* 1997; Lee *et al.,* 1997).

Like TNFα, glucocorticoid levels are increased by inflammatory stress through the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Newton, 2000). Lipophilic glucocorticoids elicit a biological response via binding to their cognate receptor, the glucocorticoid receptor (GR). The GR belongs to the steroid hormone receptor sub-family, part of the broader nuclear receptor superfamily. As an inducible transcription factor, it is found predominately in its inactive state in the cytoplasm of target cells (Picard *et al.,* 1990). While in the cytoplasm the GR is bound to a protein complex, which consists of heat shock proteins (hsp)-90, hsp70, immunophillins and other factors (Pratt & Toft, 1997). Hsp90 acts as a molecular chaperone preventing unliganded GR from translocating to the nucleus (Pratt & Toft, 1997). The classical mechanism of glucocorticoid action entails binding of the ligand to the ligand-binding

domain of the GR resulting in a conformational change of the receptor followed by the dissociation of the GR from the chaperone protein complex (Griekspoor *et al.*, 2007). The ligand-bound GR translocates rapidly to the nucleus where it recognises specific palindromic DNA sequences known as glucocorticoid response elements (GREs) in the promoters of target genes to positively regulate transcription (Zhou & Cidlowski, 2005). Glucocorticoids can also negatively regulate gene expression of target genes via either direct GR binding to DNA, at so-called negative GRE sequences, or by interacting with other transcription factors (Scheinman *et al.*, 1995; van der Saag *et al.*, 1996; De Bosscher *et al.*, 1997; De Bosscher *et al.*, 2000; Adcock & Caramori, 2001; Reichardt *et al.*, 2001; De Bosscher *et al.*, 2006; Jonat *et al.*, 1990; Nissen & Yamamoto, 2000). Ligand-activated GR has been shown to interact with NFκB, AP-1 and C/EBP transcription factors, thereby repressing a variety of immune function genes (Scheinman *et al.*, 1995; De Bosscher *et al.*, 1997; Jonat *et al.*, 1990; De Bosscher & Haegeman, 2009; Beck *et al.*, 2009).

In addition to activation by steroidal ligands, the GR has also been reported to be activated by various stimuli in the absence of ligand (Tanaka *et al.*, 1996; Eickelberg *et al.*, 1999; Kotitschke *et al.*, 2009). Ursodeoxycholic acid and the beta-adrenergic receptor agonists, salmeterol and salbutamol, were shown to induce nuclear translocation of the GR and subsequent GR-dependent transcriptional activity (Tanaka *et al.*, 1996; Eickelberg *et al.*, 1999), although the mechanism of this activation and requirement for receptors other than the GR was not established. The recent study from the Hapgood laboratory showed that gonadotropin releasing hormone (GnRH)-induced glucocorticoid-independent increase of GR phosphorylation and transcriptional activity on an endogenous promoter (Kotitschke *et al.*, 2009). It was demonstrated that GnRH-induced activation of the GR is dependent on the membrane-bound GnRH receptor and involves mitogen activated phosphatase kinases (MAPKs) (Kotitschke *et al.*, 2009). From the above-mentioned studies, it is clear that the unliganded GR can be activated by the GnRH receptor and possibly by other plasma membrane receptors. Whether the unliganded GR can be activated by TNFα has not been previously investigated. The present study investigates the roles of liganded and unliganded GR in the response to TNFα on the IL6 promoter, in the End1/E6E7 cell line as a cell model for the endocervix.

Results

TNFα is a potent inducer of IL-6 mRNA expression in End1/E6E7 cells

To study the transcriptional regulation of IL-6 gene expression in the endocervical epithelium by TNFα and DEX, End1/E6E7 cells were treated with 1 μM DEX in the absence and presence of 20 ng/μL TNFα for 24 hrs, and IL-6 mRNA levels were measured by means of quantitative real-time PCR (qPCR), and normalised to GAPDH mRNA levels. As shown in Figure 4.1, IL-6 mRNA expression is significantly ($p < 0.001$) increased in response to TNFα, by approximately 22-fold compared to EtOH control. Additionally, 1 μM DEX strongly attenuates TNFα induction of IL-6 gene expression, while DEX alone showed a slight repression of basal IL-6 expression, although this was not significant (Figure 4.1).

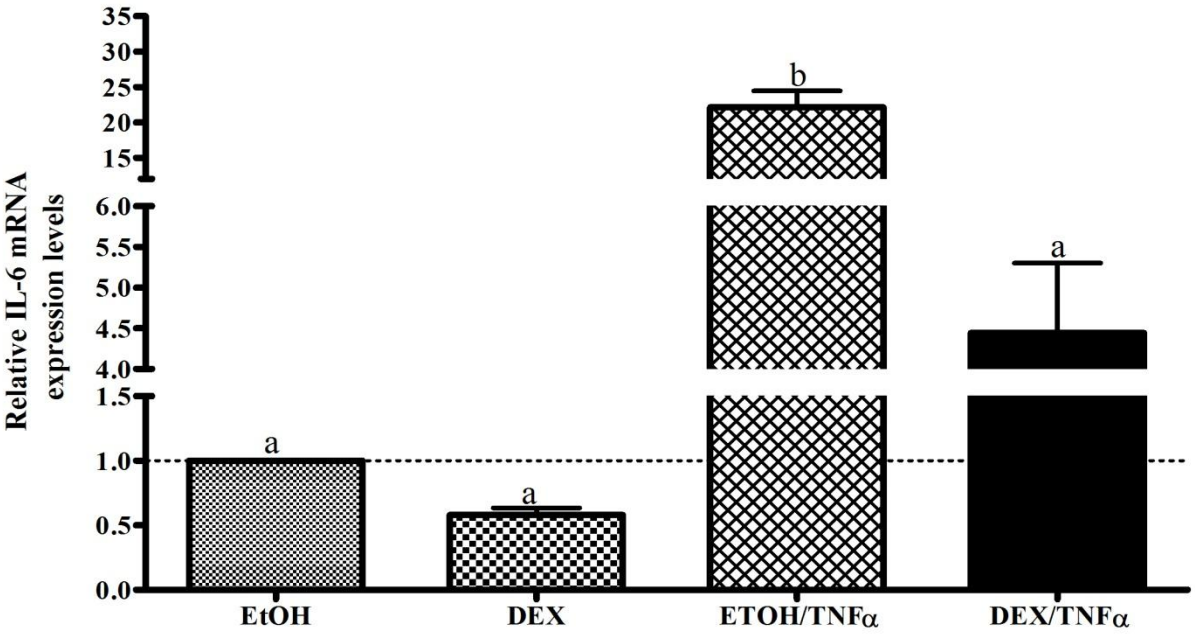


Figure 4.1: Treatment of the endocervical cell line End1/E6E7 with TNFα results in a significant increase in IL-6 mRNA expression, which is significantly repressed by DEX. End1/E6E7 cells were treated with 0.1% EtOH as vehicle control or 1 μM DEX in the absence or presence of 20 ng/μl TNFα. Relative Interleukin-6 mRNA levels were normalised to GAPDH mRNA levels, which served as an internal control. Relative IL-6 mRNA expression was calculated relative to EtOH control, which was set as 1. The graph shows results of at least five independent experiments. For statistical analysis one-way ANOVA was used and Tukey's multiple comparison post-test. Different lower-case letters indicate statistically significant differences; therefore conditions with the same letter are not statistically significantly different from each other ($p > 0.05$), while those having different letters are statistically different from each other ($p < 0.05$).

Decreased GR protein expression potentiates TNFα–induced IL-6 gene expression

It has previously been shown that the GR is expressed and is transcriptionally functional in the End1/E6E7 cells (Figure 3.3). In order to investigate the involvement of the GR in TNFα–induced IL-6 mRNA expression, End1/E6E7 cells were transfected with 10 nM validated GR-specific small interfering RNA (siRNA) oligos or scrambled non-silencing control (NSC) siRNA. The efficiency of the GR siRNA was determined by Western blotting and GR protein expression was found to decrease by to about 42% as compared to the NSC (Figure 4.2B).

The GR siRNA-transfected End1/E6E7 cells were stimulated with 20 ng/μL TNFα in the absence and presence of 1 μM DEX for 24 hrs after which IL-6 gene expression was determined by qPCR. Reduced GR expression significantly ($p < 0.001$) reversed DEX-mediated repression of TNFα– induced IL-6 gene expression, confirming a requirement of the GR in DEX-induced repression of IL-6 mRNA expression (Figure 4.2C). Interestingly, qPCR analysis revealed that diminished GR protein levels resulted in an approximately 2.6-fold increase in TNFα–induced IL-6 gene expression in the absence of GR ligand (Figure 4.2C). As expected, the liganded GR inhibits the TNFα response in the presence of DEX, while the results suggest that the unliganded GR inhibits the TNFα response in the absence of DEX on the endogenous IL-6 gene in End1/E6E7 cells (Figure 4.2C).

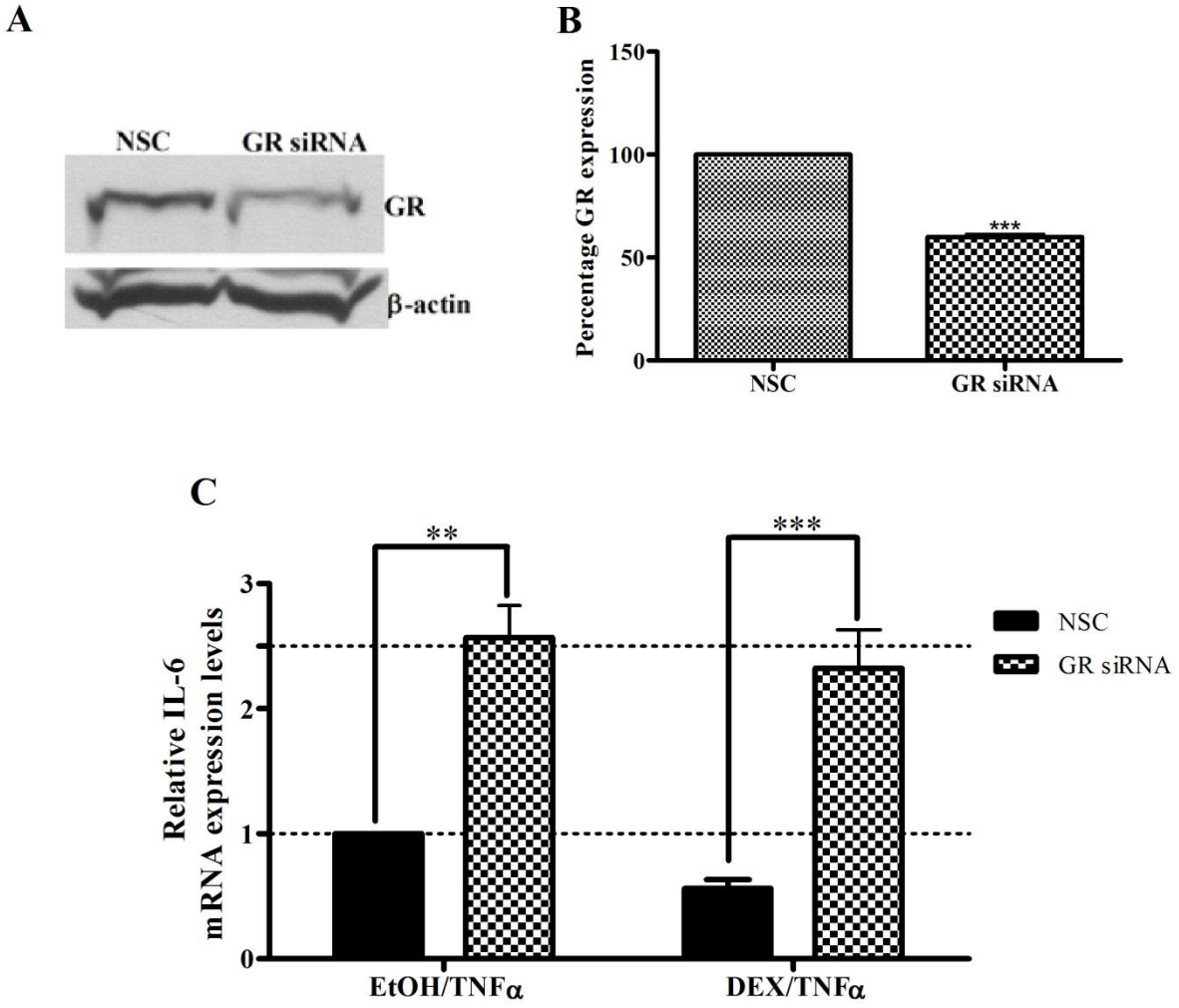


Figure 4.2: **Reduced GR protein expression results in increased TNFα–mediated induction of IL-6 mRNA expression in End1/E6E7 cells**. (A) For verification of GR knockdown, End1/E6E7 cells were transfected with 10 nM non-silencing control (NSC) or GR siRNA oligos. Forty-eight hrs after transfection cells were harvested and whole cell lysates were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane. GR-specific antibody was used for Western blotting analysis and β-actin-specific antibody as loading control. A representative blot is shown. (B) Western blots of at least four independent experiments were quantified to determine the percentage GR protein expression. For statistical analysis student *t*-test was used. p value represents $p < 0.001$; *****.** (C) In parallel, End1/E6E7 cells were transfected with either non-specific siRNA (NSC (black bars)) or with siRNA specific for the human GR (checked bars). Twenty-four hrs after transfection cell were treated with 20 ng/μL TNFα in the absence or presence of 1 μM DEX. Total RNA was isolated 24 hrs after induction, reverse transcribed and relative levels expression of IL-6 mRNA was measured by quantitative qPCR and, normalised to relative GAPDH mRNA expression. Relative fold induction of IL-6 mRNA expression was normalised to EtOH/TNFα, NSC. The graph shows pooled results of at least three independent experiments. For statistical analysis two-way ANOVA was used with Bonferroni's post-test. P-values are represented as follows: $P < 0.01$ by ** and, $p < 0.001$ by ***.

The GR antagonist RU486 augments TNFα–induced IL-6 gene expression in End1/E6E7 cells

RU486 is a well known GR antagonist with a high binding affinity for this steroid receptor (Cadepond & Ulmann, 1997). To further establish the involvement of the GR in the TNFα– mediated response on IL-6 expression, End1/E6E7 cells were treated with 20 ng/μL TNFα in the absence and presence of 1 μM DEX or 1 μM RU486. It was found that TNFα–induced IL-6 mRNA expression was significantly increased approximately 2.5-fold in the presence of 10 μM RU486 (Figure 4.3). This is consistent with the result seen in Figure 4.1, in the presence of GR siRNA. In contrast, the DEX-mediated repression of IL-6 expression remained unchanged in the presence of RU486.

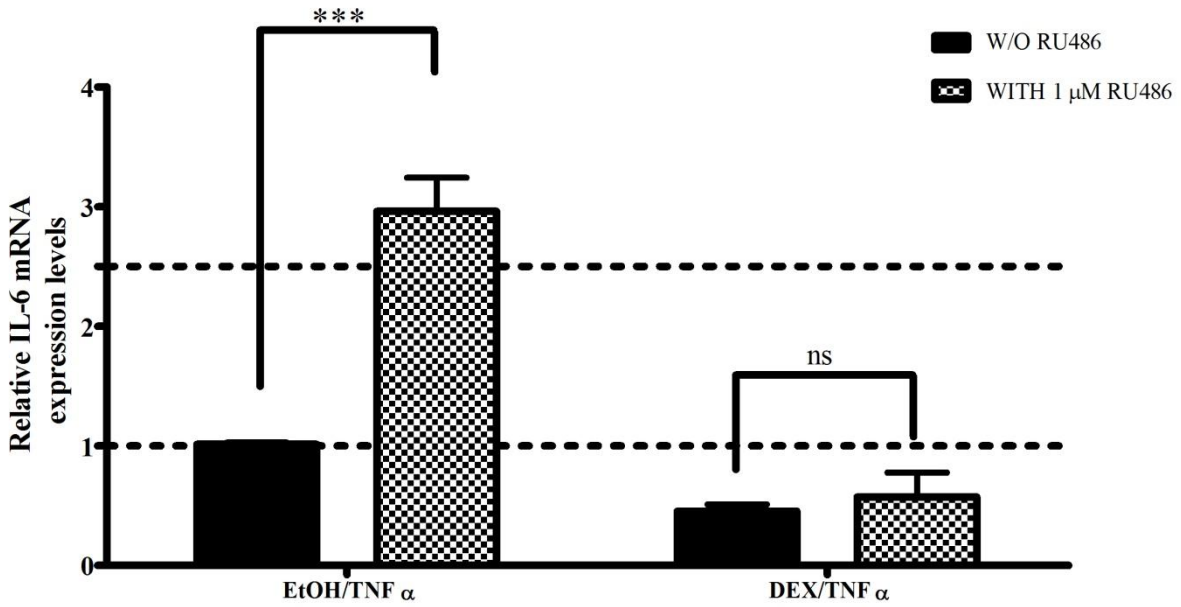
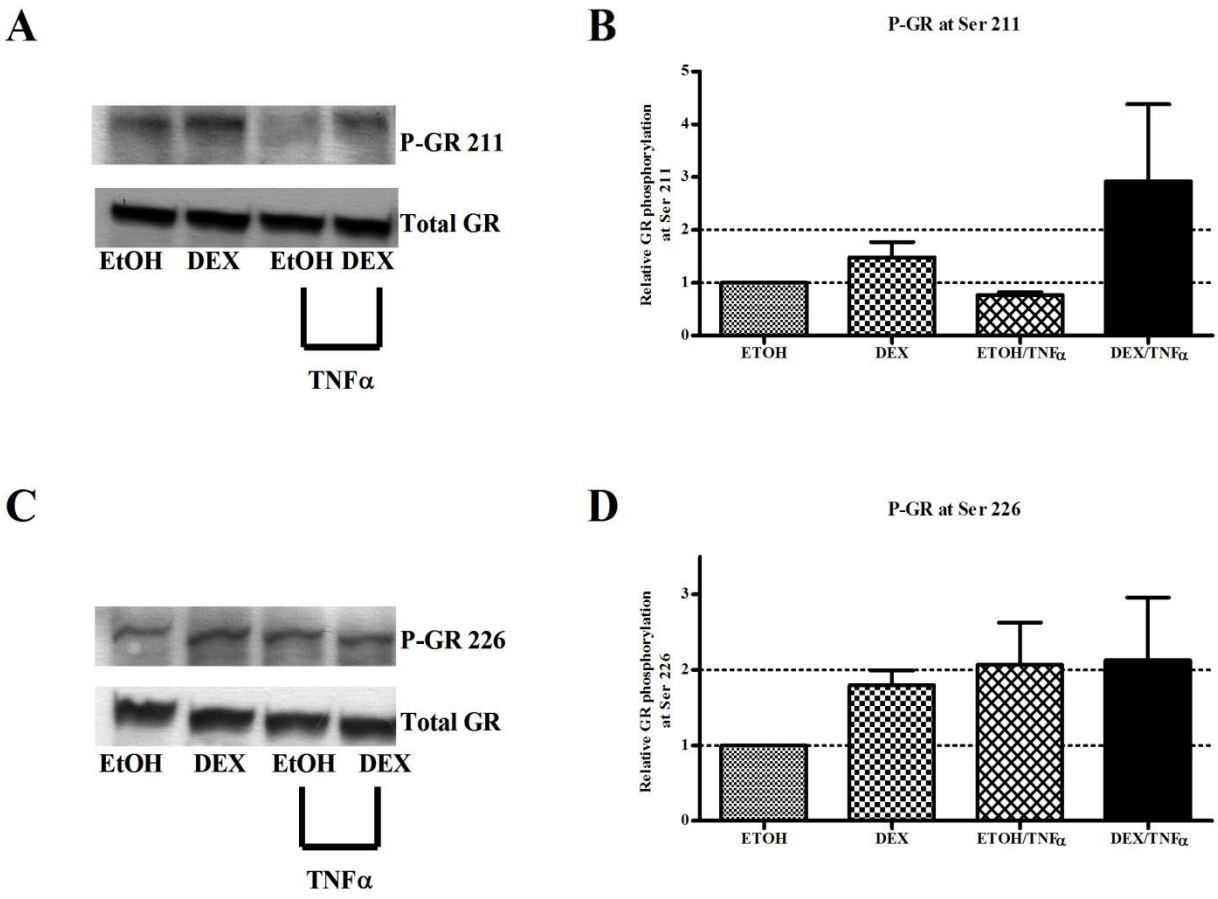


Figure 4.3: The GR antagonist RU486 potentiates IL-6 mRNA expression in the presence of TNFα. End1/E6E7 cells were induced with 20 ng/μL TNFα in the absence or presence of 1 μM DEX, and in the absence or presence of 1 μM RU486. The graph represents pooled results of three independent experiments. For statistical analysis two-way ANOVA was used with Bonferroni's post-test. P-values are represented as follows: $p < 0.001$ by ***. Abbreviations; w/o: with out; n.s: not significant.

TNFα induces phosphorylation of unliganded GR at Ser-226

Agonist-induced phosphorylation of the GR has been suggested to play an important role in GR-mediated cellular responses (Ismaili & Garabedian, 2004). A recent study also showed that ligand-induced phosphorylation is required for maximal GR activation of a GRE-containing promoter in COS-1 cells transfected with human GR, which facilitates GRIP-1 co-factor recruitment (Avenant *et al.*, 2010). Thus, TNFα-

induced phosphorylation of the GR in the absence of ligand could be a mechanism whereby TNFα activates the unliganded GR. End1/E6E7 cells were treated with 20 ng/μL TNFα in the absence or presence 1 μM DEX for 1 hr. The phosphorylation status of the endogenous GR was determined by Western blotting using specific anti-P-Ser-211- and anti-P-Ser-226 GR antibodies (Figure 4.4). The results demonstrate relatively high basal phosphorylation levels of endogenously expressed GR at both Ser-211 and Ser-226 residues (Figures 4.4A & Figure 4.4C). Although significance could not be established DEX induced ligand-dependent phosphorylation at both Ser-211 and Ser-226 of the endogenous GR in End1/E6E7 cells (Figures 4.4B & Figure 4.4D). Furthermore, TNFα in the absence of GR ligand, also induced GR phosphorylation at Ser-226 but not at Ser-211, showing that TNFα selectively induces phosphorylation of the GR in End1/E6E7 cells.



(legend to follow on next page)

Figure 4.4: TNFα induces phosphorylation of the GR at Ser-226 and not Ser-211. End1/E6E7 cell were treated with 1 μM DEX in the absence and presence of 20 ng/μL TNFα for 1 hour. Cells were harvested and whole cell lysates were separated by 8% SDS-PAGE. Phopho-GR specific antibodies raised against (A) serine 211 and (B) serine 226 respectively were used for Western blotting. Phospho-GR protein levels were normalised to total GR expressed. Total GR protein levels were measured after membrane was stripped and re-probed with an anti-GR specific antibody. Graphs are representative of pooled results from three independent experiments. The relative amounts of phosphorylation at the specific serine residues were quantified and expressed as amount phosphorylated GR relative to total GR with vehicle control (EtOH) set as one. For statistical analysis one-way ANOVA with Tukey's multiple comparison post-test was performed.

The GR only partially translocates to the nucleus in response to TNFα

The unliganded GR is mostly found in the cytoplasm (Georget *et al.,* 1997; Htun *et al.,* 1996). Upon ligand-binding, the GR is activated and translocates to the nucleus (Griekspoor *et al.,* 2007), where it regulates transcription either by direct interaction to specific DNA sequences (GREs) or directly through protein-protein interactions with other transcription factors (Nissen & Yamamoto 2000; Luecke & Yamamoto, 2005; Stein & Yang, 1995; Liden *et al.,* 1997; Zhou & Cidlowski, 2005; Wissink *et al.,* 1997). Using subcellular biochemical fractionation, it was next investigated whether TNFα could cause nuclear translocation of the GR. End1/E6E7 cells were treated with vehicle control (EtOH) or 1 μM DEX in the absence or presence of 20 ng/μL TNFα. Cells were pre-treated with EtOH or DEX for 1 hr before the addition of TNFα, after which cells were incubated for a further 2 hrs. Subsequently, cytoplasmic and nuclear fractions were prepared and separated via SDS-PAGE, followed by Western blotting with anti-GR, anti-GAPDH, and anti-histone H3 antibodies. The cytoplasmic GAPDH and nuclear H3 Western blots served as controls to (i) illustrate that pure fractions were obtained and (ii) to normalise for equal loading (Figures 4.5A & 4.5C)

As expected for DEX, decreased GR in the cytoplasmic fractions coincides with increased GR in the nuclear fractions (Figure 4.5), although some degradation of GR protein in the nuclear fractions was noted (Figure 4.5A). The results demonstrate that DEX treatment induced GR translocation into the nucleus by approximately 1.5-fold, with a corresponding 2-fold GR depletion from the cytoplasm as compared to EtOH control. (Figure 4.5B). Although not as pronounced as for DEX, TNFα, in the absence of GR ligand, also caused nuclear translocation of the GR (Figure 4.5B). In contrast to

DEX-induced translocation of the GR, TNFα mediated depletion of GR from the cytosolic fraction was not visible (Figure 4.5C).

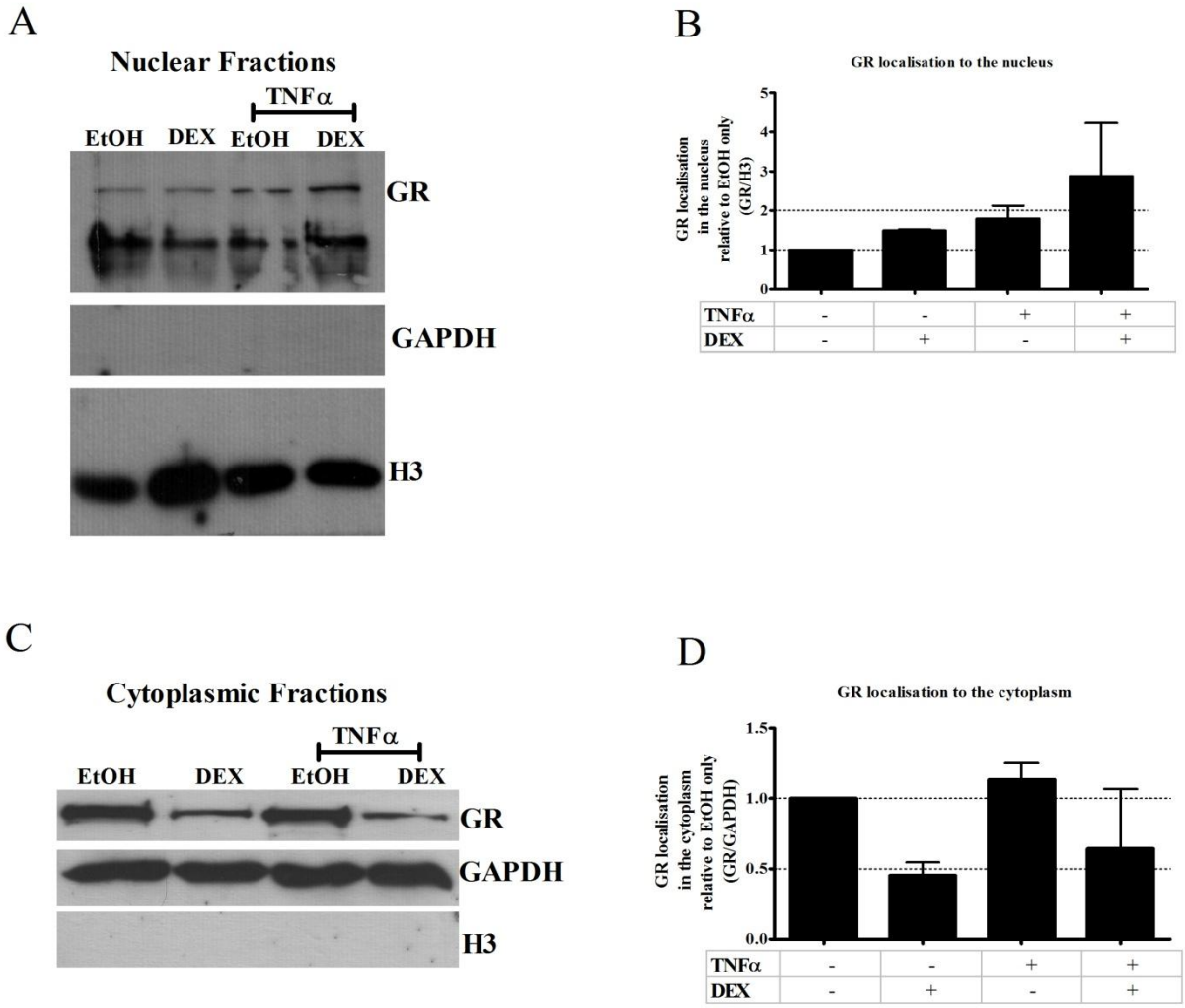


Figure 4.5: TNFα induces partial nuclear translocation of the GR in the absence of DEX. End1/E6E7 cells were pre-treated with EtOH or 1 μM DEX for 1 hr before the addition of 20 ng/μLTNFα after which cells were further incubated for 2 hrs. Subsequently, cytoplasmic and nuclear fractions were prepared and equal amounts of fractions were separated via SDS-PAGE followed by Western blotting with anti-GR, anti-GAPDH, and anti-histone (H3) antibodies. The cytoplasmic GAPDH and nuclear H3 Western blots illustrate that pure fractions were obtained (Figures 4.5A & Figure 4.5C). GR levels were quantified and normalised to H3 (A) or GAPDH (B) loading controls, and presented relative to vehicle control using Alphaease densitometry software.

The GR is recruited to the IL-6 promoter in response to TNFα in the absence of GR ligand

Having established that a decrease in GR protein results in a significant ($p < 0.001$) increase of TNFα–induced IL-6 mRNA expression and that TNFα induces unliganded GR phosphorylation at serine residue 226 as well as nuclear translocation,

recruitment of the GR to the endogenous IL-6 promoter in the presence of TNFα was next investigated in intact End1/E6E7 cells. This was done by chromatin-immunoprecipitation (ChIP) assay, using an anti-GR antibody for immunoprecipitation, and primers spanning 296 bp of the IL-6 promoter encompassing the most important regulatory elements, which include NFκB, C/EBPβ, and AP-1. Intact End1/E6E7 cells were treated with vehicle control (EtOH) or 1 μM DEX in the absence or presence of 20 ng/μL TNFα. As shown in Figure 4.6B, DEX treatment resulted in significant recruitment of the GR to the endogenous IL-6 promoter ($p < 0.05$). Similarly, DEX treatment in the presence of TNFα also induced significant ($p < 0.001$) GR recruitment (Figure 4.6B). Surprisingly, in the absence of DEX, TNFα caused recruitment of the endogenous GR to the IL6 promoter to a similar extent as that of DEX in the presence and absence of TNFα ($p < 0.01$; Figure 4.6B). Recruitment of GR to the IL6 promoter by TNFα alone was not significantly different from that induced by TNFα plus DEX. Figure 4.7 shows that an increase in GR protein expression is also not required for the response by DEX and TNFα as GR levels remained unchanged when End1/E6E7 cells were stimulated with TNFα or DEX for 3 hrs. The IgG negative control confirmed the specificity of the GR antibody and IL-6 primers (Figure 4.8A). Furthermore, agarose gel analysis showed that a single, specific product of the expected size is amplified by the PCR reaction, and that input samples result in amplification of PCR products of similar intensity (Figure 4.8A).

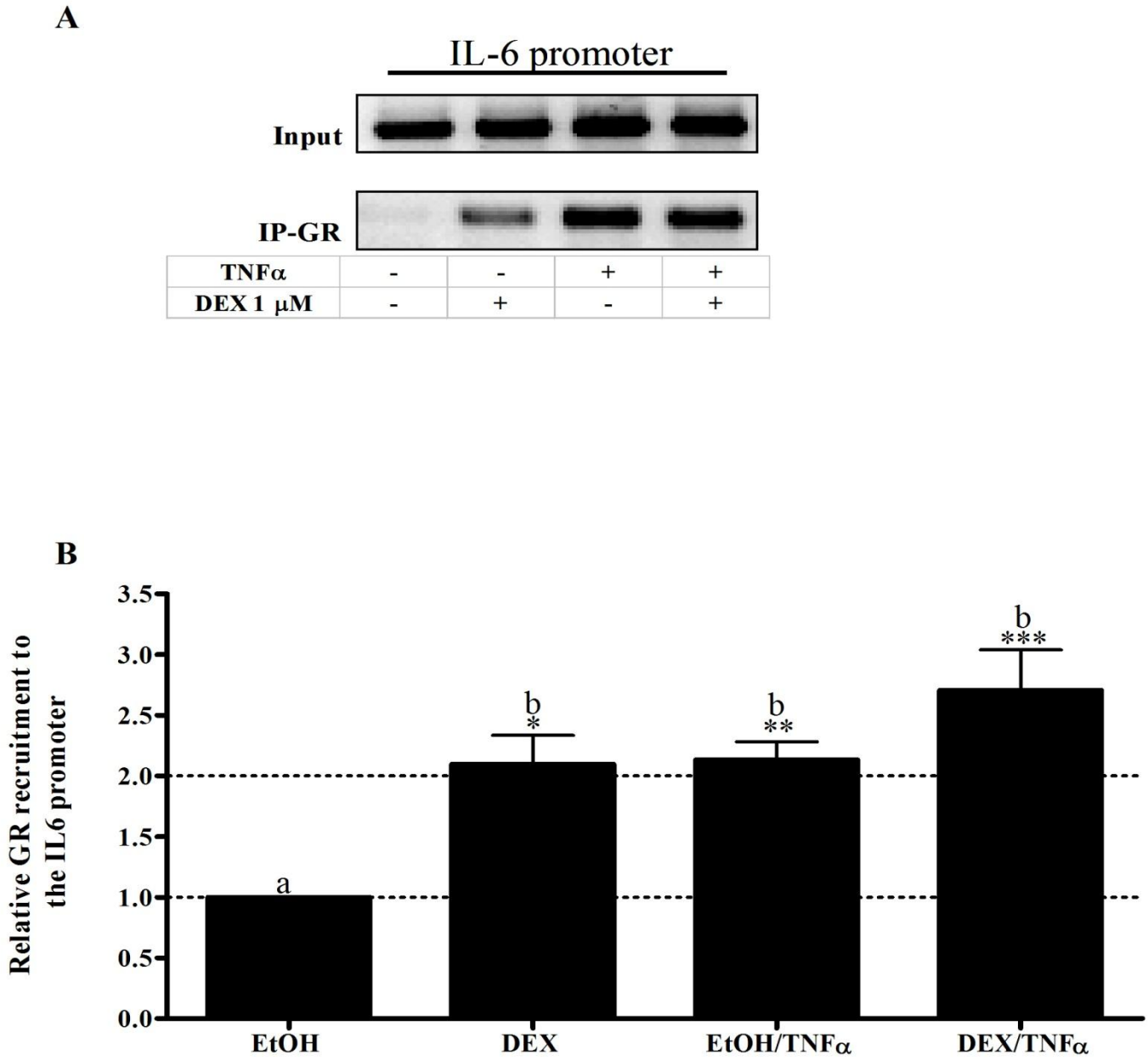


Figure 4.6: The GR is recruited to the IL-6 promoter in response to TNFα in the absence and presence of DEX. End1/E6E7 cells were pre-treated with EtOH or 1 μM DEX for 1 hr and subsequently treated with 20 ng/μLTNFα for an additional 2 hrs, followed by ChIP. Immunoprecipitated GR protein bound to the endogenous IL-6 promoter, was detected using primers specific for the IL-6 promoter encompassing the NFκB, C/EBPβ, and AP-1 regulatory elements. The co-immunoprecipitated DNA fragments and input DNA were analyzed by qPCR. (A) The qPCR products were analysed on a 2% agarose gel and a representative result is shown. (B) The graph is representative of pooled quantified results of at least 4 independent experiments and is shown normalized to input and expressed as the fold-response relative to EtOH control, which was set as 1. For statistical analysis one-way ANOVA was used and Tukey's multiple comparison post-test was performed. Different lower-case letters indicate statistically significant differences; therefore conditions with the same letter are not statistically significantly different from each other ($P>0.05$), while those having different letters are statistically significantly different from each other ($P<0.05$). P-values are represented as follows: $P < 0.05$ by *, $P < 0.01$ by ** and, $p < 0.001$ by *** and represent statistical significance compared to vehicle control (EtOH).

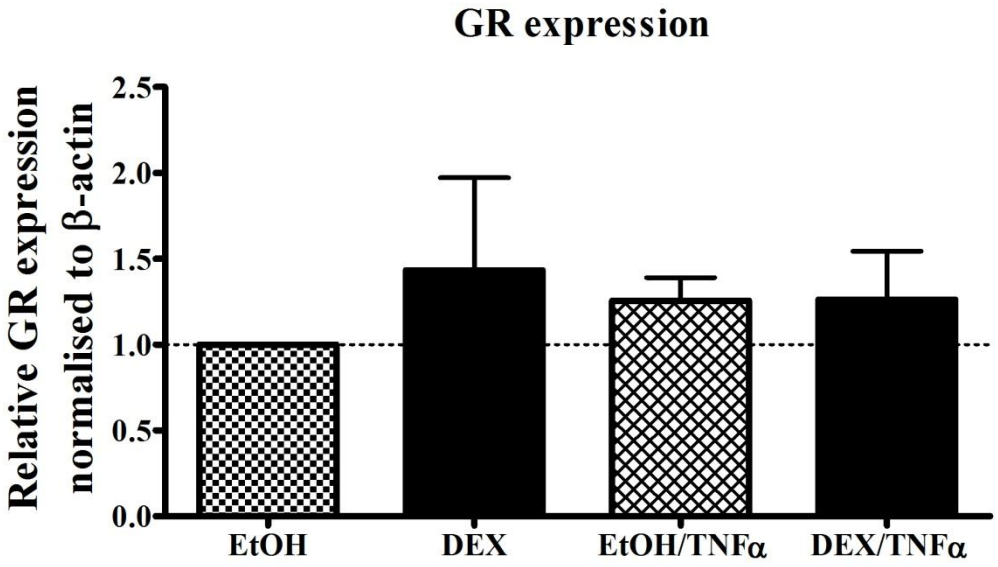


Figure 4.7: GR protein levels remain unchanged in response to TNFα in the absence and presence of DEX. End1/E6E7 cells were pre-treated with EtOH (vehicle control) or 1 μM DEX for 1 hr and subsequently treated with 20 ng/μL TNFα for an additional 2 hrs. Equal volume of whole cell extracts were separated via SDS-PAGE followed by Western blotting with anti-GR and anti-β-actin antibodies. The graph is representative of pooled results from three independent experiments with vehicle control (EtOH) set as one. For statistical analysis one-way ANOVA was used and Tukey's multiple comparison post-test. Blots were quantified and analysed relative to vehicle control using Alphaease densitometry software.

Increased GRIP-1 co-factor recruitment to the IL-6 promoter occurs in response to TNFα in the presence and absence of DEX and not with Dex alone

The finding that the GR is recruited to the endogenous IL-6 promoter to a similar extent in the presence of TNFα, DEX or DEX plus TNFα suggests that a different factor is involved in the strong DEX-induced IL-6 gene repression in the presence of TNFα (Figure 4.1). As the co-factor GR-interacting protein type 1 (GRIP-1) was recently shown to be involved in repression of both AP-1- and NFκB-driven promoters in a GR ligand-dependent manner (Rogatsky *et al.*, 2001; Rogatsky *et al.*, 2002) the involvement of GRIP-1 in GR mediated repression of IL-6 was investigated in the absence and presence of DEX and TNFα or in combination thereof.

As shown in figure 4.8B, DEX treatment alone did not induce recruitment of GRIP-1 to the endogenous IL-6 promoter ($p > 0.05$) (Figure 4.8B), while treatment with TNFα significantly ($p < 0.01$) recruited GRIP-1 to the promoter. DEX in the presence of TNFα caused the most significant recruitment of GRIP-1 to the IL-6 promoter ($p <$

0.001). These results suggest that GRIP-1 plays a role in the regulation of IL-6 expression in response to TNFα alone and to co-treatment with DEX plus TNFα.

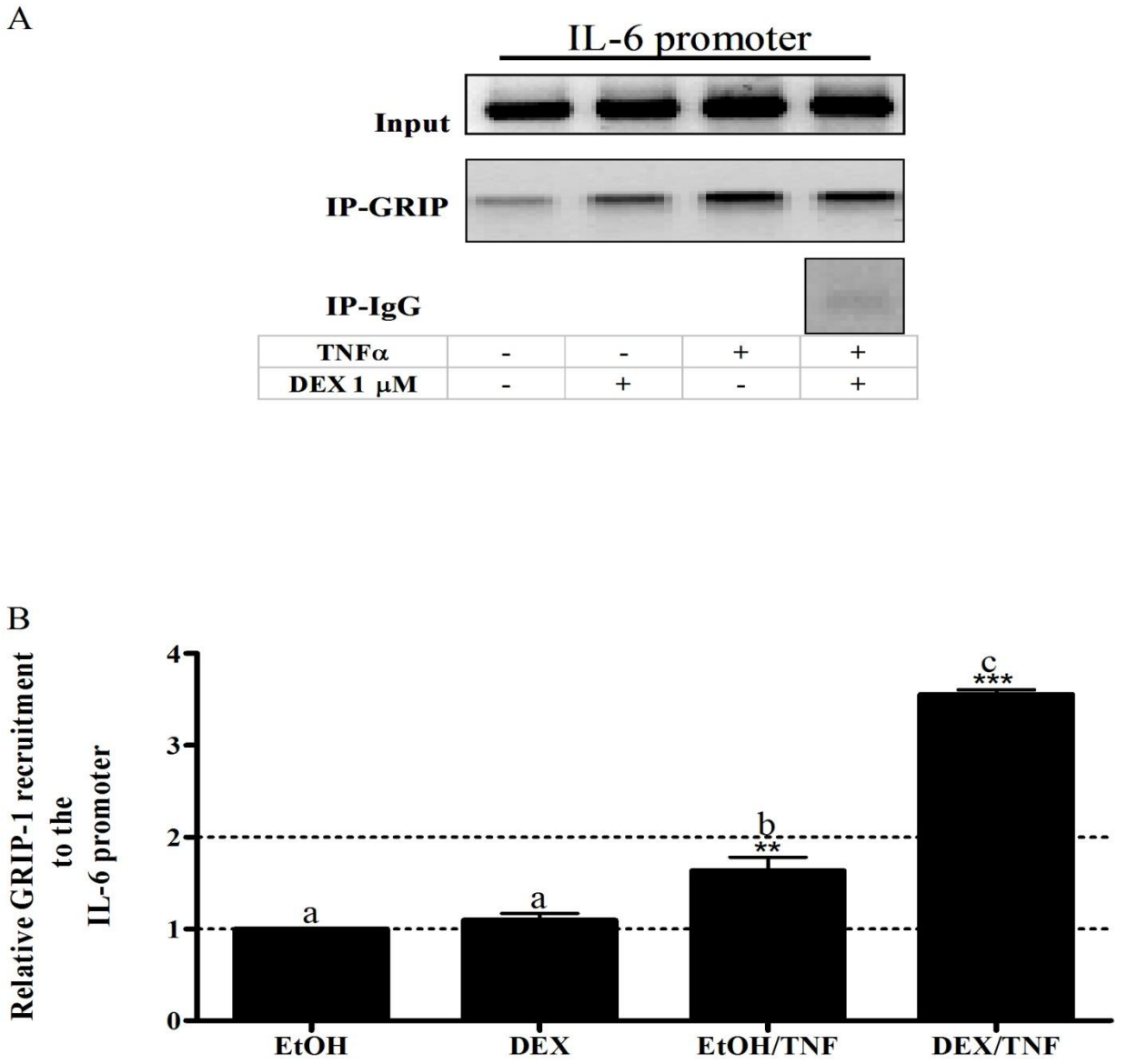


Figure 4.8: The co-factor, GRIP-1 is recruited to the IL-6 promoter in response to both TNFα and DEX/TNFα. End1/E6E7 cells were pre-treated with EtOH or 1 μM DEX for 1 hr before the addition of 20 ng/ μL TNFα for 2 hrs. Immunoprecipitated GRIP-1 protein bound to the endogenous IL-6 promoter, was detected using primers specific for the IL-6 promoter. The co-immunoprecipitated DNA fragments and input DNA were analyzed by qPCR with (A) the PCR product analysed on an 2% agarose gel. (B) The graph is representative of pooled quantified results of at least 3 independent experiments and are shown normalized to input and expressed as fold-response relative to EtOH control. For statistical analysis one-way ANOVA with Tukey's multiple comparison post-test was performed. Different lower-case letters indicate statistically significant difference; therefore conditions with the same letter are not statistically significantly different from each other ($p > 0.05$), while those having different letters are statistically significantly different from each other ($p < 0.05$). P-values are represented as follows: $p < 0.01$ by ** and $p < 0.001$ by *** and represent statistical significance compared to vehicle control (EtOH).

Overexpression of GRIP-1 protein in End1/E6E7 cells attenuates TNFα–induced IL-6 mRNA expression

As the previous results suggest that GRIP-1 is involved in the regulation of IL-6, the role of GRIP-1 in TNFα– induced IL-6 gene expression in End1/E6E7 cells was further investigated. GRIP-1 protein is recruited to the IL-6 promoter in response to 20 ng/μL TNFα. However more GRIP-1 was found to be present on the IL-6 promoter following co-incubation with DEX and TNFα, a condition which causes the most repression of IL-6 gene expression. It was thus hypothesised that GRIP-1 recruited to the IL-6 promoter in response to TNFα alone in the absence and presence of DEX acts as a co-repressor. To further investigate the role of GRIP-1 as a repressor of the TNFα-induced response, End1/E6E7 cells were transiently transfected with a GRIP-1 expression vector. Twenty-four hrs after transfection, cells were treated with 1 μM DEX in the absence and presence of 20 ng/μL TNFα for 24 hrs. Total RNA was isolated and IL-6 mRNA expression was measured by qPCR and was normalised to GAPDH mRNA levels.

Overexpression of GRIP-1 had no effect on basal (vehicle control) IL-6 mRNA expression (Figure 4.9). Increased GRIP-1 protein levels were found to moderately increase DEX-mediated repression of TNFα–induced IL-6 mRNA expression as shown in Figure 4.9, although significance was not established, which suggests that the IL-6 promoter may already be saturated with GRIP-1 protein. However, in the absence of DEX, TNFα–induced IL-6 transcription was significantly ($p < 0.01$) reduced when GRIP-1 protein was overexpressed. This reduction of IL-6 gene expression in End1/E6E7 cells in the presence of GRIP-1 expression vector was approximately 54%. Taken together with the results in figures 4.6, 4.8 & 4.9 these results suggest that in the presence of TNFα, GRIP-1 recruited to the IL-6-promoter modulates the levels of transcription of endogenous IL-6 in End1/E6E7 cells, via GR bound to the promoter, both in the absence and presence of DEX. In the absence of DEX, but in the presence of TNFα, the unliganded GR occupies the promoter and recruits some GRIP-1 which functions in this context as a co-repressor to dampen the TNFα response. In the presence of DEX and TNFα, more GRIP-1 is recruited, where it also acts as a co-repressor for the liganded GR.

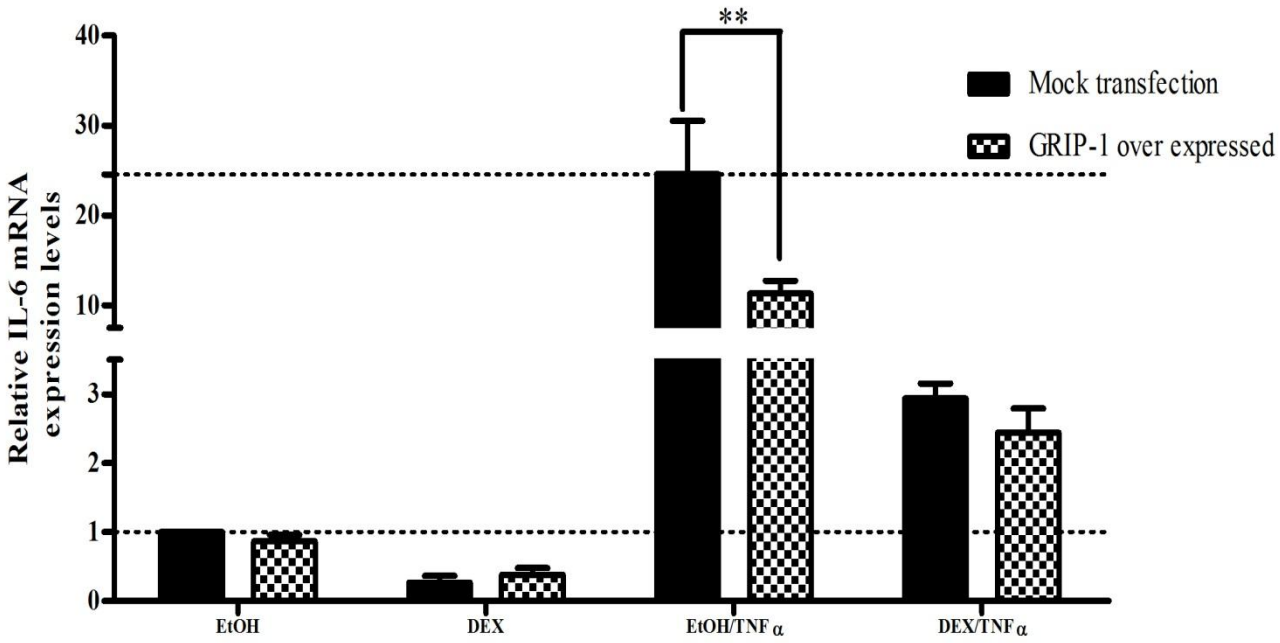


Figure 4.9: Overexpression of GRIP-1 attenuates TNFα induction of IL-6 gene expression. Twenty-four hrs after transfection with 500 ng mock plasmid (black bars) or 500 ng GRIP-1 plasmid DNA (chequered bars), End1/E6E7 cells were treated with vehicle control (EtOH) or 1 μM DEX in the absence or presence of 20 ng/μL TNFα for 24 hrs. Total RNA was isolated and 500 ng mRNA was reverse-transcribed. Relative IL-6 mRNA expression was measured by qPCR and normalised to relative GAPDH gene expression, which served as internal control. Relative IL-6 gene expression of treated samples was calculated relative to vehicle control. Graph represents pooled results of at least three independent experiments. For statistical analysis two-way ANOVA was used with Bonferroni as post-test. P-values are represented as follows: $p < 0.01$ by **.

Discussion

In the present study the role of the GR in transcriptional regulation of endogenous IL-6 in response to TNFα and DEX in End1/E6E7 cells was investigated. The results show a significant ($p < 0.001$) increase of IL-6 mRNA expression in response to TNFα, while DEX was found to result in repression of TNFα-induced IL-6 gene expression by approximately 80% (Figure 4.1). Evidence is also provided by means of siRNA technology that the DEX-mediated repression of IL-6 gene expression is mediated via the GR. Interestingly, this study also provides evidence for the first time that the unliganded-GR is involved in dampening TNFα-induced IL-6 mRNA regulation (Figure 4.2C) since RU486, the GR antagonist, decreased the TNFα response in the absence of DEX (Figure 4.3). In addition, decreasing GR protein levels by GR knockdown was found to increase TNFα-induced IL-6 expression (Figure 4.2C). Consistent with this result, TNFα induced GR nuclear translocation, albeit not as

efficiently as DEX (Figure 4.5). TNFα also selectively induced hyper-phosphorylation of the GR at Ser-226 but not Ser-211, in contrast to DEX, which elicited GR hyper-phosphorylation at both Ser-211 and Ser-226 (Figure 4.4). Moreover, the GR is recruited to the IL-6 promoter in response to TNFα in both the absence and presence of DEX. Taken together, the results show for the first time that unliganded GR represses IL-6 gene expression induced by TNFα.

TNFα is an early response pro-inflammatory cytokine released on bacterial and viral infection and is responsible for the induction of various cytokines including IL-6 (Fichorova & Anderson, 1999; Rasmussen *et al.,* 1997; De Bosscher *et al.,* 1997). The finding that TNFα dramatically increases IL-6 expression is consistent with previous reports in End1/E6E7 cells (Fichorova & Anderson, 1999). The repressive action of DEX on TNFα–induced IL-6 expression is also in accordance with previous studies in murine endothelial heart-(TC10s) and mouse fibroblast (L929A) cells (De Bosscher *et al.,* 1997; Koubovec *et al.,* 2004). This is however the first study to show ligand-independent IL-6 gene repression by the GR in response to TNFα. For both treatment conditions (TNFα alone and TNFα in the presence of DEX), GR acts as a repressor indicating that while TNFα dramatically induces IL-6 expression (~ 22-fold; Figure 4.1), it concurrently recruits the GR to dampen gene expression, possibly thereby preventing overproduction of IL-6 mRNA. This is the first report demonstrating GR recruitment to a cytokine gene promoter in response to TNFα (Figure 4.6), which occurs to the same extent as that of DEX in the presence and absence of TNFα (Figure 4.6). In contrast, although the ChIP and nuclear fractionation protocols both followed similar treatment conditions i.e incubation times and ligand concentrations, nuclear translocation in response to TNFα only was not as pronounced as for DEX alone or in co-treatment with TNFα (Figure 4.5). It is difficult to explain this apparent inconsistency. It is plausible that the amount of GR recruited to the nucleus by DEX is in huge excess over what is needed for binding to the IL-6 promoter and that binding of the GR to the IL-6 promoter could be a high affinity interaction. There is some GR present in the nuclear fractions under basal conditions (Figure 4.5), therefore a large increase in the concentration of nuclear GR may not be required for GR to elicit a nuclear response. Together this could possibly explain the similar levels of GR detected by ChIP on the IL-6 promoter, even when the extent of nuclear translocation differs

between conditions. Nevertheless, GR recruitment to the IL-6 promoter confirms GR nuclear import in response to TNFα in the absence of ligand.

Numerous studies have reported ligand-independent activation of steroid receptors including the estrogen receptor (ER) (Bunone *et al.,* 1996; El-tanani & Green 1997), progesterone receptor (PR) (Pierson-Mullany & Lange, 2004), and androgen receptor (AR) (Ueda *et al.,* 2002a; Ueda *et al.,* 2002b). However, only a few studies have investigated ligand-independent activation of the GR (Tanaka *et al.,* 1996; Eickelberg *et al.,* 1999; Kotitschke *et al.,* 2009). Results regarding nuclear translocation of the GR concur with other studies investigating unliganded GR activation (Tanaka *et al.,* 1996; Eickelberg *et al.,* 1999; Kotitschke *et al.,* 2009). Both GnRH and β2-adrenergic receptor agonist have been reported to induce nuclear translocation of unliganded GR, and both compounds were found to be less effective than DEX in eliciting translocation of GR protein from the cytoplasm (Kotitschke *et al.,* 2009; Eickelberg *et al.,* 1999), as found in the present study for TNFα. A β2-adrenergic receptor agonist induced GR translocation to the nucleus after 4 hrs stimulation as determined by means of nuclear fractionation (Eickelberg *et al*., 1999). Immunohistochemical analysis in mouse pituitary cells (LβT2) demonstrated that after 1 hr treatment with GnRH, a peptide hormone binding to the GnRH-receptor in cellular membranes (Kotitschke, Sadie-Van Gijsen, Avenant, Fernandes, & Hapgood, 2009), the GR moves to the nucleus. Similarly, in Chinese hamster ovary cells, Tanaka and co-workers (1996) reported nuclear import of the GR in response to ursodeoxycholic acid (UDCA) after 2 hrs stimulation (Tanaka *et al*., 1996). All three studies investigating activation of the unliganded GR reported increased transactivation of glucocorticoid inducible promoters whereas the present study is the first to report ligand-independent transrepression by the GR of an endogenous target gene. Ligand-independent repression by nuclear receptors has been shown for the thyroid hormone receptor (TR) (Lin *et al.,* 2002), retinoic acid receptor (RAR) (Chen & Evans, 1995), and the peroxisome proliferator-activated receptor γ (PPARγ) (Yu *et al*., 2005). However, the ligand-independent repression induced by the nuclear receptors is reversed in the presence of agonists, in contrast to the present study, which shows increased repression of IL-6 expression in the presence of ligand. The above-mentioned studies showed less co-repressors recruited in response to ligand, unlike the finding of the present study whereby increased GRIP-1 is recruited

in the presence of ligand, further supporting the hypothesis that GRIP-1 acts as a co-repressors for the GR in this context. The ability of GRIP-1 to act as a GR co-repressor via a tethering mechanism is consistent with the literature (Rogatsky *et al.*, 2001; Rogatsky *et al.*, 2002).

The GR contains a number of phosphorylation sites with Ser-203, 211 and 226 conserved between species. These become hyper-phosphorylated on ligand binding (Weigel & Moore 2007; Ismaili & Garabedian 2004). Hyper-phosphorylation at one or more of these sites was shown to be required for promoter-specific increased transcriptional activation efficacy (Avenant *et al.,* 2010). As shown in Figure 4.4, DEX induced phosphorylation of both serine residues, Ser-211 and Ser-226, in the absence and presence of TNFα, while TNFα selectively increases phosphorylation at Ser-226. Interestingly, basal phosphorylation of both serine residues investigated was high, which might suggest GR agonist present in the medium. However the selective phosphorylation induced by TNFα argues against this phenomenon. A recent study by Kotitschke and co-workers (2009), investigating ligand-independent activation of the GR by GnRH also reported selective GR phosphorylation at Ser-226 and not Ser-211 in a mouse pituitary cell line (LβT2) (Kotitschke *et al.,* 2009). Taken together these results suggest that Ser-226 plays an important role in ligand-independent activation of the GR. In addition, other phosphorylation sites may also be involved in activation of the unliganded GR. Other serine residues of the GR have been shown to be phosphorylated, e.g. Ser-203 (Weigel & Moore, 2007) and Ser-404 (Galliher-Beckley & Cidlowski, 2009), and may also undergo changes in phosphorylation status in response to non-glucocorticoid stimuli. Differential GR phosphorylation may induce dissimilar GR conformational changes that could differentially influence GR interaction with other proteins such as co-factors, thereby causing differential affects on gene transcription. Furthermore the kinase pathways responsible for GR phosphorylation at Ser-226 in an unliganded manner still need further examination. Eickelberg and colleagues (1999) showed that an increase in cellular cAMP levels resulted in the activation of the GR in lung fibroblast cells (Eickelberg *et al*., 1999). This could explain GR phosphorylation at Ser-226 by TNFα, as the PKC pathway is activated by TNFα (Comalada *et al*., 2003). In support of this hypothesis, the PKC pathway in combination with the MAPK pathways, were reported to be involved in the GnRH-induced ligand-independent

phosphorylation of the GR in LβT2 cells (Kotitschke *et al.,* 2009). However, in the human derived breast carcinoma cell line, T47D cells, GR phoshorylation status did not change in cells treated with DEX plus PKA and PKC activators, compared to DEX only (Moyer *et al.,* 1993), suggesting that the involvement of kinase pathways in GR phosphorylation is cell-specific. Activation of the JNK pathway has been shown to increase phosphorylation of the GR at Ser-226 specifically in transiently transfected COS-7 cells (Itoh *et al*., 2002). Direct interaction of the GR with JNK has also been shown without affecting JNK's interaction with c-Jun (Bruna *et al.,* 2003). This may therefore represent a possible mechanism of TNFα–induced GR phosphorylation at Ser-226, as TNFα is able to activate the JNK pathway. However, Kotitschke and colleagues (2009) showed by using JNK inhibitor, that the GR was not phosphorylated by JNK in LβT2 cells indicating that kinases involved may be cell-specific (Kotitschke *et al.,* 2009). The present author has also shown, using MAPK pathway inhibitors in the End1/E6E7 cells, that the MAPK pathways ERK, JNK, and p38 do not effect TNFα–induced IL-6 mRNA expression in the absence of DEX (Chapter 3). This suggests that in the End1/E6E7 cells, the MAPKs are not involved in phosphorylation of unliganded GR at Ser-226. The exact signalling pathway by which TNFα activates unliganded GR phosphorylation at Ser-226 and induces nuclear import still remains to be established. This and the fact that DEX induced GR recruitment to the IL-6 promoter to a similar extent (Figure 4.6) in the presence and absence of TNFα lead to the investigation of the possible role GRIP-1, in GR-mediated repression of IL-6 in response to DEX in the presence and absence of TNFα.

GRIP-1 is part of the p160 family of co-factors, which interacts with the conserved LXXLL sequence motif situated in the AF-1 region of steroid receptors (McKenna 2002). This family of co-factors is generally accepted for their role as co-activators (McKenna *et al.,* 1999). However recently, GRIP-1 has been reported to be involved in DEX-mediated repression of the AP-1 driven collegenase-3 gene (Rogatsky *et al.,* 2001) and a subsequent study also showed GRIP-1 acting as a co-repressor on a NFκB-driven promoter construct in response to DEX (Rogatsky *et al.,* 2002). TNFα induction of IL-6 expression has been reported to occur mainly via NFκB (Vanden Berghe *et al*., 1998), which recruits co-factors such as SRC-1 and CBP/p300 to the promoter region of the IL-6 gene (Vanden Berghe *et al.,* 1998; Vanden Berghe *et al.,* 1999; De Bosscher

et al., 2001). The present study shows that GRIP-1 was recruited to the IL-6 promoter in response to TNFα, not in the capacity of co-activator, but as co-repressor (Figure 4.8). This is supported by the finding that overexpression of GRIP-1 attenuated TNFα-induced IL-6 mRNA expression as shown in Figure 4.9. Co-factor interaction with other steroid receptors such as the PR, ER, and AR in the absence of ligand (in response to a non-steroid hormone stimulus) has been previously reported (An *et al.*, 2006; Tremblay & Giguère, 2001; Dutertre & Smith, 2003; Smith *et al.*, 1993; Deblois & Giguère, 2003; Zwijsen *et al.*, 1998; Lavinsky *et al.*, 1998). GnRH treatment of mouse pituitary αT3-1 cells induced progesterone response element (PRE) reporter promoter activity, which was dependent on both PR and steroid receptor co-activator (SRC)-3 expression (An *et al.*, 2006). Both PR and SRC-3 were recruited to the PRE and gonadotropin α-subunit promoter in response to GnRH (An *et al.*, 2006). In contrast to results of the present study showing that GRIP-1 acts as a co-repressor, SRC-3 knockdown studies showed that SRC-3 is required for maximal GnRH induction of the PRE (An *et al.*, 2006). Furthermore, cylin-D1 and increased cAMP levels induced the interaction of SRC-1 with the ERα in a ligand-independent manner (Kalkhoven *et al.*, 1996). Increased cAMP levels have also been shown to facilitate interaction between SRC-1 and chicken PR in a ligand-independent manner (Rowan et *al.*, 2000) and SRC-1 and AR protein-protein interaction was induced in response to IL-6 in the absence of androgens as determined by two-hybrid- and co-immunoprecipitation assays (Ueda *et al.*, 2002). Ligand-independent repression by the TR, RAR, and PPARγ has also been shown to be mediated by the co-factors nuclear receptor corepressor protein (NCoR) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT) (Horlein *et al.*, 1995; Chen & Evans 1995; Yu *et al.*, 2005). Furthermore, phosphorylation of both PR and ER increases their association with co-factors namely SRC-1 (steroid receptor co-activator-1), GRIP-1, and CREB-binding factor (CBP) (Dutertre & Smith, 2003; Rowan *et al.*, 2000). Phosphorylation of the ER at Ser-104/106/118 has also been shown to regulate SRC-1, GRIP-1 and CBP interaction with unliganded ER (Dutertre & Smith, 2003). Using a mammalian two-hybrid system in HeLa cells, mutations of Ser-104/106/118 decreased co-factor-ER interactions drastically. The authors confirmed the requirement of phosphorylation *in vitro* by the treatment of recombinant ER with phosphates, which decreased ER co-immunoprecipitation with the co-factors (Dutertre & Smith, 2003). Phosphorylation of the GR at Ser-226 might be required for the

interaction of GRIP-1 with the unliganded GR and certainly needs further investigation. This would be consistent with the findings of Avenant and co-workers (2010) that showed ligand-induced phosphorylation of transfected human GR at one or more serine residues (211, 226, or 203) is required for GRIP-1 interaction in transfected COS-1 cells (Avenant *et al.,* 2010).

The results of the present study also suggest that TNFα recruits one or more other protein e.g NFκB and/or c-Jun, which facilitates GRIP-1 recruitment to the IL-6 promoter. This is because GRIP-1 recruitment to the IL-6 promoter was only observed in the presence of TNFα with and without DEX and not with DEX alone (Figure 4.6B). This phenomenon also argues against GR agonist contamination in the treatment medium. TNFα is a potent activator of NFκB and AP-1, both of which are required for maximal induction of IL-6 gene expression (Ng *et al.,* 1994; Vanden Berghe *et al.,* 1999b). The p65 subunit of NFκB has been shown to interact with the liganded GR in *in vitro* co-immunoprecipitation assays (McKay & Cidlowski, 2000; Caldenhoven *et al.,* 1995 Scheinman, *et al.,* 1995; Ray & Prefontaine 1994) with the co-factor CBP increasing this interaction. Similarly, c-Jun has been shown to directly interact with the liganded GR (Brogan *et al.,* 1999). It is certainly plausible that these transcription factors, which are also activated by TNFα could interact with the unliganded GR in End1/E6E7 cells. Future studies certainly need to investigate what transcription factor acts as a bridging factor to recruit GRIP-1, which causes dampening of TNFα–induced IL-6 gene expression mediated by unliganded GR.

To conclude, from the present study, and other recent findings regarding the GR (Kotitschke, *et al.,* 2009), it is clear that a paradigm shift concerning the mechanism of action of the GR is developing. The present study shows that the unliganded GR plays a role in attenuating expression of IL-6 mRNA in response to TNFα in endocervical epithelial cells. Although this study is not the first to report activation of the unliganded GR (Kotitschke *et al.,* 2009; Tanaka *et al.,* 1996; Eickelberg *et al.,* 1999), this is the only study thus far to show the involvement of unliganded GR in gene repression. TNFα significantly stimulates IL-6 mRNA expression, while GR knockdown and GRIP-1 overexpression experiments suggest that TNFα also limits maximal IL-6 promoter activity by recruiting unliganded GR and GRIP-1 proteins, thereby effectively

diminishing over-production of IL-6 in the endocervical epithelial cells. While the epithelial cells lining the lower FRT are effective in protecting women against infection they do so without compromising reproductive functions such as menstruation, transport of sperm, fertilization, implantation, gestation and parturition (Wira *et al.*, 2005). Uncontrolled inflammation could have negative effects on the FRT, such as increased susceptibility to disease (Lin & Karin, 2007). The present study proposes a model whereby TNFα significantly increases pro-inflammatory IL-6 gene expression while concurrently diminishing it by recruiting unliganded GR and GRIP-1 to the IL-6 promoter. This mechanism could be described as an in-built restriction of inflammation in the FRT epithelium. Physiological relevance might also apply to other mucosal surfaces such as the respiratory- and intestinal mucosa. The prevention of excessive IL-6 mRNA expression by the GR might also apply to other cytokine genes induced by TNFα and needs to be further investigated.

CHAPTER FIVE

CONCLUSIONS AND FUTURE PERSPECTIVES

Introduction

The endocervical mucosa, which is composed of columnar epithelial cells, forms part of the lower FRT. It provides an effective physical barrier against microbial infection while at the same time playing a role in both innate and adaptive immunity (Wira *et al.,* 2005). Additionally, the endocervix as well as the rest of the FRT should be adaptable to a range of physiological events involved in reproduction. The endocervical mucosa constitutively expresses immune mediators that are significantly upregulated on TNFα and IL-1β stimulation (Fichorova *et al.,* 1999). However, overproduction of inflammatory regulators could influence the onset and progression of disease. The pro-inflammatory mediators IL-6 and IL-8 have both been implicated in replication and shedding of HIV (Alfano & Poli, 2005; Poli & Fauci, 1993; Lane *et al.,* 2001; Narimatsu *et al.,* 2005; Poli *et al.,* 1990; Schnittman *et al.,* 1991; Breen *et al.,* 1990; Gumbi *et al.,* 2008). Chronic inflammation of the endocervix also causes recruitment and activation of $CD8^+$ and $CD4^+$ lymphocytes, which are HIV host cells (Gumbi *et al*., 2008). Additionally, chronic inflammation has been implicated in promotion and/or exacerbation of cancer (Libra *et al.,* 2009; Castle *et al.,* 2001; Watts *et al.,* 2005). Besides their pro-inflammatory role, IL-8 and IL-6 also promote angiogenesis and prevent apoptosis and these cytokines have thus been implicated in cervical cancer progression (Lin & Karin, 2007; Wei *et al.,* 2001; Chauhan *et al.,* 1997; Wei *et al.,* 2001; Klein *et al.,* 1995; Rose-John *et al.,* 2006; Nilsson *et al.,* 2005). Moreover, chronic inflammation of the cervix can lead to other diseases such as endometriosis and pelvic inflammatory disease (Lusk & Konecny, 2008; Richter *et al.,* 1999). The inflammatory response of the FRT should protect against invading pathogens yet be sensitive enough to prevent overproduction of pro-inflammatory mediators that might negatively affect reproductive function.

The present study used the End1/E6E7 immortalised endocervical epithelial cell line as a cell model for the endocervical mucosa. This cell line has been reported to exhibit similar immune mediator expression and morphology to primary endocervical epithelial

cells (Fichorova *et al.*, 1997; Fichorova & Anderson, 1999). Little is known about the effects of the synthetic progestins MPA and NET-A, as compared to the endogenous hormone P4, in the endocervix, and whether or not they interfere with local endocervical epithelial immune function. In the first part of this study (Chapter 3) the effects of MPA, NET-A, and P4 on immune function gene regulation were investigated. In the second part of this study (Chapter 4), due to an interesting observation in Chapter 3, the role of the GR in TNFα regulation of IL-6 in the absence and presence of DEX was examined.

Regulation of pro-inflammatory cytokine genes by the natural hormone P4 and the synthetic progestins MPA and NET-A

MPA and NET-EN/NET-A are extensively used as contraceptives and in HRT and also at high dosages for the treatment of endometriosis and certain cancers (Wira *et al.*, 2005). Very few studies have investigated the effects of MPA and NET-A on the expression of genes involved in immune function in the endocervical muscosa. Furthermore, little is know about their mechanism of action at the cellular level. However, there is evidence to suggest that these compounds may play an important role in local immune responses. Increased susceptibility to viral and bacterial infections in the FRT of both humans and primates has been reported in response to progestin treatment (Morrison *et al.*, 2004; MacLean, 2005; Marx *et al.*, 1996; Trunova *et al.*, 2006), although some studies do not find a correlation between susceptibility to viral infection and progestin treatment (Kleinschmidt *et al.*, 2007; Myer *et al.*, 2007; Morrison *et al.*, 2007). Furthermore, an increase in both HIV and HSV shedding has been reported in subjects treated with injectable contraceptives (Mostad *et al.*, 1997; Mostad *et al.*, 2000; Wang *et al.*, 2004). MPA has also been associated with increased acquisition of cervical chlamydial and gonococcal infections, the most common bacterial sexually transmitted infections (STIs) (Morrison *et al*., 2004a).

The extent to which changes in immune function *in vivo* are due to progestin treatment is not known. In addition, little is known about the mechanism of action of synthetic progestins at a molecular level, especially in the cervical mucosae. The present study focused on the effect of P4, MPA, and NET-A on expression of the IL-6, IL-8, and RANTES genes. These cytokines were chosen specifically as their expression levels

had previously been shown to be influenced by MPA. MPA inhibited expression of IL-6 (Bamberger *et al.*, 1999; Kurebayashi *et al.*, 1999; Mantovani *et al.*, 1997; Kriek MSc thesis 2005) and IL-8 (Koubovec *et al.*, 2005; Kelly *et al.*, 1994) in several cell lines. MPA has also been reported to cause both increased and decreased expression of RANTES in different cell systems (Deng *et al.*, 2007; Zhao *et al.*, 2002)

Results presented in Chapter 3 show that MPA and NET-A both augment TNFα–induced IL-8 and RANTES gene expression and differentially regulate IL-6 gene expression (Figure 3.1). This is in contrast to P4, which only induced IL-6 expression (Figure 3.1). In the absence of TNFα, the progestins P4, MPA, and NET-A increase the expression of IL-6 although to different extents (Figure 3.2). These results would appear to be inconsistent with results by others for MPA (Koubovec *et al.*, 2004; Koubovec *et al.*, 2005; Bamberger *et al.*, 1999; Kelly *et al.*, 1994; Zhao *et al.*, 2002). However a likely cause for these discrepancies is the different cell lines in which these studies were conducted. Similarly, P4 has been reported to decrease IL-8 expression in uterine cervical fibroblasts derived from rabbits (Ito *et al.*, 1994). Although generally considered to be anti-inflammatory, the present study reports that progestins including P4, appear to play a pro-inflammatory role in endocervical epithelial cells. These results suggest that MPA and NET-A could have important implications for endocervical immune function, as pro-inflammatory cytokines are associated with elevated levels of HIV-1 shedding in the female genital tract (Gumbi *et al.*, 2008). It would be interesting to examine the effect of these compounds on viral infectability (prior and concomitant treatment with virus) in the End1/E6E7 cell line, in addition to measuring how cytokine/chemokine expression is affected in the presence of virus. In addition, both IL-6 (Wei *et al.*, 2001) and IL-8 (Rasmussen *et al*., 1997) have also been implicated in cervical cancer progression and pathogenesis which may be affected by progestin treatment.

Having shown that P4, MPA, and NET-A differentially regulate TNFα-induced IL-6 gene expression, more in-depth investigations followed, examining the molecular mechanism involved in IL-6 gene expression by the progestins in the presence of TNFα. Progestins, like other steroid hormones, mediate a biological effect by interacting with steroid receptors. Repression of IL-6 and RANTES in response to

MPA, has previously shown to be mediated by the GR and PR, respectively (Bamberger *et al.,* 1999; Koubovec *et al.,* 2004; Zhao *et al.,* 2002). Therefore the role of steroid receptors in IL-6 expression by the progestins was investigated. The endogenous steroid receptor expression profile of the End1/E6E7 cell line had not been reported, and consequently the expression of ERα, GR, PR-B, MR, and AR in these cells was investigated by Western blotting. The results showed that the ERα, GR, MR, and AR are expressed in the End1/E6E7 cells (Figure 3.3). However, only the ERα and GR were found to be transcriptionally competent on a transiently transfected synthetic steroid response element (SRE) promoter reporter construct (Figures 4A & 4B). Non-functional expressed AR has been reported in genital skin fibroblast due to abnormal AR mRNA splicing leading to the deletion of 123 nucleotides from the mRNA. Translation of the truncated mRNA resulted in an AR protein 5 kDa smaller than the wild type that is unable to induce a biological response due to ineffective ligand-binding (Ris-Stalpers *et al.,* 1990). Moreover, androgen insensitivity experienced by cancer patients is normally due to non-functional AR being expressed (Brinkmann *et al.,* 1995; Quigley *et al.,* 1995). Therefore it is plausible that a similar non-functional AR is expressed in the End1/E6E7 cell line. Surprisingly, no PR-B was detected, although immunohistochemical analysis has shown the PR to be expressed in the human cervix (Remoue *et al.,* 2003; Nair *et al.,* 2005; Konishi *et al.,* 1991). However, the expression of the PR-B has been shown to be dependent on the menstrual cycle as its expression is diminished during the follicular phase (Konishi *et al.,* 1991; Stjernholm-Vladic *et al.,* 2004). Decreased circulating P4 results in decreased total PR expression (Konishi *et al.,* 1991; Stjernholm-Vladic *et al.,* 2004). Thus since the cells are grown in the absence of P4 and also may have been established during the follicular phase, this could explain the absence of PR-B expression. However, that does not exclude the possibility that the PR-A isoform is expressed and that P4, MPA, and NET-A might also act via this receptor.

GR antagonist- and/or GR siRNA experiments showed that P4, MPA and NET-A elicit at least part of their response via the GR (Figures 3.6, 3.8B and Figure B1). GR knockdown studies (Figure 3.8B) do however suggest that the induction of IL-6 by P4, MPA, and NET-A is mainly via the GR, since complete abrogation of IL-6 induction is

observed in the presence of GR siRNA (Figure 3.8B). In agreement, results whereby various steroid receptors were overexpressed, showed that only overexpressed GR increases NET-A induced IL-6 mRNA expression (Addendum Figure B2). Interestingly, in the absence of glucocorticoids, both RU486 and decreased GR expression augmented TNFα–induced IL-6 expression and this phenomenon was further investigated in Chapter 4.

Future studies certainly need to further investigate the role of other steroid receptors such as ER in this response, since NET-A metabolites can bind to the ER albeit with low affinity (Sasagawa *et al.*, 2008). In fact, preliminary results show that NET-A-induced IL-6 mRNA decreases in the presence of overexpressed ERα, suggesting that NET-A could induce IL-6 gene repression via the ERα. Experiments using an ER-specific antagonist and ER siRNA could indicate a role of the ER in mediating induction of IL-6 by NET-A. Since both MPA and NET-A are used in combination with estrogen in HRT (Sitruk-ware, 2004), it would be of interest to determine how the expression of IL-6, IL-8, and RANTES are affected by the progestins in the presence of estrogen.

The effect of progestins on steroid receptor levels also certainly needs to be examined as increase in steroid receptor expression and turnover could have an effect on progestin-induced signalling. While the present study observed no differences in GR protein levels after 24 hrs treatments with the compounds (Figure 3.5), other steroid receptor such as PR-A and ERα levels were not monitored and future studies need to investigate this.

Another possible mechanism for the differential IL-6 regulation by the progestins could be the involvement of different MAPK signalling pathways. Further analysis of the differential activation of MAPKs by MPA and NET-A using MAPK inhibitors indicated that ERK and p38 are involved in induction of IL-6 gene expression by NET-A, but not MPA (Figure 9). Interestingly, the p38 but not the ERK pathway is involved in P4 induction of IL-6 mRNA expression (Figure 3.10). The results with the MAPK inhibitors should ideally be confirmed using other strategies, since these inhibitors can act non-specifically (Kotitschke PhD thesis, 2009). Other strategies could include 1)

overexpression of dominant negative and wild type ERK, p38, and JNK expression plasmids, and 2) knockdown of individual MAPKs using siRNA technology.

To summarise, although MPA and NET-A were created to be biologically similar to the endogenous hormone, P4, the findings presented in Chapter 3 suggest that the progestins act differently to each other and P4 depending on the cytokine gene. P4, MPA and NET-A significantly upregulate IL-8 and RANTES gene expression, unlike P4, while they differentially regulate IL-6 gene regulation. Interestingly, although P4, MPA, and NET-A differentially regulate IL-6 mRNA expression, all three compounds appear to act mainly via the GR. However, differential MAPK signalling pathways appear to be responsible for the observed differential IL-6 regulation by the progestins. A better understanding of the mechanism of differential gene regulation by the different progestins and the steroid receptors and signalling pathways involved could assist in the design of new progestins with fewer side effects.

Ligand-independent activation of the GR modulates IL-6 expression in response to TNFα

Ligand-independent activation of the GR has not been extensively studied although it has been widely reported for other steroid receptors (Cenni and Picard 1999; Weigel and Zhang 1998). Increased transcriptional activity of the unliganded GR in response to ursodeoxycholic acid, the beta-adrenergic receptor agonists salmeterol and salbutamol, and GnRH has been reported (Tanaka *et al.*, 1996; Eickelberg *et al.*, 1999; Kotitschke *et al.*, 2009). Chapter 4 focused on TNFα activation of the GR in the absence of GR ligand. Figure 5.1 summarises the results of this chapter in the form of a model for the role of the GR in modulation of the IL-6 response in response to TNFα. The results showed that the GR is required for the repression of IL-6 mRNA by DEX and interestingly that the GR also modulates the response to TNFα, in the absence of DEX (Figure 4.2C & Figure 5.1). It was also shown that TNFα induced nuclear translocation of the GR in the absence of DEX, albeit to a lesser extent than in the presence of DEX (Figure 4.5B & Figure 5.1). Nuclear translocation has also been reported for the unliganded GR in response to ursodeoxycholic acid, beta-adrenergic receptor agonists, and GnRH (Tanaka *et al.*, 1996; Eickelberg *et al.*, 1999; Kotitschke *et al.*, 2009). Consistent with activation of the GR by TNFα, TNFα induced hyper-

phosphorylation of the GR at Ser-226 and not Ser-211, unlike DEX, which induced hyper-phosphorylation of both serine residues (Figure 4.4) as depicted in Figure 5.1B versus Figure 5.1C. Selective phosphorylation of the GR in response to GnRH has previously been reported (Kotitschke *et al.*, 2009), with GnRH inducing phosphorylation of Ser-226 and not Ser-211, similar to the results presented here for TNFα. Similarly, ligand-independent phosphorylation of other steroid receptors has also been reported (Ueda *et al.*, 2002; Dutertre & Smith, 2003). Others have shown that the GR is also phosphorylated at Ser-203 (Weigel & Moore, 2007) and Ser-404 (Galliher-Beckley & Cidlowski, 2009) in response to DEX and it would be interesting to determine how TNFα affects phosphorylation at these sites by using phospho-specific antibodies as phosphorylation at Ser-226 would appear to be required for ligand-independent activation. In addition, the signalling pathway(s) responsible for the selective phosphorylation by TNFα still needs to be examined. TNFα acting via its cognate receptor activates a wide variety of signalling pathways (Barbara *et al.*, 1996). Any of these pathways might be involved in the differential phosphorylation of the GR and this certainly needs further examination. However, as already showed in Figure 3.10., the MAPK inhibitors had no effect on TNFα-induced IL-6 gene expression, which might exclude these signalling pathways although the phosphorylation status of the GR was not determined in the presence of the MAPK inhibitors. The precise mechanism of glucocorticoid-independent activation of the GR by TNFα remains an intriguing question that requires further investigation. It would be particularly interesting to examine whether phosphorylation at Ser-226 is a cause or a consequence of a key event.

Interestingly, TNFα induced the recruitment of the GR to the IL-6 promoter to the same extent as that induced by DEX, providing further confirmation of ligand-independent activation of the GR by TNFα (Figure 4.6 & Figure 5.1). Interestingly, less nuclear translocation of the GR occurs in the presence of TNFα alone than in the presence of DEX, although similar GR levels were recruited to the IL-6 promoter as shown in Figure 5.1B vs Figure 5.1C. This would suggest that the affinity of the GR for the IL-6 promoter is high and independent of excess GR present in the nucleus. Alternatively, it is possible that more GR is recruited in the presence of DEX, but that this GR is not all accessible to the antibody, due to the presence of other proteins in a

complex on the promoter. A similar situation has been described for the ERα, the presence of which is masked by GRIP-1 on the TNFα promoter (Cvoro *et al.*, 2006).

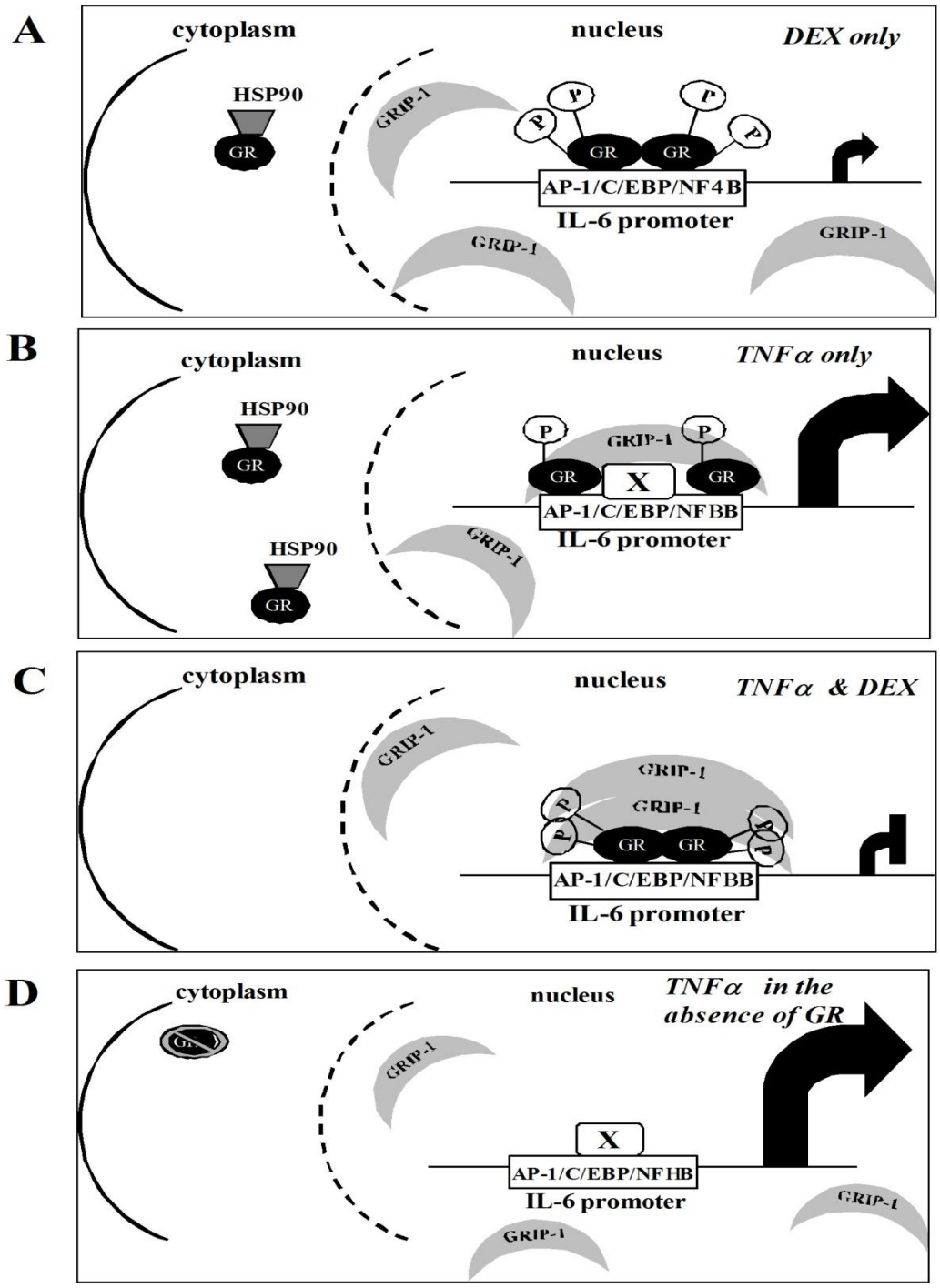


Figure 5.1: Schematic presentation of GR cross-talk with TNFα signalling and resulting modulation of IL-6 gene expression in endocervical epithelial cells. (A) DEX stimulated IL-6 promoter, (B) TNFα stimulated IL-6 promoter, (C) TNFα and DEX stimulated IL-6 promoter, (D) TNFα stimulated IL-6 promoter in the absence of GR. **Abbreviations:** c-Jun; cellular Jun, c-Fos; cellular Fos, GR; glucocorticoid receptor, GRIP-1; GR–interacting protein type 1, NFκB; nuclear factor kappa B, TNFα; tumour necrosis factor-alpha, X; unknown co-factor induced by TNFα responsible for the increase in IL-6 promoter activity, Y unknown protein, P; phosphorylation.

The involvement of the GR and co-factor GRIP-1 in DEX-dependent and DEX-independent regulation of IL-6 gene expression was investigated by ChIP analysis. Figure 5.1 summarises the results of these experiments together with the results for nuclear translocation and GR phosphoryation, which schematically illustrates the ligand-independent activation of the GR and its subsequent modulation of IL-6 expression in response to TNFα (Figure 5.1). The co-factor GRIP-1 was found to be recruited to the IL-6 promoter in response to TNFα (Figure 4.8) and on further analysis when GRIP-1 was overexpressed it was shown to be acting as a co-repressor (Figure 4.9). GRIP-1, which is generally considered to be a co-activator, has been reported to act as a co-repressor in response to DEX on both NFκB and AP-1 driven reporters (Rogatsky *et al.*, 2001; Rogatsky *et al.,* 2002) and in response to E2 on the endogenous TNFα promoter in the U2OS cell line (Cvoro *et al.,* 2006). Interestingly, only in the presence of TNFα is GRIP-1 recruited to the IL-6 promoter (Figure 4.8), which suggests that TNFα recruits a transcription factor such as NFκB and AP-1 that assists in the recruitment of GRIP-1 as schematically presented in Figure 5.1. This certainly needs further investigation.

It has been suggested that NFκB is the most important transcription factor involved in IL-6 gene expression (Libermann & Baltimore, 1990; Shimizu *et al.,* 1990; Zhang *et al.,* 1990; De Bosscher *et al.,* 1997; Vanden Berghe *et al.,* 1999; Vanden Berghe *et al.,* 2000), and a mutation in the NFκB response element has been shown to abrogate TNFα induction of the IL-6 promoter (De Bosscher *et al.,* 2001). But while NFκB is essential for IL-6 induction, activation of additional transcription factors such as AP-1 is required for maximal IL-6 induction (Vanden Berghe *et al.,* 1999). Repression of TNFα-induced IL-6 expression by liganded GR has been reported to be mediated via direct interaction between GR and NFκB (De Bosscher *et al.,* 2001). TNFα is also known to be able to activate C/EBP (Barbara *et al.,* 1996), which with NFκB and AP-1 is recruited to the IL-6 promoter (Vanden Berghe *et al.,* 2000). It could be hypothesised that ligand-independent GR modulation of IL-6 gene expression in response to TNFα in the endocervical epithelial cells also occurs via direct interaction between GR and one or all of these transcription factors. The likely tethering of the GR to these transcription factors is thus proposed as illustrated in Figure 5.1. To test

this hypothesis one could perform ChIP on ChIP analysis, using p65 (NFκB subunit) and GR specific antibodies for sequential immunoprecipitation steps. Additionally, by using primer sets designed to identify specific regulatory sites present in the IL-6 promoter, the promoter region at which the interaction occurs could be identified or more precisely mapped by ChIP. However, this strategy might not be feasible as the *cis*-regulatory sites are situated in close proximity to each other (Figure 1.2). As alternative strategy, End1/E6E7 cells could be transfected with human IL-6 promoter-reporter constructs with point mutations at the various *cis*-regulatory elements, thereby testing the requirement of the each response element for the ligand-independent GR repression on TNFα-induced promoter activity. In addition, a similar approach could also be followed to determine the possible involvement of AP-1 as shown in Figure 5.1 as the GR is also known to physically interact with AP-1 (Jonat *et al.,* 1990). These experiments will help identify the transcription factor via which the unliganded GR tethers and the particular *cis*-regulatory elements are involved. It would also be interesting to compare the molecular mechanism of ligand-independent GR repression of IL-6 to that of liganded GR.

The recruitment of regulatory proteins to the IL-6 promoter in response to TNFα must be carefully controlled to allow a significant increase in transcriptional activity and yet prevent over-stimulation of the promoter. As the results of the present study suggest GRIP-1 recruitment to the IL-6 promoter is in the capacity of co-repressor, it would be interesting to determine which co-activators are recruited in response to TNFα (Figure 5.1C, Factor X) to result in the large TNFα-induced upregulation. Co-factors CREB binding protein (CBP), p300, and steroid receptor co-factor-1 (SRC-1) have been reported to interact with AP-1 and NFκB (Gerritsen *et al.,* 1997; Perkins *et al.,* 1997; Na *et al.,* 1998; Lee *et al.,* 1998; McKay & Cidlowski, 2000; De Bosscher *et al.,* 2000) and CBP/p300 has been reported to play an important role in increasing IL-6 promoter activity through the interaction with NFκB (De Bosscher *et al.,* 2000; Vanden Berghe *et al.,* 1999). Recruitment of these co-activators to the IL-6 promoter might be blocked by ligand-activated GR and this possibility could be investigated by ChIP using co-activator specific antibodies eg. CBP, p300, and SRC-1. In contrast, CBP has been shown to increase the interaction between p65 and GR, thus augmenting ligand-dependent GR-mediated repression of the NFκB-responsive

promoter reporter (McKay & Cidlowski, 2000). CBP may therefore play a similar role in endogenous IL-6 gene regulation in endocervical epithelial cells. Overexpression or knockdown of CBP may lend support to this hypothesis.

Finally, the mechanism of action of unliganded GR studied here might not be unique to IL-6, and other cytokine genes such as IL-8 and RANTES might also be affected. This certainly needs to be considered for further investigations. Similarly, ligand-independent activation in response to TNFα might not be cell-specific and could also apply to other cells in the lower FRT such as the ectocervical and vaginal epithelial cells. Unliganded GR cross-talk with other early response pro-inflammatory cytokines such as IL-1β also needs to be investigated.

The current study has established that the activity of progestins in the context of the endocervix is primarily pro-inflammatory. This may be a mechanism whereby progestins provide protection against certain infections. However, since HIV infection requires inflammatory cells to be recruited and activated by the immune-mediators investigated, this pro-inflammatory activity could be a drawback, and actually increase susceptibility to HIV infection. This is consistent with reports that HIV infection and viral load are increased in the presence of the cytokines investigated (Gumbi *et al.*, 2008). Additionally, since progestins increase markers of inflammation, use of progestins as contraception could also lead to chronic inflammation of the endocervix, which could affect cancer progression. This possibility and certainly needs further investigation.

In the second part of this study it was established that in endocervical epithelial cells, unliganded GR restricts the over production of IL-6 mRNA in response to TNFα. The present study is the first study to report a role for the unliganded GR in a transrepression model and to suggest an auto-regulatory mechanism involving restriction of IL-6 expression by the unliganded GR. This finding suggest a mechanism whereby the FRT should be immune-sensitive in order to prevent infection by invading pathogens, while the immune response should not impair reproductive processes. Modulation of TNFα induction of IL-6 by unliganded GR presents an attractive mechanism to allow both processes.

ADDENDUM A

EXPERIMENTAL OPTIMIZATION

A.1: Quantitative real time PCR (qPCR) optimisation

Isolation of RNA is a crucial first step in the analysis of gene expression. It is important to obtain high quality, intact RNA as poor quality RNA can compromise experimental results obtained by qPCR, which is very time-consuming, labour-intensive, and expensive. Therefore, RNA quality was routinely assessed by two methods prior to reverse transcription. The first method was measuring the optical density (OD) with a spectrophotometer at 260 nm (specific for nucleic acids) and 280 nm (specific for proteins). The OD 260/280 ratio greater than 1.9 is generally considered acceptable and indicative of good quality RNA (Sambrook *et al.*, 1989). However, the 260 nm OD reading, which is also used for concentration determination, can be compromised by the presence of genomic DNA. Therefore, the isolated RNA was secondly analysed on a denaturating formaldehyde agarose gel. The 28S RNA band should be approximately twice as intense as the 18S RNA band. Thus a 2:1 ratio is a good indication that the RNA is intact. A smear is indicative of degraded RNA, and genomic DNA generally remains in the well due to its high molecular weight. Figure A1.1 shows an example of such a gel, showing intact RNA uncontaminated by genomic DNA.

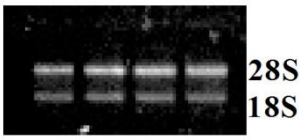


Figure A1.1: Representative of a 0.5 μg RNA agarose gel showing the quality of RNA. RNA was isolated from End1/E6E7 cells using TriReagent® (Sigma-Aldrich).

Following RNA isolation and assessment of the RNA integrity, cDNA was synthesised as described in Chapter 2. However, initial qPCR experiments were found to be very irreproducible. On investigation it was found that the cDNA synthesis step and not the actual PCR step was at fault. Three different cDNA synthesis kits from three different manufacturers were therefore tested to solve this problem. These kits were compared in triplicate parallel reactions, from a single RNA sample. The relative GILZ mRNA

expression was measured for each sample, as shown in Figure A1.2. A high degree of variability was detected for kits B and C. Kit B, which had been used initially was found to exhibit large error in the efficiency of cDNA synthesis between parallel reactions Kit A exhibited the highest degree of reproducibility and it was therefore decided to use Kit A for subsequent experiments.

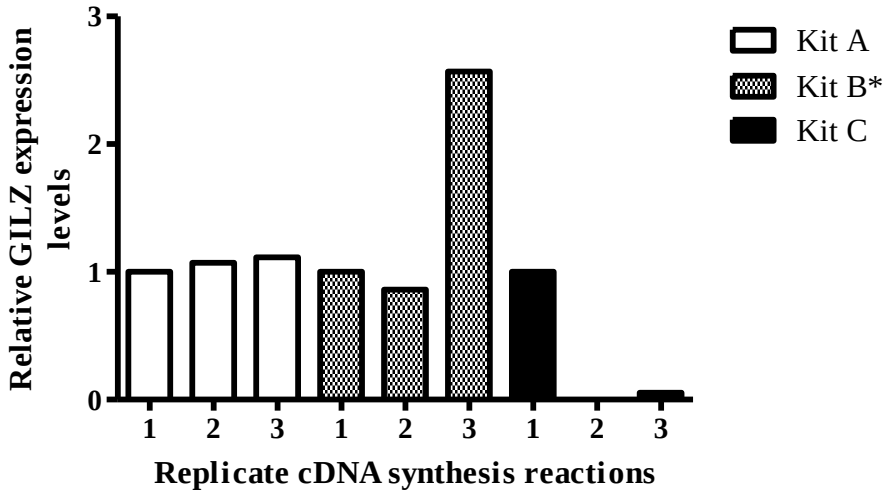


Figure A1.2: Comparison of different cDNA synthesis kits. cDNA synthesis from a single RNA sample was performed in triplicate, using three different cDNA synthesis kits designated A, B, and C. Relative GILZ mRNA expression was subsequently measured by qPCR in duplicate and normalised to relative GAPDH mRNA expression. The average crossing-point of the duplicate PCR samples amplified with GILZ primers was normalised to the average crossing point of duplicate PCR samples analysed with GAPDH primers to give the relative GILZ expression levels.
*Kit P was initially used and the likely source of the irreproducibility experienced at the outset

The relative expression of the genes of interest was calculated using the Pfaffl equation (Pfaffl, 2001), as shown below and expressed relative to the expression levels of the untreated control.

$$\textit{Relative expression levels} = \frac{(E_i)^{\Delta CP\ (control\ -\ sample)}}{(E_r)^{\Delta CP\ (control\ -\ sample)}}$$

E_i is the amplification efficiency of the gene of interest, and E_r the efficiency of the reference gene. The difference in crossing points (ΔCP) between the vehicle control

and the treated samples is calculated for the gene of interest and the reference gene (Pfaffl MW, 2001).

As the method of Pfaffl (2001) was used to calculate the relative gene expression of the various cytokines, the primer efficiencies of the primer sets used (i.e. IL-6, IL-8, RANTES, and GAPDH) were determined. This was calculated by creating serial dilutions of a single cDNA sample, and performing triplicate qPCR reactions for each dilution. A dilution curve is generated by plotting the crossing points for each dilution (Y-axis) against the log of cDNA concentration (X-axis) as shown in Figure A.1.3.

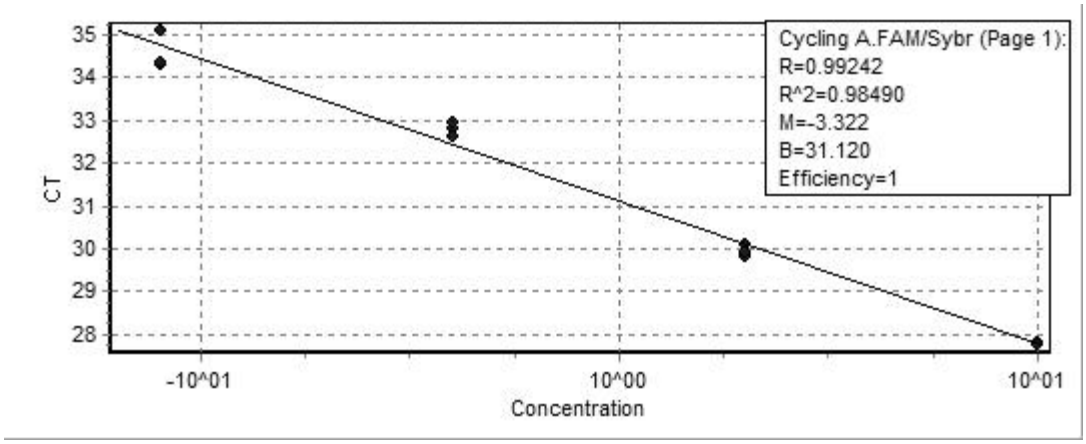


Figure A1.3. Example of dilution series data generated by Rotorgene software for IL-6 amplification products.

The slope of the curve as given by the Rotorgene software (annotated as M) is used to calculate the primer efficiency, by means of the following equation (Pfaffl, 2001).

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

The final primer efficiencies used of the cytokine genes together with the reference gene, GAPDH, was determined by pooling the primer efficiencies determined from three independent experiments (Table A1.1).

Table A1.1. Primer efficiency determination the various cytokine genes investigated together with the reference gene GAPDH

	Primer set			
	IL-6	**IL-8**	**RANTES**	**GAPDH**
1	2.00	2.05	1.96	2.04
2	2.43	2.07	2.12	1.55
3	2.23	1.94	1.89	2.32
Average	**2.22**	**2.02**	**1.99**	**1.97**

However, before commencing on calculating the relative gene expression levels, it is important to perform a melting curve analysis at the end of each qPCR reaction. The position of the peak of the melting curve is unique for each PCR product and is used to distinguish between different amplification products and therefore essential to confirm amplification specificity. An example of such a curve is shown below (Figure A1.4).

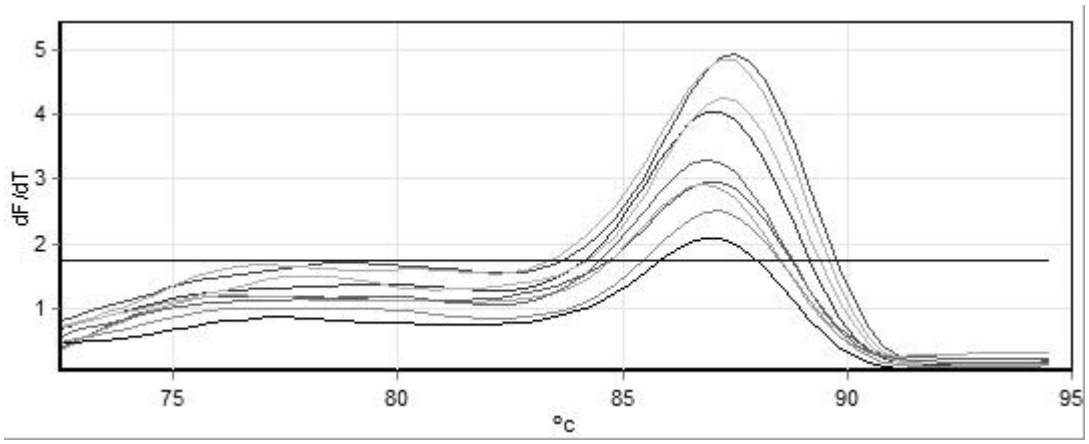


Figure A.1.4. Example of melting curve analysis generated by the Rotogene software for IL-6 amplification products.

A.2. ChIP assay optimisation

ChIP assays are used to investigate the association of proteins with specific DNA regions. This technique involves crosslinking of proteins with chromatin, fragmentation by sonication, and immunoprecipitation with an antibody specific for the protein of interest. The DNA sequence associated with the protein of interest is then identified and quantified by means of PCR. Sonication of the chromatin is a critical step in the ChIP

protocol as it not only aids in further lysing of crosslinked cells, but also determines the average size of the DNA fragments for immunoprecipitation. The optimal DNA fragment sizes should be between 300 bp and 800 bp (Ma *et al.*, 2003) as very small fragments may be lost during the DNA purification step and large chromatin fragments might not allow for efficient immunoprecipitation. The sonication step therefore needs to be optimised for different cell lines, to yield appropriately sized chromatin fragments. Pilot experiments using different settings and times were performed. The size of the fragmented chromatin was evaluated by agarose gel electrophoresis after crosslinking reversal. In Figure A2.1 End1/E6E7 cells were sonicated using the Misonix Sonicator® set on power 3, for 10 cycles at 20 sec per cycle, with 40 sec intervals between pulses, producing chromatin fragments with a average size of 400 bp. Brief pulses of sonication were used because sonication generates heat. Proteins are heat sensitive and long sonication intervals could cause overheating and denature proteins.

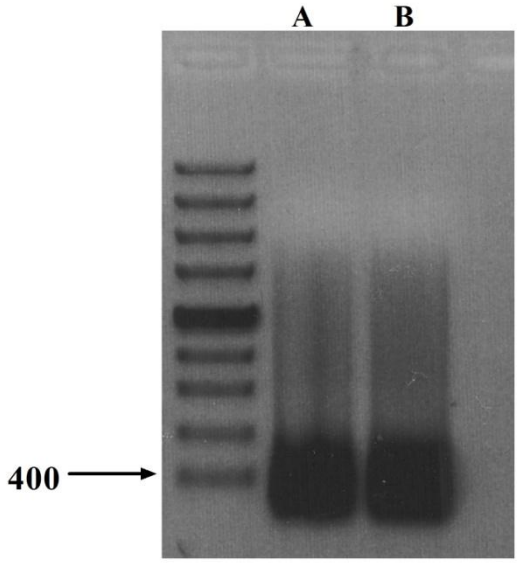


Figure A.2.1: Example of gel-electrophoresis analysis of chromatin after sonication. End1/E6E7 cells were treated with (A) EtOH or (B) 1 mM DEX for 2 hrs after which cells were crosslinked, harvested and sonicated at Power 3 for 10 cycles at 20 sec per cycle, with 40 sec intervals between pulses using a Misonix Sonicator®. Subsequently crosslinking was reversed overnight and DNA was purified using QiaQuick® columns. Sonicated chromatin was analysed by 1% agarose gel electrophoresis.

In addition, the qPCR product from the ChIP analysis was routinely analysed by melting curve analysis and agarose gel electrophoresis to confirm the amplification a single specific product. An example of such an analysis is shown in Figure A2.2.

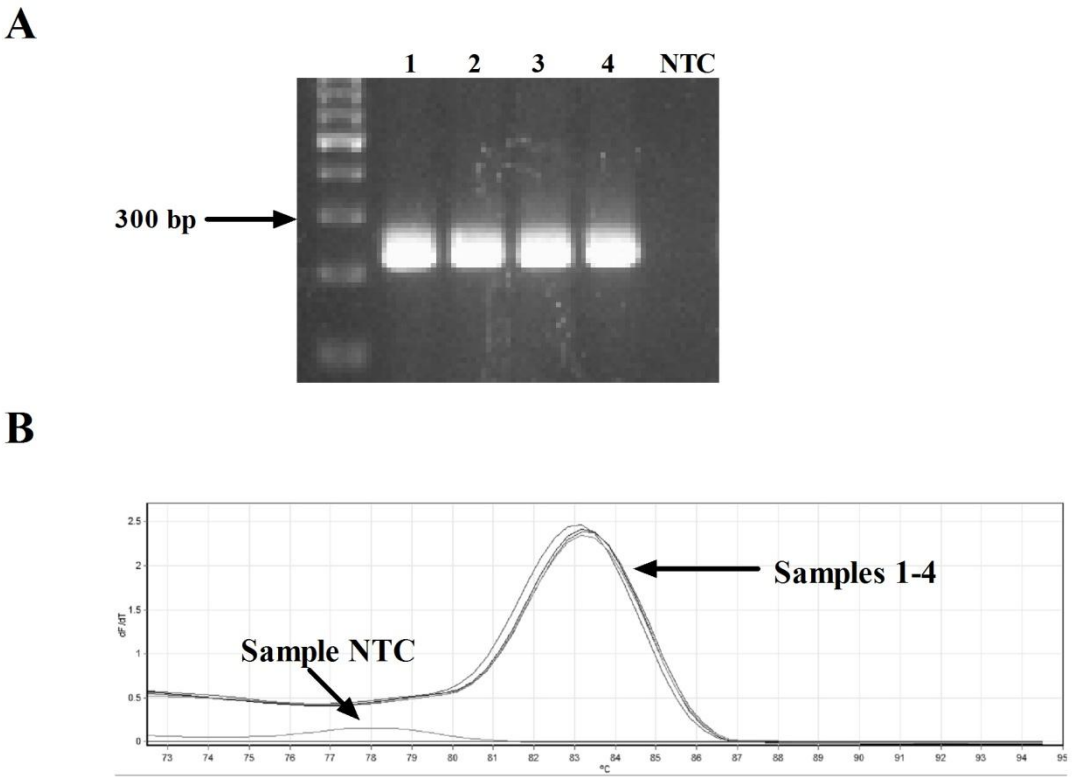


Figure A.2.2: Verification of amplification of a single product of expected size. End1/E6E7 cells were treated with (1) EtOH or (2) 1 mM DEX for 3 hrs or pre-treated with (3) EtOH or (4) 1 μM DEX for 1 hr and subsequently treated with 20 ng/μLTNFα for an additional 2 hrs, followed by ChIP. Results shown are those of input samples only. PCR products were analysed by (A) 2% agarose gel electrophoresis and (B) melting curve analysis. Representative figures are shown.

ADDENDUM B

SUPPLEMENTARY RESULTS PERTAINING TO CHAPTER 3

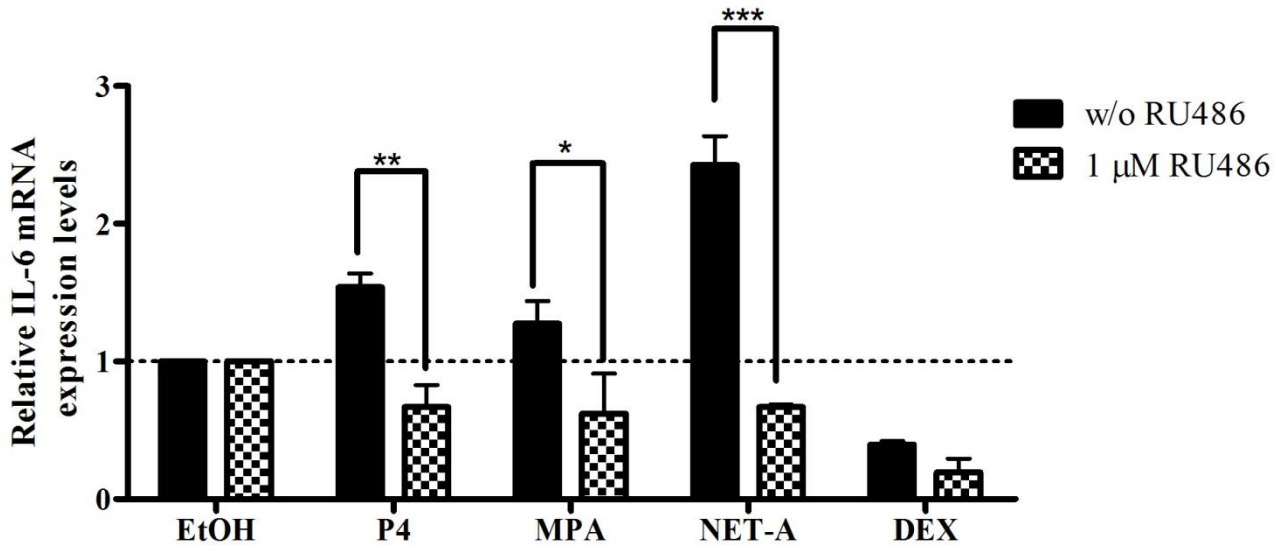


Figure B1: The role of the GR in IL-6 gene expression in response to P4 and the synthetic progestins MPA and NET-A in the endocervical cell line End1/E6E7. End1/E6E7 cells were treated with 20 ng/μL TNFα and 1 μM P4, MPA, NET-A, or DEX in the absence or presence of 1 μM RU486. Total RNA was isolated after 24 hrs and 500 ng mRNA was reverse-transcribed. Relative IL-6 mRNA expression was measured by quantitative real-time PCR and normalised to relative GAPDH gene expression, which served as internal control. Relative IL-6 gene expression of treated samples was calculated relative to vehicle control, which was set at 1. Graphs represent pooled results of at least three independent experiments. For statistical analysis two-way ANOVA was used with Bonferroni as post-test (***; $p < 0.001$).

Overexpression of various steroid receptors

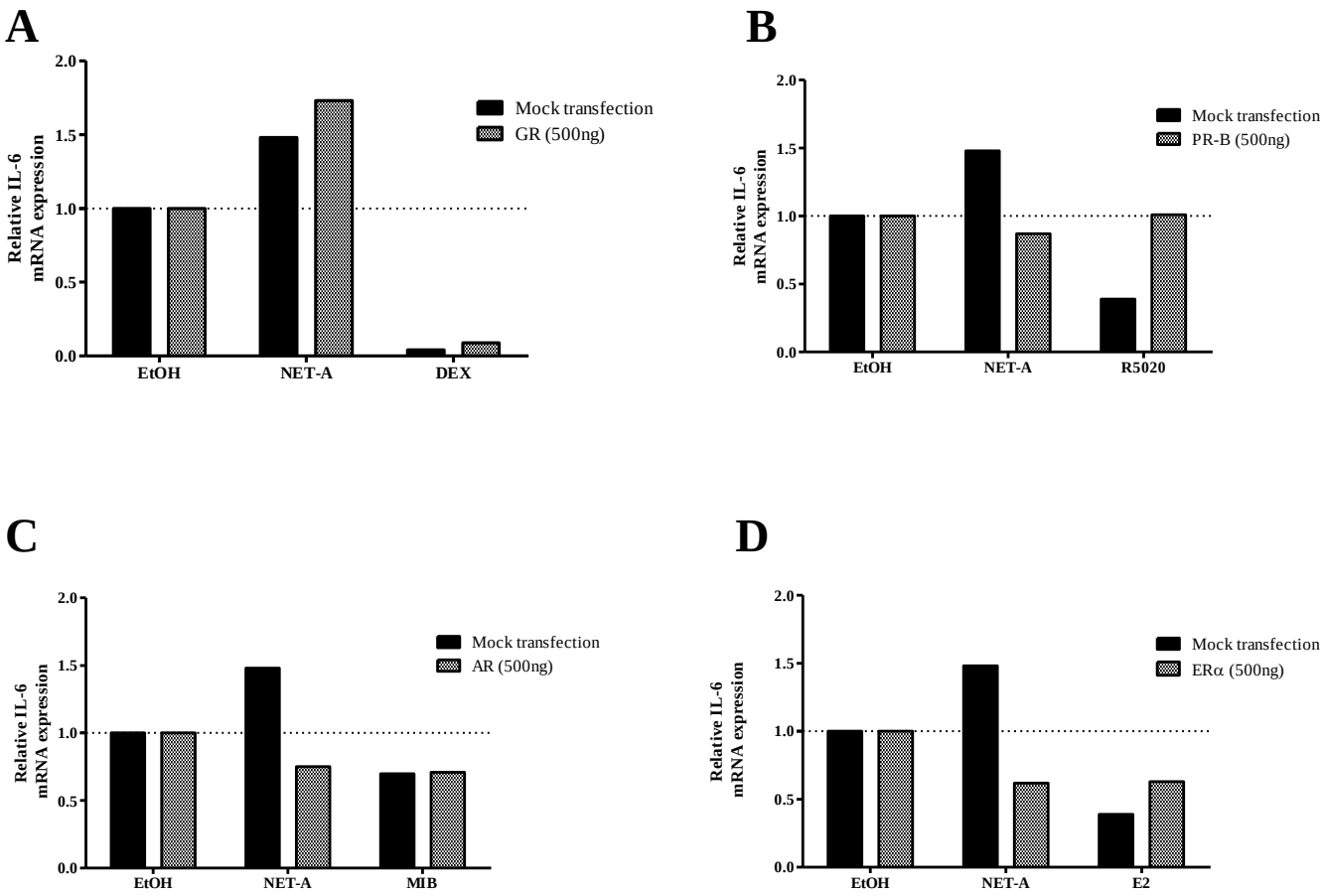


Figure B2: End1/E6E7 cells were transiently transfected with 500ng empty vector (pGL2-basic) (solid bars) or (A) GR (pCMV-HA-human GR), (B) PR-B (pSG5hPR-B), (C) AR (pSVARo), and (D) ERα (pSG5-hERα) expression vectors (checked bars). Twenty-four hrs after transfection cells were treated with EtOH (0.1%) or 1 μM steroid receptor specific agonists for 24 hrs. Total RNA was isolated after 24 hrs treatments and 500 ng total RNA was reverse-transcribed. Relative IL-6 mRNA expression was measured by quantitative real-time PCR and normalised to relative GAPDH mRNA expression, which served as internal control. Relative IL-6 gene expression of treated samples was calculated relative to vehicle control. Graphs represent one experiment.

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