Synergism between immune activators and progestogens: differential effects on gene expression and HIV-1 replication and the role of the glucocorticoid receptor

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Declaration

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

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Abstract

Globally, women account for more than half of the 36.9 million people living with HIV-1. In some regions particularly Sub-Saharan Africa, women of child-bearing age are at great risk of being infected with HIV-1 than men of the same age group. Transmission is mainly through penetrative vaginal intercourse with an infected male partner. Current evidence suggests that women using certain types of progestin-only hormonal contraceptive methods have a higher risk of HIV-1 infection. In addition, there is evidence suggesting that genital tract infections (GTIs), which have high prevalence in Sub-Saharan Africa, also modify the risk of HIV-1 infection in women. Incidentally, regions were progestogen-only hormonal contraceptives use and genital tract infections are prevalent also have with high rates of HIV-1 prevalence in women. This raises the question whether medroxyprogesterone acetate (MPA), norethisterone enanthate (NET) or levonorgestrel (LNG), the active compounds in progestogen-only hormonal contraceptives, cooperate with inflammation associated with GTIs to further elevate the risk of HIV-1 acquisition in women. Current evidence suggests MPA, LNG and NET differentially the expression of immune function genes relevant for HIV-1 infection in the female genital tract (FGT). In addition, there is evidence suggest that MPA and LNG impairs the integrity of mucosal barrier of the FGT.

This study investigated the central hypothesis that MPA, NET and LNG acting alone or in synergy with immune activators [tumour necrosis factor-alpha (TNF) and lipopolysaccharide (LPS)] via the glucocorticoid receptor (GR) regulate the expression of genes of relevant for maintaining the integrity of genital tract mucosal surfaces, mucosal permeability, select immune function genes and HIV-1 replication. This hypothesis was investigated in the endocervical epithelial End1/E6E7 cell, ectocervical tissues explants from pre-menopausal women, primary genital tract epithelial cells, peripheral blood mononuclear cells (PBMCs) and the TZM-bl indicator cell line. Gene expression analysis in responses to progestogen alone or in combination with immune activators was performed by real-time PCR, ELISA, Luminex assays and western blotting. The role of the GR was investigated using RU486 or GR siRNA knockdown. Mucosal barrier integrity and permeability was assessed by confocal microscopy and transepithelial electrical resistance measurements. For infection assays, TZM-bl cells were exposed to HIV-$1_{Bal\text{-}Rellina}$ infectious molecular clones (IMCs) before treatment progestogen alone or in combination with immune activators. In addition, TZM-bl cells were pre-conditioned with supernatants from PBCs treated alone with progestogen or in combination with immune activators before exposed to HIV-$1_{Bal\text{-}Rellina}$ IMCs.

In the absence of TNF, MPA at physiologically relevant doses acted via the GR to downregulate claudin-4 mRNA expression in End1/E6E7 cells, with MPA behaving like a partial GR agonist when

compared to dexamethasone (DEX). Similarly, MPA acted via the GR to selectively upregulate CCL20 mRNA expression in End1/E6E7 cells, with MPA behaving like a full GR agonist when compared to hydrocortisone (CORT). It was also observed that MPA upregulated toll-like receptor (TLR)2 mRNA expression, but reduced interleukin (IL)6 and IL1β mRNA expression in End1/E6E7 cells. Neither NET not LNG regulated claudin-4 and immune function expression in End1/E6E7 cells. The addition of TNF, on the other hand, did not alter the effect of MPA on claudin-4 expression, suggesting that there is no cooperativity between MPA and TNF in regulating this gene. Neither NET nor LNG was found to cooperate with TNF to regulate claudin-4 expression in End1/E6E7 cells. However, MPA unlike NET acted synergistically via the GR with TNF or LPS to upregulate C-C motif chemokine ligand (CCL)20 expression in End1/E6E7 cells. This was not an isolated event as MPA was also found to enhance TNF-induced expression of TNF receptor 2 (TNFRSF1B). The occurred against the backdrop of MPA but not NET repressing TNF-induced expression of IL6, IL1β and CCL5. In ectocervical tissues, MPA like NET did not regulate basal claudin-4 expression, but unlike NET downregulated desmoglein-1 expression. This suggest that MPA may increase the permeability of the endocervix and ectocervix via different mechanisms. This study was unable to established whether MPA and TNF synergistically upregulate CCL20 in ectocervical tissues. In PBMCs, however, MPA selectively enhanced LPS-induced expression of CCL20, but suppressed LPS-induced expression of IL6, IL8, IL1β and CCL5. Finally, MPA unlike NET was found to act alone or additively with TNF or LPS to increase HIV-1 replication in TZM-bl cells. While this suggest MPA directly affects HIV-1 replication, it was observed that the effects may also be indirect as secretions from PBMCs cotreated with MPA and LPS but not NET and LPS enhanced HIV-1 replication in TZM-bl cells.

Taken together, the results shown in this study provides new insight into plausible mechanisms by MPA but not NET acting via the GR may enhance the susceptibility of the endocervix to HIV-1. Firstly, they suggest that MPA unlike NET may increase the permeability of the endocervix via a mechanism that is different from the ectocervix. Secondly, they suggest that the upregulation of select immune mediators and innate immune receptors by MPA but not NET in the endocervix in the absence of immune activation may render the endocervix vulnerable to HIV-1 infection. Thirdly, that MPA unlike NET is more likely to synergise with immune activators to further upregulate the expression of select immune function, but not tight junction genes in the endocervix. Collectively, this suggest MPA unlike NET can cooperate with GTIs to further increase the risk of HIV-1 acquisition in women residing in high risk regions. In this setting, NET-EN but not DMPA-IM would be a safer choice of injectable progestin-only contraceptive.

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Thesis Outline

This thesis is comprised of six chapters as well as three appendices for supplementary data.

Chapter One: Literature Review. This chapter is the literature review and it gives a brief overview of the effects of progestogens used in hormonal contraception on HIV-1 acquisition risk in women. It also focuses on the molecular mechanisms, detailing the role of the glucocorticoid receptor.

Chapter Two: **Materials and Methods**. Describes the materials and methods used to obtain the results presented in the thesis.

Chapter Three: Medroxyprogesterone acetate, like glucocorticoids but unlike other select progestins, regulates select tight junction and immune function target genes, including CCL20, alone and in combination with pro-inflammatory stimulators, in a gene-specific manner in an endocervical epithelial cell line. This chapter focuses on whether glucocorticoids and progestogens differ in the manner in which they regulate gene expression in the absence and presence of immune activators. More specifically, this chapters attempts to establish whether glucocorticoids and progestogens cooperate with immune activators to regulate the expression of genes relevant for HIV-1 infection and pathogenesis in an endocervical epithelial cell line.

Chapter Four: The glucocorticoid receptor mediates the potent partial agonistic MPA-induced downregulation of claudin-4 gene expression as well as the gene-specific and synergistic interactions between MPA and immune activators that upregulate CCL20 gene expression in the endocervical epithelial cell line. This chapters focuses on whether the effects of glucocorticoids and select progestogens on the expression of select tight junction and immune function genes in an endocervical epithelial cell line occur at physiologically relevant concentrations and whether they are mediated by the glucocorticoid receptor . The chapter also attempts to establish the biocheracter of select progestogens via the glucocorticoid receptor relative to reference glucocorticoids.

Chapter Five: MPA unlike NET downregulates desmoglein-1 mRNA levels in ectocervical tissue explants, positively cooperate with immune activators to increase HIV-1 infection in TZM-bl cells and augments CCL20 expression in PBMCs. This chapter attempts to establish whether the effects seen in the cell line model can be replicated in vitro using physiologically relevant primary models (ectocervical tissue explants, PBMCs and primary genital epithelial cells). This chapter also focuses on the effects of glucocorticoids and select progestogens alone or in combination with immune activators on HIV-1 replication in the TZM-bl HIV-1 indicator cell line.

Chapter Six: Discussion, Conclusions and Future Perspective. This chapter focuses on the short-comings of the study, physiological relevance of the results, possible molecular mechanisms

involved and draws conclusions based in the context of the field. This chapter also proposes future research questions

References: Provides a list of the literature reviewed throughout this study.

Appendix A: Contains supplementary results for Chapter Three

Appendix B: Contains supplementary results for Chapter Four

Appendix C: Contains supplementary results for Chapter Five

The following are peer reviewed publication arising from this thesis:

Hapgood, J, P., Avenant, C. and Moliki, J.M. Glucocorticoid-independent modulation of GR activity: Implications for immunotherapy. *Pharmacology and Therapeutics*. 165:93-113.

The present author wrote section 5 and 7 of the review.

List of Abbreviations

AF	Activation Function
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AP	Activation protein
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
AREs	AU-rich elements
BCG	Bacillus Calmette–Guérin
BV	Bacterial vaginosis
C/EBPβ	CCAAT-enhancer-binding protein-beta
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CO_2	Carbon dioxide
COC	Combined oral contraceptives
CORT	Hydrocortisone
CXCL	C-X-C chemokine ligand
DCs	Dendritic cells
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DMPA-IM	Depot medroxyprogesterone acetate intramuscular
DMPA-SC	Depot medroxyprogesterone acetate subcutaneous
DNA	Deoxyribonucleic acid
E2	Estrogen
EC_{50}	Effective concentration required for 50% of maximal response

ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular regulated kinases 1/2
FCS	Foetal calf serum
FGT	Female genital tract
FOXA1	Forkhead Box A1
FOXO1	Forkhead box protein O1
GAPDH	Glycerealdehyde-3-phosphate dehydrogenase
GCs	Glucocorticoids
G-CSF	Granulocyte-colony stimulating factor
GILZ	Glucocorticoid-induce leucine zipper
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GRBS	Glucocorticoid receptor binding sites
GREs	Glucocorticoid response elements
GTIs	Genital tract infections
H_2O_2	Hydrogen peroxide
HC	Hormonal contraception
HIV-1	Human immunodeficiency virus type 1
IFN	Interferon
IHCs	Injectable hormonal contraceptives
IL	Interleukin
IMCs	Infectious molecular clones
IRAK-M	Interleukin-1 receptor-associated kinase M
IRF	Interferon regulatory factor
IUD	Intrauterine device
IU	Infectious Units

IκBα	Inhibitor of kappa B-alpha
JNK	c-Jun N-terminal kinase
Kd	Dissociation constant
kg	Kilogram
LBD	Ligand binding domain
LNG	Levonorgestrel
LNG-IUD	Levonorgestrel intrauterine device
LPS	Lipopolysaccharides
MAPK	Mitogen activated protein kinase
MKP-1	Mitogen-activated protein kinase inhibitor
MPA	Medroxyprogesterone acetate
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NET	Norethisterone
NET-EN	Norethisterone enanthate
NFκB	Nuclear factor-kappa B
nGREs	Negative glucocorticoid response elements
NK	Natural killer
NTD	N-terminal domain
p38	p38 MAPK
p65	NFκB p65 subunit
PBMCs	Peripheral blood mononuclear cells
pDCs	Plasmacytoid dendritic cells
PGECs	Primary genital epithelial cells
PI3K/AKT	Phosphatidyl inositol 3-kinase/protein kinase B
RLU	Relative light units
RNA	Ribonucleic acid

RPMI	Roswell Park Memorial Institute
SDF1	Stromal cell-derived factor 1
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Ser	Serine
SHIV	Simian human immunodeficiency virus
siRNA	Small interfering RNA
SKM	Stemline Keratinocyte Medium
SLPI	Secretory leukocyte protease inhibitor
STAT3	Signal transducer and activator of transcription 3
TER	Transepithelial electrical resistance
T h	T helper
TBS	TRIS-buffered saline
TBST	TRIS-buffered saline-tween
TGFβ	Tissue growth factor-beta
TJs	Tight junctions
TLR	Toll-like receptor
TNF	Tumour necrosis factor-alpha
TNFAIP3	TNF Alpha Induced Protein 3
TNFRSF1B	TNF receptor 2
TTP	Tristetraprolin
U	Units
UN	United Nations
v/v	Volume per unit volume
w/v	Weight per unit volume
ZO	Zonula occluden

Chapter One

Literature Review

1.1: Introduction

Human immunodeficiency virus type 1 (HIV-1) transmission is the transfer of infectious viral particles from an infected host to another regardless of whether they were previously infected. In women, this occurs primarily in the female genital tract (FGT) where HIV-1 target cells become infected after exposure to pathogenic cell-free or cell-bound viral particles in semen from an infected male partner (Sewankambo *et al*., 1987). The probability of male-to-female HIV-1 transmission after a single episode of unprotected vaginal sex ranges from 1:200 to 1:2000 (Hladik & McElrath, 2008). This may vary around the world, with developing countries usually having higher transmission rates than developed countries (Boily *et al*., 2009). This low probability of transmission per coital act suggests that the FGT is endowed with innate protective mechanisms that protect against infection. However, the FGT can be compromised and rendered vulnerable to infection by factors the roles of which have been controversial in HIV-1 disease acquisition and transmission.

1.2: The FGT mucosal and immune systems – understanding the first line of defence against HIV-1 infection

Mucosal surfaces of FGT are the main sites of heterosexual transmission of HIV-1 infection. They comprise the vagina, ectocervix, endocervix, endometrium and fallopian tubes (**Figure 1.1**). They provide a physical barrier against infection by pathogens including HIV-1. The vagina and ectocervix constitute the lower portion of the FGT and are lined by multiple layers of squamous epithelial cells. On the other hand, the endocervix like the rest of the upper FGT is lined by a single layer of columnar epithelial cells (Blaskewicz *et al*., 2011). The epithelial cells rest on a basement membrane and remain as a single layer (or stratum) in the upper FGT mucosa. In the lower FGT, however, they proliferate to form several strata of cells at different stages of differentiation. An intact lower FGT epithelium can restrict the passage of HIV-1 viral particles (Blaskewicz *et al*., 2011; Carias *et al*., 2016). However, productive infection can still occur in the lower FGT if the mucosal surface is impaired (Carias *et al*., 2013). Because it is lined by a single layer of columnar epithelial cells, HIV-1 viral particles are thought to be more efficiently transported across mucosal surfaces of upper than those of the lower FGT.

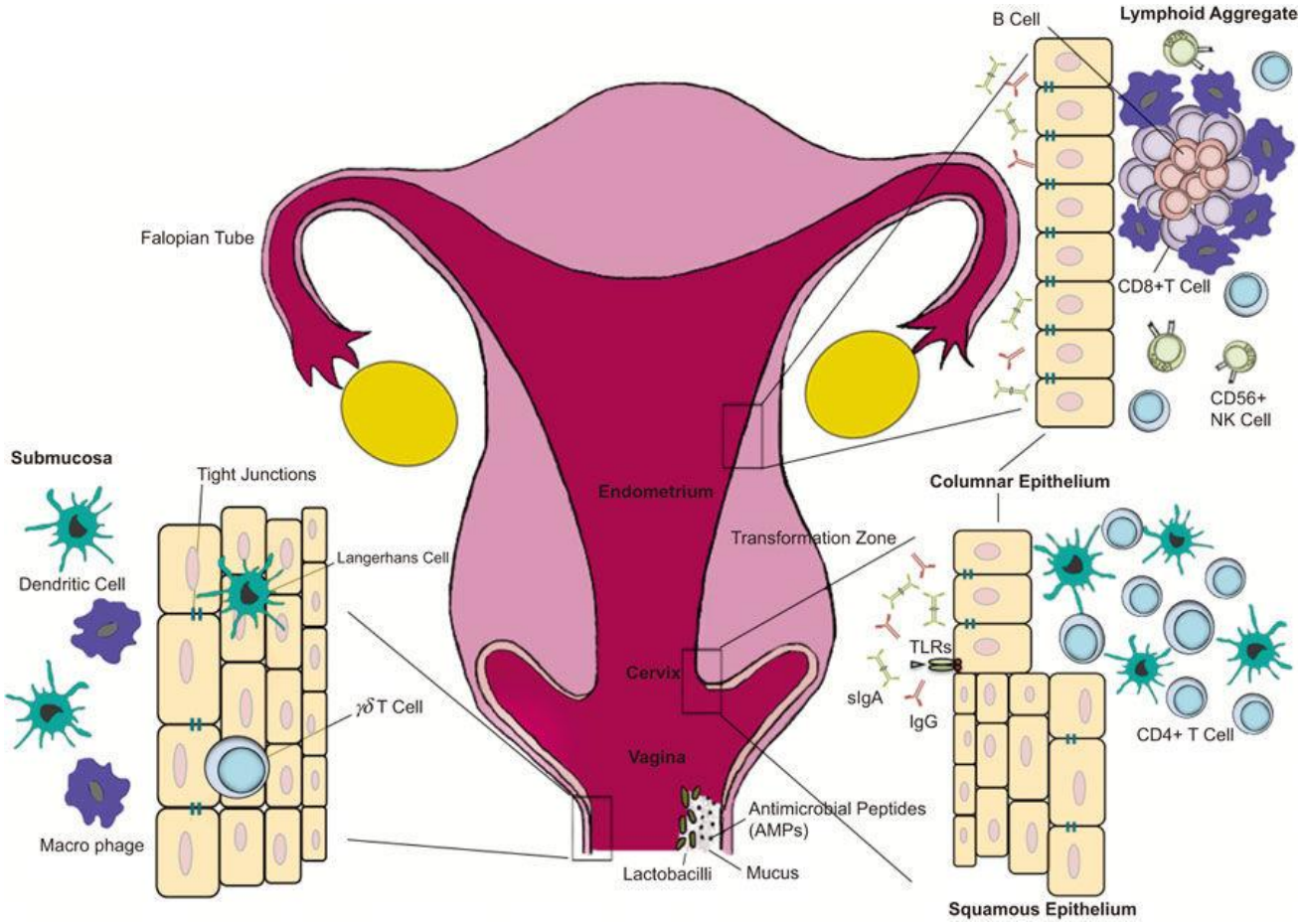

Figure 1.1: A schematic representation of the FGT showing cellular components of mucosal epithelia and immune systems. Source of image: Nguyen *et al*., 2014.

In healthy women, the vagina and ectocervix are colonised by lactic acid- and hydrogen peroxide (H_2O_2)-producing bacteria, which are predominantly of the genus *Lactobacillus*. These bacteria metabolize glycogen made by epithelial cells under the control of estrogen (E2) to produce lactic acid. This allows the FGT to maintain an acidic or low pH that is hostile to HIV-1 (Boskey *et al*., 1999). It has been reported that low pH reduces the net negative charge on the surfaces of cell-free HIV-1 viral particles. This interferes and disrupts interactions between the viral envelop and host cell receptors and explains why cell-free viral particles are less infectious at low pH (Martin *et al*., 1985; Lai *et al*., 2009). In addition, HIV-1 target cells are less activated at low pH and as a result are less susceptible to infection (Hill & Anderson, 1992; Olmsted *et al*., 2005). Moreover, in vitro studies have found that lactic acid inhibits the expression of pro-inflammatory cytokines associated with HIV-1 acquisition in the FGT (Hearps *et al*., 2017). Despite the presence of these protective mechanisms, HIV-1 infection can still be established in the FGT and the mechanisms involved are not fully understood.

Underneath the mucosal epithelial tissues of the FGT are cells of the innate and adaptive immune system (Thurman *et al*., 2016; Nguyen *et al*., 2014). The following cell types have been identified; cluster of differentiation (CD)4^+ T cell, $CD8^+$ T cells, macrophages ($CD68^+$), monocytes ($CD14^+$), neutrophils ($CD177^+$), natural killer cells (NK, $CD56^+$), Langerhans ($CD1a^+$) and dendritic cells ($CD11c^+$), with $CD4^+$, $CD1a^+$, $CD11c^+$ and $CD14^+$ expressing cells constituting targets for HIV-1 infection. Most of these cells reside in the lamina propria, but Langerhans cells and sometimes $CD4^+$ T cells and dendritic cells (DCs) can be found in intraepithelial spaces (Thurman *et al*., 2016; Nguyen *et al*., 2014). It has recently been reported that the risk of HIV-1 infection in humanised murine models is associated with the frequency of $CD45^+CD4^+$ T cells in the FGT (Nguyen *et al*., 2017). However, the majority of $CD4^+$ T cells in the FGT are effector memory T [C-C chemokine receptor $(CCR7)^-$ $CD45^-$] cells (Smith-McCune *et al*., 2017), although the proportion of central memory $CD4^+$T ($CCR7^+CD45^-$) cells may increase during the luteal phase of the menstrual cycle (Swaims-Kohlmeier *et al*., 2016). Central memory like naïve ($CCR7^+CD45^+$) $CD4^+$ T cells especially of CCR6+Th17 phenotype have been shown to be very susceptible to HIV-1 infection (Saleh *et al*., 2007; Gosselin *et al*., 2010; Gosselin *et al*., 2017; Stieh *et al*., 2016; Rodriquez-Garcia *et al*., 2014). This suggest that changes in the frequency of specific $CD4^+$ T cell subsets in the FGT may significantly alter the risk of HIV-1 infection

1.2.1: The FGT mucosal epithelial barrier function and HIV-1 infection

One of the main functions of the mucosal systems of the FGT is to restrict the exchange of material between the luminal and basal surfaces of the structures they protect (Kaushic *et al*., 2011). This function depends on junctional proteins which are strands of interconnecting transmembrane protein complexes expressed between neighbouring epithelial cells. They regulate paracellular flux and alterations in their composition and structure results in changes in paracellular permeability. There are three major junctional proteins; desmosomes, adherens junctions and tight junctions. All three types of junctional proteins are expressed in epithelial cells of the upper FGT, whereas only stratum basal epithelial cells of the lower FGT express these proteins (Blaskewicz *et al*., 2011).

Epithelial tight junctions (TJs) are usually formed on the apical surfaces of polarised epithelial cells. This arrangement maintains the polarity of epithelial cells as well as the integrity of the mucosal surfaces they line (Shin *et al*., 2006). However, these are very dynamic complexes, the structure and composition of which are regulated by physiological stimuli including growth factors, steroids and cytokines (Sekiyama *et al*., 2012; Ogawa *et al*., 2012; Al-Sadi *et al*., 2009). Three main families of TJ proteins have been identified. This include zonula occludens (ZO), occludin and claudins. ZO and occludin are mainly scaffolding proteins that are stabilised by claudins. Compared to ZO and occludin,

claudins are the most diverse TJ protein comprising at least 27 members (Günzel & Fromm, 2012). Based on their barrier forming properties, claudins are categorised as sealing and leaky. Sealing claudins include claudin-1, -4, -5 and -8, whereas leaky claudins include claudin-2, -7 and -10 (Shen *et al*., 2011). Overexpressing claudin-4 has been shown to enhance the mucosal barrier functions of epithelial cells (Balda *et al*., 1996; van Itallie *et al*., 2001). On the other hand, overexpressing claudin-2 in epithelia cells has been found to induce channel formation within the junctional complexes, which renders them porous and so more permeable (Luettig *et al*., 2015). Interestingly, claudin-2 and claudin-4 have been found to be incompatible and are inversely regulated at TJs (Capaldo *et al*., 2014). It has been reported that TJ genes are differential expressed and distributed in mucosal surfaces of the FGT (Blaskewicz *et al*., 2011). However, little is known concerning their regulation and how this affects mucosal barrier function and susceptibility to infection.

Cell- free or cell-associated HIV-1 viruses are likely to enter the body through cuts and micro-lesions in the mucosal surface (Dickerson *et al*, 1996). However, others have shown that these are not always required for infection as cell-free HIV-1 can most likely compromise the mucosal surface through mechanisms, which are not fully understood (Miller *et al*, 1994; Spira *et al*, 1996). Evidence from in vitro studies suggests that during heterosexual transmission in the FGT, cell-free HIV-1 viral particles might breach the mucosal barrier by opening up TJs between epithelial cells (Nazli *et al*., 2010; Nazli *et al*., 2013; Ferreira *et al*., 2015a; Ferreira *et al*., 2015b). Cell-free HIV-1 viral particles or viral proteins such as gp120 have been shown to downregulate the expression of TJ genes including ZO-1, occludin, claudin-1, claudin-2, claudin-3, claudin-4 and claudin-5 in primary genital epithelial cells (PGECs) (Nazli *et al*., 2010). Similarly, HIV-1 Tat has been shown to downregulate claudin-1, -3 and -4, while upregulating claudin-2 expression in retinal epithelial cells (Bai *et al*., 2008). This may allow viral particles to access target cells in the lamina propria via paracellular penetration. Alternatively, some studies have suggested that cell-free HIV-1 viral particles can be ferried across intact epithelial cells in a process called transcytosis; however, this is an inefficient process (Bohardt *et al*., 2007; Ferreira *et al*., 2015a). The study by Bohardt and colleagues showed that only 0.02% of the initial inoculum traverses the epithelium by transcytosis (Bohardt *et al*., 2007). This low efficiency can be explained by the fact that viral particles are recycled back to the apical surface as soon as they are translocated to the basolateral surface (Ferreira *et al*., 2015a). However, there is ex vivo and in vitro evidence showing that cell-associated HIV-1 virus is transmitted more efficiently across the mucosal surfaces compared to cell-free HIV-1 virus (Kolodkin-Gal *et al*., 2013; Lawrence *et al*., 2013).

In summary, the mucosal epithelia lining the FGT presents a physical barrier against HIV-1 infection. However, HIV-1 viral particles can breach this barrier by disrupting junctional proteins. In addition,

viral particles can be internalised by epithelial cells from the luminal surface and translocated across to the laminar propria.

1.2.2: The role of FGT and systemic inflammation in HIV-1 acquisition and disease progression

Inflammation is a process that involves the production of soluble immune mediators in response to an invading pathogen and the subsequent recruitment of immune cells to the site of invasion (Stankov, 2012). Evidence from animal studies suggests that inflammation plays a very important role in simian HIV-1 (SHIV) infection (Li *et al*., 2009; Shang *et al*., 2017). These studies observed that when macaques are challenged intravaginally with SHIV infectious molecular clones (SHIV IMCs), target cells are recruited to the site of exposure, most likely attracted by chemokines secreted by epithelial cells in responses to encountering viral particles (Li *et al*., 2009; Shang *et al*., 2017).

Current evidence also suggests that pre-existing inflammation associated with genital tract infections (GTIs) increases the risk of HIV-1 acquisition in women (Mirmonsef *et al*., 2012; Passmore *et al*., 2016; Alcaide *et al*., 2017). Pre-infection cervicovaginal lavages (CVL) from HIV-1 seroconverters have been found to have high levels of mostly pro-inflammatory immune mediators compared to non-converters (Masson *et al*., 2015; Selhorst *et al*., 2017). Some of these such as interleukin (IL)1β, IL6, IL8, chemokine (C-C motif) ligand (CCL)3, CCL4 and chemokine (C-X-C motif) ligand (CXCL)10 have been associated with an increased risk of HIV-1 acquisition (Mlisana *et al*., 2012; Masson *et al*., 2015).

The exact roles of these cytokines in HIV-1 infection in the human FGT are not known. Nonetheless, it has been suggested that they may enhance the recruitment of HIV-1 target cells, disrupt mucosal barrier function and modulate processes involving viral entry, integration and replication (Levine *et al*., 1998; Fichorova *et al*., 2001; Reddy *et al*., 2004; Masson *et al*., 2014). It has also been shown that HIV-1 IMCs replicate more readily in ectocervical tissue explants with high levels of IL1β, IL6, CCL2, CXCL10 and CXCL1 (Rollenhagen & Asin, 2011). In addition, highly infectious variants have been isolated from seroconverters with no FGT inflammation whereas lowly infectious strains have been isolated from seroconverters with FGT inflammation (Selhorst *et al*., 2017). This suggest that less infectious viral particles, unlike highly infectious clones, may not be able to establish infection in the absence of pre-existing inflammation.

Systemic inflammation and immune activation may influence the risk of HIV-1 acquisition in the FGT (Lehman *et al*., 2014). One study found that women who seroconvert have high levels of pro-inflammatory immune mediators in pre-infection blood samples compared to non-seroconverters, and

some of these cytokines namely CXCL10, tumour necrosis factor-α (TNF) and IL1β show significant positive association with risk of HIV-1 acquisition (Naranbhai *et al*., 2012; Kahle *et al*., 2015; Liebenberg *et al*., 2017). In addition, seroconverters had more activated NK and $CD8^+$ T cells in circulation than non-converters (Naranbhai *et al*., 2012). In contrast, HIV-1 exposed seronegative women have very low serum levels of pro-inflammatory cytokines and show low levels of immune activation compared to HIV-1 unexposed or HIV-1 positive women (Lajoie *et al*., 2012; Jaumdally *et al*., 2017).

Taken together, these studies suggest that elevated systemic inflammation and immune activation increases the risk of HIV-1 acquisition. However, it is not fully understood how systemic inflammation influences risk of HIV-1 acquisition in the FGT. Liebenberg and colleagues recently observed that if FGT inflammation superseded systemic inflammation, a cytokine gradient is created that attracts target cells from circulation to the FGT (Liebenberg *et al*., 2017). These authors computed the difference between FGT and systemic inflammation and found that certain cytokines such as CXCL10, CCL2, IL8 and CCL4 had positive gradients and were significantly associated with an increased risk of HIV-1 acquisition (Liebenberg *et al*., 2017).

Taken together, results from these studies suggest that inflammation in very critical for HIV-1 infection and disease progression. However, it is not fully understood how inflammatory genes are regulated in the FGT and blood.

1.2.2.1: Lipopolysaccharides and FGT or systemic inflammation

Lipopolysaccharide or LPS is the major component in the cell walls of Gram-negative bacteria. LPS is the ligand for toll-like receptor 4 (TLR4), which is expressed on peripheral blood mononuclear cells (PBMCs) and on genital epithelial cells (Pivarcsi *et al*., 2005; Fazeli *et al*., 2005; Schaefer *et al*., 2004; Lashkari & Anumba, 2017). Evidence in the literature suggests that LPS is a marker for immune activation in patients with acute and chronic HIV-1 infection (Vassallo *et al*., 2012; Zhang *et al*., 2015). Compared to controls, HIV-1 positive individuals have been reported to have high levels of LPS in plasma, with a few studies reporting concentrations ranging between 87.2 pg/mL and 170.8 pg/mL (Brenchley *et al*., 2006; Funderburg *et al*., 2012; Valiathan *et al*., 2014). It has been demonstrated that gut bacteria are the source of plasma LPS during active HIV-1 infection (Brenchley *et al*., 2006).

LPS in the FGT is contributed mainly by bacterial vaginosis (BV), which is an asymptomatic pathological condition characterised by a decrease in the number of lactic acid-producing bacteria

and an increase in the number of anaerobic gram-negative bacteria in the FGT (Woodman, 2016). Women with BV have been found to have high levels of LPS in vaginal washes, with one study reporting concentrations as high as 323.5 ng/mL (Aroutcheva *et al*., 2008). Evidence from epidemiological studies suggests that BV increases the risk of HIV-1 infection in women (Cohen *et al*., 1995; Sewankambo *et al*., 1997; Taha *et al*., 1999; Martin *et al*., 1999; Myer *et al*., 2005; van de Wijgert *et al*., 2013; Gosmann *et al*., 2017). Several pro-inflammatory immune mediators have been reported to be elevated in CVL from women with BV (Spandorfer *et al*., 2001; Hedges *et al*., 2006; Ryckman *et al*., 2008; Kyongo *et al*., 2015). Current evidence suggests that the severity of inflammation depends on diversity of the microbiome (Lennard *et al*., 2017) and could explain the heterogeneity in pro-inflammatory cytokine expression profiles reported by different studies (Spandorfer *et al*., 2001; Hedges *et al*., 2006; Rid *et al*., 2008; Ryckman *et al*., 2008; Doerflinger *et al*., 2014; Kyongo *et al*., 2015; Deese *et al*., 2016).

1.3: Hormonal contraceptives and the risk of HIV-1 acquisition and disease progression

According to the United Nations (UN), progestogen-only injectable hormonal contraceptives (IHCs) are the most prevalent contraceptive method used by women of child-bearing age in Sub-Saharan Africa (UN, 2015). Two types of IHCs are used in the region: medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN). MPA is marketed as Depo-Provera® (DMPA) and is administered as a three-monthly 150 mg intramuscular (IM) injection (DMPA-IM). MPA is also administered as a three-monthly low-dose 104 mg subcutaneous (SC) injection (DMPA-SC). NET-EN is provided as a two-monthly 200 mg intramuscular injection and marketed as Nur-Iterated®. Other progestins used in contraception include levonorgestrel (LNG), administered in implants (Jardell®) delivering 150 mg LNG or in an LNG-releasing intrauterine device (LNG-IUD). LNG (implants or IUD) is sparsely used in Sub-Saharan Africa (UN, 2015). MPA, NET, LNG and P4 are collectively referred to as progestogens (**Figure 1.2**).

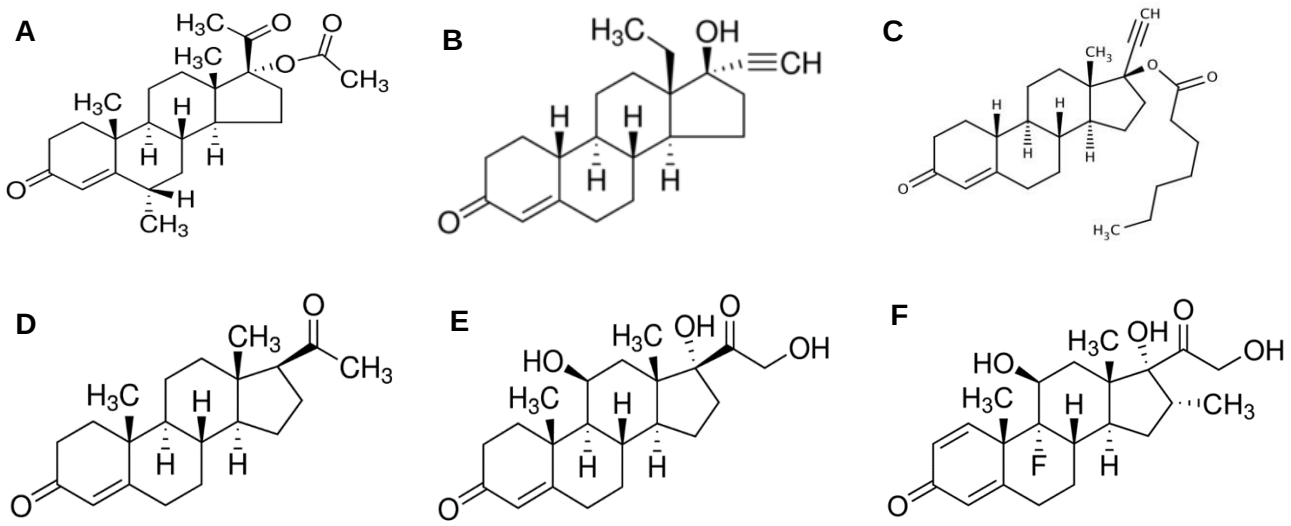

Figure 1.2: Chemical structures of some progestogens and glucocorticoids. (**A**) medroxyprogesterone acetate (MPA), (**B**) levonorgestrel (LNG), (**C**) norethisterone enanthate (NET), (**D**) progesterone (P4), (**E**) hydrocortisone (CORT) and (**F**) dexamethasone (DEX). (Source: Sigma Aldrich website)

1.3.1.1: Evidence from clinical and observational studies

Evidence from epidemiology studies suggest that women using DMPA-IM have an increased risk of HIV-1 acquisition (Martin *et al*., 1998; Baeten *et al*., 2007; Leclerc *et al*., 2008; Wand & Ramjee, 2012; Heffron *et al*., 2012; Crook *et al*., 2014; Noguchi *et al*., 2015). In contrast, others have not found any significant association between DMPA-IM use and HIV-1 acquisition (Kapiga *et al*., 1998; Kiddugavu *et al*., 2003: Myer *et al*., 2007; Morrison *et al*., 2007; Morrison *et al*., 2012; McCoy *et al*., 2013; Lutalo *et al*., 2013; Whiteman *et al*., 2016; Balkus *et al*., 2016; Hofmeyr *et al*., 2017). However, recent systematic reviews and meta-analyses of higher quality published data have found a significant association between DMPA-IM use and HIV-1 infection risk (Ralph *et al*., 2015; Morrison *et al*., 2015; Brind *et al*., 2015; Polis *et al*., 2016). These studies have also found that the risk of HIV-1 acquisition is more elevated in commercial sex workers and women in serodiscordant relationships with HIV-1 positive male partner, irrespective of their herpes simplex virus type 2 (HSV-2) status or age (Ralph *et al*., 2015; Morrison *et al*., 2015).

Unlike DMPA-IM, clinical studies have found no significant association between NET-EN or combined oral contraceptive (mainly containing LNG) use and HIV-1 acquisition in women (Wand & Ramjee, 2012; Myer *et al*., 2007; Noguchi *et al*., 2015; Crook *et al*., 2014; Morrison *et al*., 2007; Morrison *et al*., 2012; Kleinschmidt *et al*., 2007; McCoy *et al*., 2013; Wall *et al*., 2015; Lavreys *et al*., 2004; Ralph *et al*., 2015; Morrison *et al*., 2015). Furthermore, systematic reviews with meta-analyses have found

no significant association between NET-EN and the risk of HIV-1 acquisition in high risk populations (Ralph *et al*., 2015; Morrison *et al*., 2015). In contrast, head-to-head comparisons between the different types of IHCs showed that women using DMPA-IM unlike those using NET-EN are more susceptible to HIV-1 infection (Noguchi *et al*., 2015; Morrison *et al*., 2015).

It has recently been reported that women with BV using DMPA-IM are 6.5 times more likely to become infected with HIV-1 than women with BV not using DMPA-IM (Haddad *et al*., 2018). This suggests that DMPA enhances the risk of HIV-1 acquisition associated with BV in women, but the mechanisms are not fully understood. It is possible that DMPA-IM use favours the growth of *Lactobacilli* species such as *L. iners* that produce low hydrogen peroxide and lactic acid (Eschenbach *et al*., 1989; Hillier *et al*., 1992; Hillier *et al*., 1993; Tachedjian *et al*., 2017). However, this is unlikely the case as some studies have shown that DMPA-IM use specifically cause a decline in *L. iners* (Achilles *et al*., 2018) and does not alter the overall vaginal microbiome (Achilles *et al*., 2018; Mitchell *et al*., 2014). On the other hand, prolonged use of DMPA-IM might induce hypoestrogenism, which could result in a decrease in lactic acid production in the FGT (Hickey *et al*., 2016; Miller *et al*., 2000). In addition, DMPA use has been shown to alter the levels of pro-inflammatory immune mediators in cervical secretion from women with BV (Cherpes *et al*., 2008). However, it remains unclear whether the effects of DMPA use on genital inflammation are the consequence of DMPA modifying the genital microbiome. Taken together, this suggests that the mechanisms by which DMPA use enhance the risk of HIV-1 in women with BV are complex and may include changes in the pH and levels of pro-inflammatory immune mediators in the FGT.

It remains unknown whether prolonged use of LNG-IUD or NET-EN can induce hypoestrogenism. In addition, it remains unclear whether LNG-IUD or NET-EN use can modify the risk of HIV-1 infection associated with BV. This is very unlikely as it has been reported that neither LNG-IUS nor NET-EN alters the vaginal microbiome (Donders *et al*., 2011; Hashway *et al*., 2014; Jacobson *et al*., 2014; Brooks *et al*., 2017; Bassis *et al*., 2017; Achilles *et al*., 2018). These results suggest that LNG-IUD and NET-EN might be safer to use in women with BV.

Evidence from some clinical observational studies or systematic reviews with meta-analyses also suggest that DMPA-IM use does not accelerate disease progression to acquired immune deficiency syndrome (AIDS) in HIV-1 infected women (Whiteman *et al*., 2016; Morrison *et al*., 2011; Heffron *et al*., 2013; Richardson *et a*l., 2007; Phillips *et al*., 2016). However, results from other studies did suggest that DMPA-IM use might accelerate HIV-1 disease progression to AIDS, but these studies

were flawed by high rates of DMPA-IM failures (Stringer *et al*., 2007; Stringer *et al*., 2009a; Stringer *et al*., 2009b). The effects of NET-EN or LNG-IUD on HIV-1 disease progression remain unknown.

Taken together, the clinical evidence indicates that DMPA-IM, unlike NET-EN or combined oral contraceptives (COCs)-containing LNG, increases the risk of HIV-1 acquisition in women but does not accelerate disease progression to AIDS. However, the mechanisms for DMPA-IM may occur at multiple levels and are currently unclear. It remains unknown whether LNG used in an IUD or implant for contraception modifies the risk of HIV-1 acquisition or disease progression in women.

1.3.1.2: Evidence from animal and in vitro studies

Evidence from animal studies further supports the link between DMPA-IM use and the risk of HIV-1 acquisition. Studies conducted in pigtail and rhesus macaques showed that animals treated with DMPA-IM are more susceptible to SHIV infection (Trunova *et al*., 2006; Radzio *et al*., 2014; Carias *et al*., 2016). In addition, studies involving the use of the mouse and humanised mouse models have shown that animals treated with DMPA-IM as well as LNG and exogenous progesterone (P4) were more susceptible to HSV-2 and HIV-1 infection, respectively (Quispe-Calla *et al*., 2016; Quispe-Calla *et al*., 2018). This is consistent observations that female macaque monkeys are more susceptible to infection with SHIV in the luteal phase than in the follicular phase, with the window for infection determined as the period during or just after the surge in serum P4 levels (Vishwanathan *et al*. 2011; Kersh *et al*., 2014; Sodora *et al*., 1998). Taken together, this suggest that a progestogen-dominant state increases the vulnerability of laboratory animals to infection.

Because MPA is metabolised and cleared quicker in macaques than in humans, some studies used doses higher than that used in humans. Considering that the average macaque weighs 7 kg, this equates to 4.3 mg/kg as opposed to 2 mg/kg for a woman with the average body weight of 75 kg (Trunova *et al*., 2006; Carias *et al*., 2016). Thus, peak serum concentrations of MPA may differ drastically between macaques and humans. This raises questions as to the relevance of these studies when assessing the association between DMPA and the risk of HIV-1 acquisition. However, other studies have used lower doses (3 mg) and found that the average peak serum concentration is 6.98 nM (3.62 – 12.41), which equates peak serum levels in humans (Radzio *et al*., 2014; Butler *et al*., 2016). Similarly, doses used in mice achieve the serum concentrations that approximate those measured in women using DMPA-IM (Vicetti *et al*., 2012; Quispe-Calla *et al*., 2018). Therefore, reported increase in SHIV or HIV-1 infection in animal models is very unlikely to be an artifact caused by suprapharmalogical serum concentrations.

Additionally, evidence from in vitro studies support the association between DMPA and the risk of HIV-1 acquisition t (Huijbregts *et al*., 2013; Sampah *et al*., 2015; Ferreira *et al*., 2015a; Irvin & Herold, 2015; Tasker *et al*., 2017; Maritz *et al*., 2018). It has previously been reported that MPA doses ranging between 7.79 pM and 12.93 nM enhance HIV-1 infection in unactivated $CD4^+$ T cell (Sampah *et al*., 2015). This is consistent with findings from a more recent study that showed for the first time that MPA enhances viral replication in $CD4^+$ T cells and TZM-bl indicator cells exposed to HIV-1 (Maritz *et al*., 2018). These effects were observed at doses as low as 10 nM, suggesting that DMPA at physiologically relevant doses may enhance the risk of HIV-1 infection in women (Maritz *et al*., 2018). In fact, findings from another recent study showed that in the presence of DMPA $CD4^+$ T cells are rendered more vulnerable to infection in vivo (Tasker *et al*., 2017). This study showed that PBMCs isolated from women using DMPA-IM are more readily infected ex vivo with HIV-1 IMCs compared to PBMCs obtained before DMPA-IM injection (Tasker *et al*., 2017). However, it has also been reported that MPA at 1 µM but not 100 nM can increase HIV-1 infection in activated $CD4^+$ T cells (Huijbregts *et al*., 2013). Taken together, these findings suggest that MPA at physiologically relevant doses renders unactivated $CD4^+$ T cells vulnerable to infection, whereas higher doses do the same for activated $CD4^+$ T cells. MPA at 10 nM has been shown to induce that activation of $CD4^+$ T cells (Maritz *et al*., 2018). In contrast, higher doses of MPA have been shown to prevent the deactivation of activated $CD4^+$ T cells (Huijbregts *et al*., 2013). Thus, it appears MPA utilises different mechanisms to increase the susceptibility of unactivated and activated $CD4^+$ T cells.

The studies above suggest that the effects of MPA on PBMCs are direct. However, there is also evidence suggesting that the effects may be indirect. It has been reported that supernatants from PGECs treated with 1 nM MPA in the absence or presence of HIV-1 IMCs enhances *trans* infection of Jurkat T cells (Ferreira *et al*., 2015a). *Trans* infection is when HIV-1 traverses across an epithelial mucosal barrier to access and infection target cells. Suprapharmalogical doses of MPA ranging between 38.81 µM and 388.1 µM), but not a 100-times less were shown to enhance trans HIV-1 infection of Jurkat-Tat-CCR5 (JT-CCR5) cells (Irvin & Herold, 2015). In addition, it has been demonstrated that supernatants from vaginal epithelial cells treated with 388.1 µM, but not 38.81 µM enhanced HIV-1 replication in a chronically infected cell line (Irvin & Herold, 2015).

Evidence from animal studies show LNG enhances the susceptibility of humanise mice to infection (Quispe-Calla *et al*., 2016; Quispe-Calla *et al*., 2018). It remains to be established whether LNG can enhance HIV-1 infection in vitro. There is paucity of data from animal studies for NET. However, findings from in vitro studies suggest that NET does not enhance the risk of infection in HIV-1 target cells and ectocervical tissue explants (Maritz *et al*., 2018; Ray *et al*., manuscript under review). Therefore, head-to-head studies are needed to compare the effects of MPA, LNG and NET on HIV-1

infections. Taken together, these findings suggest that a progestogen-dominant state increases the susceptibility to infection. It remains unclear whether all progestins can enhance the risk of HIV-1 infection. However, these findings suggest that MPA unlike NET enhances HIV-1 infection of target cells and that it occurs at physiologically relevant doses. This suggests that some progestogens might be safer than others.

1.3.2: The influence of injectable hormonal contraceptives on FGT epithelial thickness and mucosal permeability

It remains unclear whether the vaginal epithelium of female macaques becomes thinner in the luteal phase compared to the follicular phase (Hadzic *et al*., 2014; Butler *et al*., 2015; Hild-Petito *et al*., 1998; Radzio *et al*., 2014; Butler *et al*., 2016). However, it has been reported that treating luteal phase macaques with DMPA or exogenous P4 reduces the thickness of the ectocervical or vaginal epithelial tissues (Marx *et al*., 1996; Smith *et al*., 2000; Hild-Petito *et al*., 1998; Radzio *et al*., 2014; Butler *et al*., 2015; Carias *et al*., 2016; Butler *et al*., 2016). It has also been reported that DMPA-treated mice, like those treated with LNG, have thinner vaginal epithelia compared to mice in estrus phase (Quispe-Calla *et al*., 2016). Most studies have shown that DMPA- as well as LNG-treated animals are more susceptible to infection by SHIV, HSV-2 and HIV-1 (Gillgrass *et al*., 2003; Radzio *et al*., 2014; Butler *et al*., 2016; Carias *et al*., 2016; Quispe-Call *et al*., 2016; Quispe-Calla *et al*., 2018; Bosinger *et al*., 2018). It has also been reported that LNG in COC at 66% human dose caused a reduction in the vaginal epithelial thickness of pigtail macaques (Dietz Ostergaard *et al*., 2015). Together, these findings suggest that DMPA and LNG compromise the integrity of the vaginal and ectocervical epithelial mucosal tissues and renders them more vulnerable to infection.

In humans, however, it remains unclear whether DMPA-IM use alters the thickness of the vaginal and ectocervical epithelia (Mauck *et al*., 1999; Kuhn *et al*., 1999; Bahamondes *et al*., 2000; Chandra *et al*., 2013; Bahamondes *et al*., 2014; Mitchell *et al*., 2014; Ildgruben *et al*., 2003). Also controversial is the question whether the luteal phase alters the thickness of vaginal and ectocervical epithelial tissues in women (Patton *et al*., 2000; Miller *et al*., 2000; Thurman *et al*., 2016). The discordance between human and animal studies may results from differences between the biology and structures of monkey and human FGT.

It remains unclear what the effects of LNG and DMPA are on genital epithelial thickness. One study found that women using DMPA, COC and LNG implants had thicker vaginal epithelia compared to controls (Ildgruben *et al*., 2003). Another study found that women on LNG-IUD had thinner ectocervical stratum corneum compared to COC users or non-HC controls (Tjernlund *et al*., 2015).

However, the study by Tjernlund and colleagues also reported no difference in the overall thickness of the ectocervix between the groups ((Tjernlund *et al*., 2015). Thus, it remains unclear whether the mode of administration of LNG elicits different effects on genital epithelial thickness. Nonetheless, it has recently been reported that ectocervical mucosal tissue biopsies from women using DMPA-IM and LNG-IUD are very porous (Quispe-Calla *et al*., 2016; Quispe-Call *et al*., 2017). This could indicate thinning of the epithelium; however, this was not investigated by the authors. On the other hand, they reported that tissues from DMPA-IM and LNG-IUD users expressed low levels of desmoglein-1 and desmocollin compared to controls (Quispe-Calla *et al*., 2016; Quispe-Call *et al*., 2017). Unlike DMPA and LNG, there is paucity on the effects of NET-EN on genital epithelial thickness in the literature.

Collectively, the data suggest that MPA and LNG can disrupt the integrity of mucosal tissues of the lower FGT. In animal models, this is achieved via reducing the thickness of mucosal epithelial tissues and down-regulating the expression of desmosomal junctions. In humans, however, this may be achieved only by down-regulating desmosomes.

1.3.3: The influence of injectable hormonal contraceptives on the activation and distribution of HIV-1 target cells in the FGT

Evidence from human and animal studies collectively suggests that DMPA-IM use causes HIV-1 target cells to infiltrate the FGT, including $CD4^+$ T cells (Ildgruben *et al*., 2003; Carias *et al*., 2016; Quispe-Calla *et al*., 2018; Goode *et al*., 2014; Smith-McCune *et al*., 2017), macrophages (Smith-McCune *et al*., 2017; Ildgruben *et al*., 2003) and monocytes (Quispe-Calla *et al*., 2016). Some of these studies also show that $CD4^+$ T cells recruited to the FGT are activated (Smith-McCune *et al*., 2017) and express the mucosal tissue homing marker α4β7 (Goode *et al*., 2014). Findings from recent studies showed that DMPA-IM or MPA enhances the expression of α4β7 and/or CCR5 on $CD4^+$ T cells (Tasker *et al*., 2017; Maritz *et al*., 2018). This suggests that MPA prepares systemic $CD4^+$ T cells to home to mucosal tissues. A recent study showed that women using DMPA-IM had more $CCR5^+CD4^+$ T cells in the endometrium compared to controls (Smith-McCune *et al*., 2017). However, another study found DMPA-IM use caused a decrease in the proportion of $CCR5^+CD4^+$ T cells in the vagina (Mitchell *et al*., 2014). This suggests that effects of DMPA-IM on the homing of $CCR5^+CD4^+$ T cells in the FGT are compartment specific. This is consistent with evidence from a study that reported the frequency of $CCR5^+CD4^+$ T cells in DMPA-treated macaques was higher in vaginal tissue, but lower in endocervix (Goode *et al*., 2014).

It has also been reported that women using LNG-IUD have a high proportion of $CCR5^+CD4^+$ T cells in circulation (Sciaranghella *et al*., 2015). In addition, LNG-IUD use has been reported to increase the

proportion of activated $CCR5^+CXCR4^+CD4^+$ T cells in the FGT (Shanmugasundaram *et al*., 2016). In contrast, it is currently unclear whether NET-EN use changes the frequency of systemic $CCR5^+CD4^+$ T cells and induces their homing to mucosal tissues of the FGT. A recent study reported women using IHC (without distinguishing between type) had more $CCR5^+CD4^+$ T cells in circulation and in the cervix compared to controls (Byrne *et al*., 2016). However, this study did not differentiate between DMPA-IM and NET-EN. Current evidence from an in vitro study suggests that unlike MPA, NET does not alter CCR5 levels and the activation status of $CD4^+$ T-cells (Maritz *et al*., 2018). Thus, it is possible that effects reported in the Byrne study were DMPA-IM induced.

Taken together, these observations suggest that DMPA and LNG-IUD, unlike NET, activate and induce the migration of systemic $CD4^+$ T cells to mucosal surfaces of the FGT. This may render the FGT more susceptible to HIV-1 infection.

1.3.4: Physiological relevant concentrations of MPA, NET-EN and LNG

A key question regarding the effects of contraceptives on HIV-1 acquisition/infection is the dose and hence concentration-dependency of such possible effects. Several studies have been undertaken to measure MPA levels in serum after an DMPA-IM injection is administered (recently reviewed by Hapgood *et al*., 2018). Pooled data from the literature suggest that an average serum concentration of 21 nM is attained within 20 days after administration, but this may range between 3 nM to 100 nM. At day 30 post-administration, serum concentrations level off at 2.6 nM (Hapgood et al., 2018). The time it takes to reach maximum serum concentration (t_{max}) as well as the maximum serum concentration (C_{max}) vary greatly (Hapgood *et al*., 2018). This may reflect differences in the different methods used to quantify serum levels of steroids as well as bioavailability (Hapgood *et al*., 2018). It has also been determined that peak serum concentrations of NET range between 10 nM and 50 nM, whereas those of LNG-IUD range between 0.2 and 2.4 nM, although higher concentrations have been reported for LNG implants and LNG in COC (Hapgood *et al*., 2018).

The biological effects of DMPA-IM are most probably observable at peak serum concentrations. This could explain why studies in which biological samples obtained from women just before the next DMPA-IM injection often reported that DMPA-IM had no effect of on the parameters being investigated. For example, the average serum concentration of DMPA-IM reported in two studies that investigated its effects on human vaginal epithelial thickness was 2.59 nM (Bahamondes *et al*., 2000; Bahamondes *et al*., 2014). Because the effects of DMPA on vaginal epithelial thickness in monkeys are dose-and time-dependent (Radzio *et al*., 2014; Butler *et al*., 2016), it is possible that significant changes in this parameter can only be observed at peak serum concentrations. In macaques,

significant changes in vaginal epithelial thickness have been found to occur within two weeks after MPA administration and are sustained for an additional 7 weeks before the effect is lost (Radzio *et al*., 2014). This suggests that the effects of MPA on vaginal epithelial thickness are transient and can be observed only at specific time points corresponding to time of peak serum concentration. While tissue biopsies are obtained from macaques between injections, in humans they are obtained just before the next MPA shot is administered (Mauck *et al*., 1999; Kuhn *et al*., 1999; Bahamondes *et al*., 2000; Chandra *et al*., 2013; Bahamondes *et al*., 2014; Mitchell *et al*., 2014; Radzio *et al*., 2014; Butler *et al*., 2016). This raises the question whether MPA-induced thinning of the human vaginal epithelium can be observed if tissue sampling is done around the time peak serum concentrations are reached in humans. Until this is done, the effects of MPA on the thickness of human FGT mucosal epithelia will remain elusive. Thus, it remains unclear whether this is one of the mechanisms by which MPA enhances the risk of HIV-1 acquisition in women. Nonetheless, in vitro studies have shown MPA can affect HIV-1 transcytosis and/or infection at physiological concentrations: 1 nM (Ferreira *et al*., 2015a), 7.79 pM to 12.93 nM (Sampah *et al*., 2015) and 10 nM to 100 nM (Maritz *et al*., 2018).

In summary, there are wide variations in peak serum concentrations of the different progestogen-only hormonal contraceptives. Biological effects of the different progestogen are most likely to be observed at the time peak serum concentrations are attained. However, this may be different in different individuals.

1.4: Glucocorticoids and the glucocorticoid receptor

Glucocorticoids (GCs) bind to and activate the glucocorticoid receptor (GR). Hydrocortisone (CORT) is the natural GCs in humans. Several synthetic GCs also exist including prednisolone, dexamethasone (DEX), budesonide (Bud), fluticasone propionate (FP), etc (Barnes, 2014). Endogenous GCs are steroid hormones produced by the adrenal cortex under the influence of the hypothalamus-pituitary-adrenal (HPA) axis in response to stress (Zhou & Cidlowski, 2005). The HPA axis is activated by invading pathogens or stress to release GCs that resolve inflammatory responses associated with the inducing stimuli. Consequently, both natural and synthetic GCs are used widely to treat inflammatory and autoimmune diseases (Hapgood *et al*., 2016). However, GCs show substantial variations in their biological effects with synthetic GCs being more potent that natural GCs (Meikle & Tyler, 1977; Langhoff & Ladefoged, 1983).

Through alternative splicing, the human GR gene encodes several isoforms with the main ones, by relative abundance, being GRα, GRβ and GR$^{\gamma}$ (Zhou & Cidlowski, 2005; Cian & Cidlowski, 2017; Rivers *et al*., 2009; Morgan *et al*., 2016). More isoforms are generated through alternative start codon

usage (Lu & Cidlowski, 2005; Zhou & Cidlowski, 2005; Nicolaides *et al*., 2010; Nicolaides *et al.*, 2014). However, GRα is the transcriptionally active isoform and will be referred to as the GR (Oakley & Cidlowski, 2013; Vandevyver et al., 2014). Structurally, the GR is a modular protein made up of four distinct domains; an N-terminal domain (NTD), a deoxyribonucleic acid (DNA)-binding domain (DBD), a hinge region and a C-terminal ligand-binding domain (LBD) (Oakley & Cidlowski, 2013; Cruz-Topete & Cidlowski, 2015). The NTD contains a transcriptional activation function 1 (AF-1) domain that is required for basal as well as maximal transcriptional activity in the presence of GCs (Kumar & Thompson, 2005; Heitzer *et al*., 2007). In addition, the NTD recruits co-regulators and is subject to site-specific phosphorylation - both events can occur in the absence or presence of GCs (Godowski *et al*., 1987; Faus & Haendler, 2006; Verhoog *et al*., 2009; Hapgood *et al*., 2016). The DBD contains two zinc-finger motifs that bind to specific DNA sequences called glucocorticoid response elements (GREs) in the promoter or enhancer regions of target genes (Freedman *et al*., 1988; Dahlman-Wright *et al*., 1991). The DBD and LBD are separated by a hinge region which allows the receptor to change conformation (Picard & Yamamoto, 1987). In addition, the hinge contains a nuclear localisation signal and is required for receptor dimerization (Picard & Yamamoto, 1987; Bledsoe *et al*., 2002). The LBD on its part contains a hydrophobic ligand-binding pocket, a second nuclear localisation signal and a transcriptional activation function 2 (AF-2) domain. The AF-2 region recruits co-regulator in a ligand-dependent manner (Oakley & Cidlowski, 2013; Cruz-Topete & Cidlowski, 2015).

1.4.1: Classic GR signaling pathway (genomic effects)

In the absence of ligands, the GR is sequestered in the cytoplasm as part of a multi-protein complex (Pratt *et al*., 2004; Baschant & Tuckermann, 2010). This complex comprising heat-shock proteins and immunophilins keeps the GR in an inactive conformation, but one that allows it to recognise and bind GCs. Ligand binding causes the GR to become phosphorylated at specific serine residues namely Ser-203, Ser-211 and Ser-226 (Beck *et al*., 2009; Avenant *et al*., 2010a; Avenant *et al*., 2010b). It also induces a conformational change GR that allows the GR to dissociate from the complex and translocate to the nucleus where it regulates gene expression. The liganded GR, acting via its LBD, recognises and binds to GREs in the promoter regions of target genes and activates their expression (Meijsing *et al*., 2009). This process is known as transactivation and results in the expression of multiple genes including those involved in inflammation (Chinenov *et al*., 2013; Hapgood *et al*., 2016; Cain & Cidlowski, 2017), maintaining mucosal barrier function (Felinski & Antonetti, 2005) and metabolism (Garabedian *et al*., 2017). On the contrary, gene expression is inhibited by a mechanism known as transrepression. This can involve the liganded GR tethering other transcription factors namely nuclear factor kappa beta (NFκB), activator protein (AP)-1 or signal transducer and activator of transcription (STAT) and inhibiting the transcriptions of genes activated by these transcription factors (De Bosscher & Haegeman, 2009; Langlais *et al*., 2012; Ratman *et al*., 2013). This interaction

may sometimes occur in the cytoplasm and result in the sequestration of NFκB subunits by the GR (Widen *et al*., 2003). The liganded GR can also interact through composite binding with other transcription factors. Such interactions may inhibit or activate gene expression depending on the transcription factors (Cain & Cidlowski, 2015; Cruz-Topete & Cidlowski, 2015). Alternatively, the liganded GR may bind to negative GREs (nGREs) or GRE half-sites in the promoter of target genes to inhibit their expression (Surjit *et al*., 2011; Schiller *et al*., 2014). Binding of the liganded GR to GR binding sites (GRBS) present in promoter or enhancer regions of target genes remodels the chromatin around those regions and makes it more accessible to other transcription factors (Jubb *et al*., 2017; King *et al*., 2012). In addition, that the presence of other transcription factors in the promoter or enhancer regions have been shown to affect GR occupancy (John *et al*., 2011). For instance, it has been reported that, in the absence of GCs, the majority of GRBs are occupied by transcription factors such AP-1, CCAAT/Enhancer Binding Protein Beta (C/EBPβ) and Forkhead box A1 (FoxA1) and GR recruitment to chromatin in the presence of GCs is reduced when these factors are prevented from binding to DNA (Grøntved *et al*., 2013; Belikov *et al*., 2009; Biddie *et al*., 2012). This suggest that these factors direct the recruitment of the GR to target genes and by so doing modulate the responses mediated by the GR in the presence of GCs.

The GR is largely a ligand-activated transcription factor, but it has been found to be transcriptionally active in the absence of GCs (Kotitschke *et al*., 2009; Verhoog *et al*., 2011; Hapgood *et al*., 2016). Some studies have demonstrated that knocking down or over-expressing the GR results in changes in basal gene expression (Verhoog *et al*., 2009; Ritter *et al*., 2012; Robertson *et al*., 2013a; Robertson *et al*., 2013b; Ritter *et al*., 2014). In addition, stimuli such as TNF, gonadotropin-releasing hormone (GnRH) and the cell-cycle have been shown to phosphorylate the unliganded GR at Ser-206, Ser-211 and Ser-226, thereby increasing its basal transcriptional activity (Kotitschke *et al*., 2009; Verhoog *et al*., 2011; Matthews *et al*., 2015). This might sensitise the unliganded GR for subsequent GC-dependent activation (Hapgood *et al*., 2016). For example, one study found that in the absence of GCs Interleukin (IL)13 increases phosphorylation of the GR at Ser-203 and Ser-211 and nuclear translocation without evoking DNA binding and transcription (Hu *et al*., 2013). In the presence of GCs, Hu and colleagues showed that these initial events prompted by IL13 are augmented as well as DNA binding (Hu *et al*., 2013). On the other hand, some non-GC stimuli cannot modify the unliganded GR on their own. However, they can enhance GC-induced modification of the GR by activating other signalling pathways such as mitogen activated protein kinases (MAPKs) that enhance the GC-induced effects. Such is the case with tissue growth factor β (TGFβ) which has been shown to augment DEX-induced phosphorylation of the GR at Ser-211, and hence upregulated the expression of plasmogen activator inhibitor 1 in human ovarian epithelial cells (Pan *et al*., 2015). On the other hand, some non-GC stimuli may desensitise and make the unliganded GR less responsive to GC-dependent activation. Pro-inflammatory cytokines such as TNF, IL1α and IL1β have been shown to reduce GC-

binding, nuclear translocation and DNA-binding abilities of unliganded GR (Pariante *et al*., 1999; Wang *et al*., 2005; Escoll *et al*., 2015). Furthermore, GC-independent cleavage of the unliganded GR by caspase-1 or the GC-independent phosphorylation of the GR at Ser-134 have been shown to reduce GC-mediated transcription by the GR (Galliher-Beckley *et al*., 2011; Paugh *et al*., 2015). Taken together, these observations suggest that the microenvironment in which the GR operates determine the outcome of the responses mediated by the receptor.

1.4.2: Non-classical GR signalling pathway (non-genomic effects)

The above mechanisms constitute the genomic effects of GCs. However, several GC-induced effects are rapid, do not require the GR to bind to DNA or TFs and cannot be prevented by inhibitors of transcription or protein synthesis (Sanden *et al*., 2000; Buttgereit & Scheffold, 2002; Buttgereit *et al*., 1998; Pérez *et al*., 2013; Liu *et al*., 2005). These are termed non-genomic effects. Non-genomic effects are induced by GC doses higher than 1 μM, but doses as low as 1 nM have been found to induce non-genomic effects as well (Urbach *et al*., 2002). It has been suggested that higher GC doses elicit very rapid non-specific effects that occurs within seconds, whereas lower doses elicit specific non-genomic effects that occur within minutes (Buttgereit *et al*., 2002). A membrane-bound GR (mGR) that is structurally different from cytosolic GR (cGR) mediates non-genomic effects of GCs (Pérez *et al*., 2013; Mitre-Aguilar *et al*., 2015). However, the latter has also been localised in the plasma membrane (Oppong *et al*., 2014) and mitochondrial membrane (Sionov *et al*., 2006). This suggests that non-genomic effects can also be mediated by cytosolic GR. Unlike mGR, non-genomic effects mediated by cytosolic GR are sensitive to RU486 and GR siRNA (Kotitschke *et al*., 2009).

1.4.3: Anti-inflammatory effects mediated by the GR

Glucocorticoids substantially inhibit the expression and action of several pro-inflammatory cytokines and chemokines (Mukaida *et al*., 1994; Meduri *et al*., 2002; de Kruif *et al*., 2008; Abou-Raya *et al*., 2014; Leigh *et al*., 2016; Dantas *et al*., 2017). The liganded GR inhibits the expression of many inflammatory mediators through its interactions with transcription factors such as NFκB and AP-1 (Mukaida *et al*., 1994; De Bosscher & Haegeman, 2009; Langlais *et al*., 2012; Ratman *et al*., 2013). Alternatively, GCs acting via the GR have been shown to reduce the stability of pro-inflammatory mRNAs (Lee *et al*., 1988; Amano *et al*., 1993). The mechanisms involved include the liganded GR binding to the 5'-untranslated regions of messenger ribonucleic acid (mRNA) transcripts and initiating their decay (Ishmael *et al*., 2011). Chemokines regulated this way include CCL2 and CCL7. In addition, GCs have been shown to induce the expression of the mRNA destabilising protein tristetraprolin (TTP), which recognises specific transcripts through adenylated/uridylated rich elements (AREs) in the 3' untranslated region (Ishmael *et al*., 2008; Smoak & Cidlowski, 2006;

Stojadinovic *et al*., 2007). Inflammatory mediators targeted by TTP include CCL2, CCL20, IL6, TNF, IL1α and IL1β (Brooks & Blackshear, 2013; Patial *et al*., 2016). Another mechanism by which GCs suppress inflammation is by inducing the expression of anti-inflammatory genes such as glucocorticoid-induced leucine zipper (GILZ), inhibitor of NFκB (IκBα), mitogen-activated protein kinase phosphatase (MKP)-1, interleukin-1 receptor-associated kinase-M (IRAK-M) and TNF Alpha Induced Protein 3 (TNFAIP3) to control inflammation (Govender *et al*., 2014; Kassel *et al.*, 2001; Vandevyver *et al*., 2012; Altonsy *et al*., 2014; Newton *et al*., 2017; Miyata *et al*., 2015). Activation and transcriptional activities of NFκB and AP-1 is triggered by mitogen-activated protein (MAP) kinases namely p38, extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK). IκBα like GILZ and TNFAIP3 have been reported to antagonise NFκB and AP-1 thereby inhibiting inflammatory responses mediated by these transcription factors (Ronchetti *et al*., 2015; Ayroldi & Riccardi, 2009). Furthermore, GC-induced MKP-1 expression and activity has been reported to abrogate NFκB and AP-1 activation by inactivating MAP kinases (Kassel *et al*., 2001; Vandevyver *et al*., 2012; Lasa *et a*l., 2002). IRAK-M is a negative regulator of myeloid differentiation factor 88 (MyD88), which is a critical downstream adaptor molecule of all TLRs, except TLR3. It has recently been reported that GC-induced expression of IRAK-M attenuates TLR-mediated expression of pro-inflammatory immune mediators in murine macrophages (Miyata *et al*., 2015).

1.4.4: Pro-inflammatory effects mediated by the GR

While they are mostly anti-inflammatory, GCs may also promote pro-inflammatory immune responses in some cell types by enhancing the expression of select pro-inflammatory genes (Sorrells & Sapolsky, 2007; Bellavance & Rivest, 2014; Cruz-Topete & Cidlowski, 2014; Duque Ede & Munhoz, 2016). The context under which this occurs is not well established. Some studies have shown that cells pre-treated with the GCs and thereafter stimulated with specific immune activators express more pro-inflammatory cytokine and chemokines compared to controls (Homma *et al*., 2004; Busillo *et al*., 2011; Yeager *et al*., 2009; Loram *et al*., 2011; Munhoz *et al*., 2010; Barber *et al.*, 1993; Smyth *et al*., 2004; Zhang *et al*., 2017; van de Garde *et al*., 2014). This suggested that some cells are primed by GCs to respond more robustly to specific immune activators. In fact, it has been reported that GCs induce the expression of innate immune receptors such as nod-like receptor family, pyrin domain containing **(**NLRP)1, NLRP3, TLR2 and TLR4 in some cell types, which makes them more sensitive to specific immune activators (Sakai *et al*., 2004; Homma *et al*., 2004; Pace *et al*., 2015; Su *et al*., 2017; Busillo *et al*., 2011; Zhang *et al*., 2017). However, in some cell types, concurrent treatment with GCs and immune activators resulted in the upregulation of CCL20 (Zijlstra *et al*., 2014). This suggest that mechanisms other than priming are involved in GC-induced expression of pro-inflammatory genes. Some authors have proposed that low doses of GCs are pro-inflammatory, whereas high doses are anti-inflammatory (Sorrells & Sapolsky, 2007). This view is supported by evidence from a study that

showed CORT at 27.59 mM, but not 275.9 mM enhanced basal and LPS-induced expression of TLR2, TLR4, IL6 and IL8 in human corneal epithelial cells (Xie *et al.*, 2011). In addition, hydrocortisones doses between 0.01 pM and 100 pM, but not doses between 0.01 µM and 1 µM have been found to induce the expression of macrophage migration inhibitory factor (MIF) in murine RAW 264.7 (Calandra *et al*., 1995). However, some studies suggest that 1 nM DEX can enhance TNF-induced expression of CCL20 in human airways epithelial cells, whereas at the same dose DEX represses nontypeable *Haemophilus influenzae*-induced expression of pro-inflammatory cytokines in human primary macrophages. This suggest that the effects of GC dose on the expression of pro-inflammatory gene expression are gene- as well as cell-specific.

The molecular mechanisms by which GCs upregulate the expression of pro-inflammatory genes are not well understood. It has been demonstrated that genes such as *TLR2* and *CCL20* have GREs in their promoter or enhancer regions (Hermoso *et al*., 2004; Wang *et al*., 2004). This suggests that they can be transactivated by the liganded GR. However, other genes including *CCL2*, *CCL7, IL8, IL1B* and *CCL8* have not been reported to contain GREs and the GR transactivates these genes by interacting with other transcription factors. Even for genes with known GREs, the GR has been found to interact with other TFs to regulate gene expression. For instance, the GR has been reported to interact with the GRE, STAT and NFκB binding sites in the *TRL2* minimal promoter (Hermoso *et al*., 2004). Hermoso and colleagues found that DEX on its own could neither transactivate the minimal nor a full-length promoter that contained two more GREs (Hermoso *et al*., 2004). CCL20 has been reported to contain a GRE site that is located +4000 kb from the transcription start site (Wang *et al*., 2004). In addition, its minimal promoter has been found to contain NFκB, AP-1, C/EBPβ and STAT binding elements (Miao *et al*., 2012). It remains unknown whether the GR interacts with these sites to upregulate basal-, TNF- or LPS-induced CCL20 expression.

1.4.5: Synergistic interactions between glucocorticoids and immune activators affects gene expression and HIV-1 infection

GCs can crosstalk and modulate signalling pathways activated by immune activators (Adcock & Caramori, 2001; Van Bogaert *et al*., 2010; Arango-Lievano & Jeanneteau, 2016). Conversely, several immune activators can modulate and influence the activity of the GR. While GCs and immune activators are mostly antagonistic towards each other, evidence in the literature suggest that they can coregulate gene expression in a synergistic manner (Imasato *et al*., 2002; Shuto *et al*., 2002; Sakai *et al*., 2004; Homma *et al*., 2004; Shibata *et al*, 2009; Lannan *et al*., 2012; Miyata *et al*., 2015; Altonsy *et al*., 2014).

Synergism involves two ligands inducing a response that is larger than the sum of the individual responses induced by each ligand. When one ligand does not induce a response but causes an increase in the response elicited by another, the effect is termed potentiation or enhancement (Chou, 2010). Immune activators have been reported to induce the expression of anti-inflammatory genes such as TNFAIP3 (A20) and MKP-1 to control inflammatory responses (Boone *et al*., 2004; Altonsy *et al*., 2014; Zhang *et al*., 2012; Talavera *et al*., 2015). GCs, in some cell types, have been reported to synergise with immune activators to upregulate the expression of these genes. For example, DEX has been found to synergise with NTHi to upregulate the expression of IRAK-M in murine as well as human primary macrophages, via a mechanism that required the GR and p65 (Miyata *et al*., 2015). Similarly, DEX has been reported to synergise with LPS to upregulate (sphingosine kinase) SphK1 expression in murine macrophages, requiring the GR and p38-activated mitogen- and stress-activated protein kinase-1 in the process (Vettorazzi *et al*., 2015). It has also been reported that DEX and LPS synergise to upregulate MKP-1 expression in murine cardiomyocytes to inhibit p38-dependent expression of TNF (Zhang *et al*., 2012). Collectively, these examples constitute negative feedback mechanisms that most likely control inflammatory responses to protect against tissue damage from excessive inflammation (Ruland, 2011)**.**

Alternatively, synergistic interactions between GCs and immune activators may also result in the upregulation of pro-inflammatory genes (Lannan *et al*., 2012). Several studies have reported that GCs can synergise with TNF, IL1β or NTHi to upregulate TLR2 expression in human airways epithelial cells, endocervical epithelial cells and keratinocytes (Homma *et al*., 2004; Shuto *et al*., 2002; Imasato *et al*., 2004; Sakai *et al*., 2004; Shibata *et al*., 2009; Hermoso *et al*., 2004). This occurs via a mechanism involving the GR, p65, STAT and MPK-1-dependent inactivation of p38 and JNK (Hermoso *et al*., 2004; Sakai *et al*., 2004; Shibata *et al*., 2009; Imasato *et al*., 2004). Homma and colleagues additionally showed that airways epithelial cells concurrently pre-treated with DEX and TNF and thereafter stimulated with TLR2-ligands secreted more pro-inflammatory immune mediators than control cells (Homma *et al*., 2004). This suggests that GCs and immune activators can prime and sensitise airways epithelial cells towards specific immune responses. It has also been reported that FP synergises with LPS to upregulate CCL2, CCL7, CCL8 and CCL20 expression in monocyte-derived macrophages (van de Garde *et al*., 2014). One study found that the GR and ADAM (A Disintegrin And Metalloproteinase Domain)-7 were involved in the synergistic upregulation of CCL20 expression by Bud and TNF in human bronchial epithelial cells (Zijlstra *et al*., 2014). Taken together, these observations suggest that the GR can interact with other signalling pathways in a context-specific manner to upregulate the expression of select pro-inflammatory genes. Therefore, synergism between GCs and immune activators may exacerbate specific inflammatory responses.

It has also been reported that CORT synergises with IL7 to increase the proliferation and survival of activated systemic $CD4^+$ T cells (Cima *et al*., 2006). Because activated $CD4^+$T cells are vulnerable to HIV-1 infection, this suggests that GCs acting via the GR may enhance the risk of HIV-1 infection in the presence of specific immune activators. Interestingly, it has been reported that GCs synergistically interact with TNF or IL6 to upregulate HIV-1 replication in a chronically infected cell line (Kinter *et al*., 2001; Bressler *et al*., 1997). Moreover, it has been reported that DEX enhances HIV-1 infection and replication in $CD4^+$ T cells (Huijbregts *et al*., 2013). However, it remains unclear whether this effect is via the GR, and whether GCs acting via the GR and immune activators can synergise to increase de novo HIV-1 infection in target cells.

1.4.6: The GR and select synthetic progestins

MPA, unlike NET and LNG, binds to the GR with a relatively high affinity similar or greater to that of CORT and can activate the receptor (Stanczyk *et al*., 2013; Bamberger *et al*., 1999; Koubovec *et al*., 2004; Ronacher *et al*., 2009; Hadley *et al*., 2011; Hapgood *et al*., 2013). It has been reported that MPA is a partial to full GR agonist for transactivation and transrepression (Ronacher *et al*., 2009). Moreover, MPA has been shown to induce the phosphorylation of the GR at Ser-211 and Ser-226, resulting in enhanced GR nuclear translocation and turnover (Avenant *et al*., 2010a; Hadley *et al*., 2011). NET and LNG, on the other hand, have no partial or full GR agonist activity (Stanczyk *et al*., 2013; Ronacher *et al*., 2009; Hapgood *et al*., 2018). Taken together, these reports show that MPA, NET and LNG differentially regulate gene expression via the GR.

1.4.6.1: The influence of MPA, NET and LNG on FGT and systemic inflammation

Evidence from in vitro studies shows that MPA is pre-dominantly anti-inflammatory and suppresses the expression of pro-inflammatory cytokines and chemokines in the FGT and blood (Govender *et al*., 2014; Huijbregts *et al*., 2013; Mantovani *et al*., 1997; Huijbregts *et al*., 2013; Huijbregts *et al*., 2014; Hapgood *et al*., 2014b; Kleynhans *et al*., 2011). These findings corroborate results from a single clinical study that showed DMPA-IM suppressed IL8 and IL6 protein levels in the FGT (Roxby *et al*., 2016). It has been reported that the in vitro immunosuppressive and anti-inflammatory effects of MPA are mediated by the GR (Govender *et al*., 2014; Hapgood *et al*., 2014a; Koubovec *et al*., 2005). This suggests that MPA acting via the GR might reduce the ability of the immune system in the FGT and blood to protect against HIV-1 infection.

However, there also exist evidence suggesting that MPA is pro-inflammatory i.e. it can induce or enhance the expression of select pro-inflammatory genes in the FGT (Louw du Toit *et al*., 2014;

Francis *et al*., 2016; Deese *et al*., 2016; Quispe-Calla *et al*., 2016; Irvin & Herold, 2015; Zheng *et al*., 2012; Woods *et al*., 2018). In addition, some studies have shown that MPA can selectively induce or enhance the expression of some pro-inflammatory genes, while at the same time repressing others (Ferreira *et al*., 2015a; Cordeaux *et al*., 2010; Goldfien *et al*., 2015; Smith-McCune *et al*., 2017; Africander *et al*., 2011; Goode *et al*., 2014; Kleynhans *et al*., 2013; Fichorova *et al*., 2015; Morrison *et al*., 2014). By selectively upregulating pro-inflammatory immune mediators, MPA may create an immune microenvironment that is conducive to specific HIV-1 target recruitment (Woods *et al*., 2018; Deese *et al*., 2016).

Results from clinical study suggests that LNG-IUD increases the levels of pro-inflammatory immune mediators in the FGT (Shanmugasundaram *et al*., 2016). Similarly, endometrial tissue biopsies from women using LNG-IU have been found to express more pro-inflammatory genes compared to controls (Goldfien *et al*., 2015). These findings suggest that LNG-IUD induces a pro-inflammatory response in the FGT. However, evidence from in vitro studies show that LNG does not regulate cytokine expression by activated systemic mononuclear and pDC cells and ectocervical tissue explants (Huijbregts *et al*., 2014; Ray, 2015). This suggests that the effects of LNG are compartment specific.

Unlike MPA and LNG, evidence from some in vitro studies show that NET does not regulate the expression of pro-inflammatory immune mediators in epithelial cells from the FGT (Govender *et al*., 2014; Africander *et al*., 2011) and in PBMCs (Hapgood *et al*., 2014a; Tomasicchio *et a*l., 2013; Maritz *et al*., 2018). Conversely, others have shown that NET suppresses TNF-induced expression of pro-inflammatory immune mediators in endometriotic stromal cells in vitro (Grandi *et al*., 2016). This suggests that the effects of NET on gene expression are cell-specific and if there is an effect it likely to be anti-inflammatory. However, findings from a recent clinical study show that women using NET-EN have high levels of select pro-inflammatory immune mediators in the FGT but is less pro-inflammatory than DMPA-IM (Deese *et al*., 2016). This suggests NET-EN might be pro-inflammatory to some extent, but more studies are needed to confirm this observation.

Given the contradictory data for DMPA-IM, and paucity of data for NET-EN and LNG, it is possible that their effects on immune function in FGT and blood in women may be confounded by intrinsic confounding factors and differences in study design. Therefore, more careful head to head clinical studies are needed.

1.4.6.2: The role of specific pro-inflammatory immune mediators in HIV-1 infection and their regulation by MPA

1.4.6.2.1: CCL20/CCR6 Axis

One of the main functions of CCL20 is to direct the migration of $CCR6^+$ immune cells to the site of inflammation. CCR6 is the sole receptor for CCL20 and is expressed on a variety of immune cells notably Th17 cells, macrophages, Langerhans cells and neutrophils (Acosta-Rodriguez *et al*., 2007; Annunziato *et al*., 2007; Gosselin *et al*., 2010; Greaves *et al.*, 1997; Liao *et al*., 1999; Lee & Körner, 2017). Systemic $CCR6^+CD4^+$ T cells have been shown to be more permissive to HIV-1 infection than $CCR6^-CD4^+$ T cells (Gosselin *et al*., 2010). In addition, it has been reported that $CCR6^+CD4^+$ T cells, which constitute HIV-1 latent reservoirs, are mostly central memory cells of Th17 or Th1Th17 phenotype (Gosselin *et al*., 2017). Besides expressing CCR6, central memory Th17 cells also express α4β7 and CXCR4, but not CCR5 (Alvarez *et al*., 2013; Gosselin *et al*., 2017). The majority of $CD4^+$ T cells in FGT mucosal tissues are effector memory cells, but populations of naïve and central memory T cells have also been identified (Smith-McCune *et al*., 2017). It has recently been found that Th17 cells are present in the FGT and like systemic Th17 cell genital Th17 cells are also more permissive to HIV-1 infection than non-Th17 cells (Rodriguez-Garcia *et al*., 2014). Furthermore, it has been reported that SIV preferentially infects genital Th17 than non-Th17 cells (Stieh *et al*., 2016). Thus, the infiltration of Th17 or other $CCR6^+$ cells in the FGT could substantially elevate the risk of HIV-1 acquisition in women.

Besides trafficking of immune cells, CCL20 may also regulate events in HIV-1 infection within target cells. It has been reported that CCL20 enhances latent HIV-1 infection in resting $CD4^+$ T cells and may thus contribute to the dissemination of infected cells from the site of infection (Cameron *et al*., 2010; Gosselin *et al*., 2010). In fact, evidence from some studies show that CCL20 inhibits production of new virus particles in the FGT of HIV-1 positive women (Mauck *et al*., 2016). In addition, it has been reported that CCL20 suppresses HIV-1 replication in $CD4^+$ T cells (Lafferty *et al*., 2010) and in TZM-bl cells (Ghosh *et al*., 2010). The mechanism involves CCL20 inducing the expression of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), an enzyme that prevents the accumulation of HIV-1 transcripts in the cytoplasm of infected cells (Lafferty *et al*., 2010). Taken together, this suggests that CCL20 facilitates HIV-1 entry and integration into target cells but does allow new viruses to be made by creating a microenvironment that degrades nascent HIV-1 mRNA transcripts.

It has recently been reported that that MPA at 1 nM increases CCL20 mRNA levels in primary genital epithelial cells (Woods *et al*., 2018). This result suggests that MPA-induced expression of CCL20

might be involved in the trafficking of $CCR6^{+}CD4^{+}$ T cells that are very permissive to HIV-1 infection in the FGT. In addition, this finding suggests that these cells might become latently infected in an MPA-induced CCL20-rich microenvironment. However, evidence from clinical studies show that DMPA-IM use does not alter CCL20 protein levels in genital secretions obtained by cervical wicks from women irrespective of GTI status (Morrison *et al*., 2014; Fichorova *et al*., 2015). While this effect might be compartment specific, this nonetheless suggests that there might be differences in the way MPA regulates CCL20 mRNA and protein levels in the FGT. Thus, it remains unclear how MPA regulates CCL20 expression in the FGT.

1.4.6.2.2: CCL5/ CCR5 Axis

There has been an interest in determining whether MPA regulates CCL5 and CCR5 expression in HIV-1 target cells and in the FGT. Findings from some clinical studies show that women using DMPA-IM have high levels of CCL5 protein in the FGT (Fichorova *et al*., 2015; Morrison *et al*., 2014; Deese *et al*., 2016). In addition, MPA has been shown to enhance CCL5 expression in PGECs in the presence of HIV-1 (Ferreira *et al*., 2015a), in vaginal epithelial Vk2/E6E7 cells at suprapharmalogical doses (Irvin & Herold, 2015) or in vaginal mucosal tissues from mice infected with HSV-2 (Zheng *et al*., 2012). CCL5 is one of the natural ligands of CCR5 co-receptor for HIV-1 entry into target cells, the others being CCL2 and CCL4 (Gross *et al*., 2003; Secchi *et al*., 2012). This implies that high levels of CCL5 in the FGT may protect against HIV-1 infection (Hirbod *et al*., 2006; Novak *et al*., 2007). Conversely, because CCL5 is a chemoattractant, increases in its expression might result in the infiltration of CCR5 expressing cells in the FGT (Iqbal *et al*., 2005; Kaul *et al*., 2008). In this scenario, the likelihood of infection occurring will depend on the ratio of CCR5 to CCL5.

However, there exists evidence from in vitro studies that MPA suppresses CCL5 expression in epithelial cells from the FGT (Govender *et al*., 2014; Africander *et al*., 2011) and in PBMCs (Hapgood *et al*., 2014a; Ray, 2015). This suggests that MPA reduces the ability of HIV-1 target cells in the FGT or blood to protect themselves or neighbouring cells against infection (Alvarez *et al*., 2013). This might render the FGT or blood more vulnerable to HIV-1 infection. This also suggests that MPA might prevent the recruitment of CCR5 expressing cells in the FGT. Results from a recent in vitro study showed that MPA increases the expression of CCR5 on $CD4^{+}$ T cells from blood (Maritz *et al*., 2018). Furthermore, the Hapgood group has found evidence that MPA increases $CCR5^{+}$ expression in ectocervical tissue explants (Ray *et al*., manuscript under review). Thus, MPA might be involved in recruiting new $CCR5^{+}$ cells to the FGT, but it may change the density of CCR5 on target cells already present in the FGT.

Taken together, these findings suggest that MPA enhances the expression of CCR5 on $CD4^+$ T cells. In contrast, the effects of MPA on CCL5 expression remains controversial. However, it is very likely that the effects are heterogeneous as well as cell-, tissue or subject-specific.

1.4.6.2.3: IL8, IL1β and IL6

It has been reported that IL1β and IL6 increase HIV-1 replication in chronically infected cells in vitro, with IL6 synergising with IL1β or TNF (Poli *et al*., 1990; Poli *et al*., 1994; Granowitz *et al*., 1995; Rollenhagen & Asin, 2011; Irvin & Herold, 2015). IL8 like CCL5 and CCL20 is a chemoattractant that induces the migration of HIV-1 target cells (Chuntharapai *et al*., 1994; Appay & Rowland-Jones, 2001; Stanford & Issekutz, 2003; Takata *et al*., 2004). In addition, IL8 has been found to regulate viral replication in cells or tissues infected with HIV-1 (Lane *et al*., 2001; Rollenhagen & Asin, 2010). Furthermore, it has been suggested that IL8, like IL1 and IL6, helps to spread HIV-1 in mucosal tissues following infection (Guha & Ayyavoo, 2013). Thus, regulating the expression of these immune mediators in the FGT and blood may affect the outcome of HIV-1 infection in these tissues.

Several studies have investigated the effects of MPA on IL6, IL8 and IL1β levels in the FGT and the results are conflicting. Some clinical studies have reported that DMPA-IM use increases IL6 protein levels in genital secretions (Deese *et al*., 2015; Francis *et al*., 2016), whereas others have found the opposite effect (Roxby *et al*., 2016; Smith-McCune *et al*., 2017). Yet others have reported that DMPA-IM use does not affect IL6 levels in genital secretions (Morrison *et al*., 2014; Fichorova *et al*., 2015) as well as in serum (Batista *et al*., 2017). The variability of the results may indicate inter-individual variations as well as differences in study design and methods. However, the majority of animal and in vitro studies have shown that MPA suppresses IL6 expression in the simian vaginal swabs (Goode *et al*., 2014), PBMCs (Kleynhans *et al*., 2011; Hapgood *et al*., 2014a; Huijbregts *et al*., 2013), human primary endometrial epithelial cells (Ferreira *et al*., 2015a) and endocervical epithelial End1/E6E7 cell line (Govender *et al*., 2014). However, one in vitro study found MPA at supraphysiological doses enhanced IL6 in vaginal epithelial cells (Irvin & Herold, 2015). This suggests that effects of MPA on the regulation of IL6 is cell-specific and dose-dependent.

A clinical study recently reported that DMPA-IM use increases IL8 in genital secretions (Deese *et al*., 2014). This is consistent with findings from some in vitro studies that MPA induces IL8 expression in endometrial epithelial cells (Woods *et al*., 2018; Ferreira *et al*., 2015a), vaginal epithelial cells (Irvin & Herold, 2015), ectocervical epithelial cells (Africander *et al*., 2011) and in endometrial stroma cells (Arici *et al*., 1999). In contrast, other clinical studies have reported DMPA-IM use reduces genital IL8 protein levels over time (Roxby *et al*., 2016). This has also been demonstrated in some in animal and

in vitro studies: simian vaginal swabs (Goode *et al*., 2014), endocervical epithelial cells (Govender *et al*., 2014), PBMCs (Kleynhans *et al*., 2011; Hapgood *et al*., 2014a; Huijbregts *et al*., 2013) and endometrial explants (Kelly *et al*., 1994). However, there also is evidence from some clinical studies that DMPA-IM does not alter IL8 levels in genital secretions (Smith-McCune *et al*., 2017; Francis *et al*., 2016; Morrison *et al*., 2014; Fichorova *et al*., 2015).

Evidence from a single clinical study showed that DMPA-IM use increased IL1β levels in genital secretions (Francis *et al*., 2016). However, findings from others clinical studies revealed DMPA-IM use may (Smith-McCune *et al*., 2017) or may not reduce IL1β levels in genital secretions (Deese *et al*., 2014; Morrison *et al*., 2014; Fichorova *et al*., 2015). It has recently been shown that DMPA increases IL1β mRNA expression in murine vagina in vivo, whereas in human it has been reported that MPA suppresses IL1β expression in BCG-treated PBMCs in vitro (Kleynhans *et al*., 2011).

Collectively, evidence from in vitro studies suggests that MPA suppresses IL6, IL8 and IL1β expression. However, the in vivo effects of MPA on the expression of these immune mediators in the FGT are heterogeneous due to inter-individual variations. Moreover, these effects may be compartment–specific and further confounded by factors such as the presence of GTIs, age and duration of exposure to MPA.

1.4.7: Epithelial TJs are regulated by GCs and MPA

It has been reported that DMPA-IM or LNG-IUD use increases the permeability of the ectocervix and vagina in women (Tjernlund *et al*., 2015; Quispe-Calla *et al*., 2016; Quispe-Call *et al*., 2017). Similarly, DMPA-IM and LNG have been found to increase the permeability of murine ectocervix and vagina, acting via the progesterone receptor (PR) (Quispe-Calla *et al*., 2016; Quispe-Calla *et al*., 2018). It is currently known that MPA and LNG downregulate the expression of specific junctional genes (Tjernlund et al., 2015; Quispe-Calla *et al*., 2016; Quispe-Call *et al*., 2017; Quispe-Calla *et al*., 2018). However, it remains unclear whether these progestins target the same genes in the different compartments of the FGT to increase mucosal permeability. One study found that ectocervical tissue biopsies from women using LNG-IUD had reduced ZO-1 mRNA levels compared to controls, whereas claudin-1, occludin and E-cadherin mRNA levels remained the same between the groups (Tjernlund *et al*., 2015). In vaginal tissues from women or mice, neither DMPA-IM nor LNG-IUD treatment altered ZO-1, claudin-1, occludin and E-cadherin expression levels (Chandra *et al*., 2013; Quispe-Calla *et al*., 2016). On the other hand, desmoglein-1α and desmocollin-1 mRNA levels have been found to be reduced in ectocervical tissues from women and in vaginal tissues from mice treated with DMPA-IM or LNG (Quispe-Calla *et al*., 2016; Quispe-Calla *et al*., 2017; Quispe-Calla *et al*., 2018). Taken

together, this suggests that LNG downregulates both desmosomes and TJs in ectocervical mucosal tissues, but only desmosomes in vaginal mucosal tissues. It also suggests MPA downregulates desmosomes in ectocervical mucosal tissues, but it remains unclear whether it can regulate TJs in this tissue.

Another study reported that MPA like P4 might increase the permeability of primary endometrial and endocervical epithelial monolayers in vitro (Ferreira *et al*., 2015a). However, a recent study found that DMPA-IM increases claudin-8 mRNA levels in endometrial mucosal tissues (Goldfien *et al*., 2015). Additionally, MPA has been found to increase claudin-5 mRNA levels in myometrial tissue explants (Cordeaux *et al*., 2010). Because claudin-5 and -8 are sealing TJs, this suggests that MPA might increase rather the decrease the barrier functions of the endometrium. Nonetheless, the findings show that MPA can regulate the expression of TJ genes in the endometrium. It remains unclear whether and how MPA regulates the expression of TJ genes in the endocervix and whether the GR is involved.

It has been reported that GCs acts via the GR to increase the mucosal barrier functions of intestinal, mammary and airways epithelial cells (Singer *et al*., 1994; Boivin *et al*., 2007; Fischer *et al*., 2014; Kielgast *et al*., 2016). GCs have been reported to increase the expression of claudin-8 in human tracheal epithelial cells (Kielgast *et al*., 2016), claudin-4 in human colonic Caco-2 epithelial cells (Fischer *et al*., 2014) and ZO-1 in human mammary 31EG4 epithelial cells (Singer *et a*l., 1994). In addition, GCs have been shown to transactivate occludin plasmid constructs transiently transfected into COS-7 cells (Förster *et al*., 2005; Felinski *et al.*, 2008). Conversely, GCs have also been reported to supress claudin-2 expression in human colonic Caco-2 epithelial cells (Fischer *et al*., 2014). This suggests that GCs inversely regulate sealing and leaky TJs to enhance the barrier functions of mucosal tissues. Moreover, there is evidence suggesting that the effects of GCs on mucosal barrier function and expression of TJ genes are tissue-specific (Zheng *et al*., 2013). Recently, it has been reported that treating mice exogenously with methylprednisolone did not alter mucosal barrier properties of the lower FGT (Quispe-Calla *et al*., 2016). However, these authors did not investigate the serum concentrations of this GC. It remains unknown whether GCs acting via the GR can regulate mucosal barrier functions and TJ gene expression in the human FGT, as well as the dose-dependency and steroid-specific effects.

Taken together, the finding suggests that MPA increases the mucosal permeability of the lower FGT by downregulating desmosomes, but not TJs. However, it remains unclear whether MPA can alter the permeability of the endocervix and if it does what junctional genes are targeted.

1.5: Hypothesis and Aims

1.5.1: General hypothesis

The main hypothesis is that medroxyprogesterone acetate (MPA), like glucocorticoids (GCs), acts via the glucocorticoid receptor (GR) to synergise with immune activators to decrease female genital tract (FGT) mucosal barrier function via the downregulation of tight junction (TJ) genes and increase the expression of select pro-inflammatory cytokines and chemokines in the FGT and blood, thereby synergistically increasing HIV-1 infection.

1.5.2: Specific aims

A. What are the effects of GCs and progestogens on the expression of select TJ and immune function genes in an endocervical epithelial cell line?
B. Do these ligands cooperate with immune activators to regulate this gene expression?
C. What are the efficacies, potencies and biocharacters of these ligands for gene expression?
D. Is this gene regulation dependent on the GR?
E. Does this regulation occur in physiologically relevant primary tissue and primary cells from the FGT and blood?
F. What are the effects of these ligands alone or in combination with immune activators on mucosal barrier function and HIV-1 infection?

Chapter Two

Materials and Methods

2.1: Ethical Statement

This thesis was done as part of a larger study investigating whether synthetic progestins enhance HIV-1 infection in human cervical tissue explants and PBMCs. Ethical approval for both studies was obtained the Human Research Ethics Committee at the University of Cape Town, with reference number HREC 210/2011 and HREC 021/2018. Signed informed consents was obtained from individuals donating tissue and blood samples used in this study.

2.2: Compounds and antibodies

The following compounds were obtained from Sigma Aldrich, South Africa: (11β,6α)-9-Fluoro-11,17, 21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione (dexamethasone, DEX, catalogue #D4902); 4-Pregnene-3, 20-dione (progesterone, P4, catalogue #P0130); 17α-Ethynyl-17β-hydroxy-19-nor-4-androsten-3-one (norethindrone, NET, catalogue #N4128); 13β-Ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one (levonorgestrel, LNG, catalogue #N2260); 11β-(4-Dimethylamino)-phenyl-17β-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one (mifepristone, RU486, catalogue #M8046); 11β,17α,21-Trihydroxypregn-4-ene-3,20-dione (hydrocortisone, CORT, catalogue #H0888); 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT, catalogue #M5655); Lipopolysaccharides (LPS, *E. coli* O111:B4, catalogue #L4391). Recombinant human TNF was obtained from Peprotech, United States of America (USA, catalogue #300-01A); Bovine serum albumin (BSA, R&D Systems, USA, catalogue #DY995. Interleukin 2 (IL2) was obtained from Gentaur, Belgium (catalogue #04RHIL2-08E02). Phytohaemoagglutinin (PHA) was obtained from Remel (USA, catalogue #HA16/30852801)

Steroids were made up in absolute ethanol (EtOH). Human TNF was made up in 0.1% (v/v) BSA, whereas LPS was made in sterile Milli-Q water. TNF and LPS are referred to in this thesis as immune activators. All ligands working stocks were stored at -20°C. MMT was made up in Milli-Q water, filtered with a 0.22 µM filter and stored at 4°C.

The following primary antibodies against human GR (H-300; 8992), PR (C-20; sc-539) and GAPDH (0411; sc-47724) were obtained from Santa Cruz Biotechnology, United States of America (USA). Antibodies against hyaluronic acid (HA, catalogue #12CA5) were obtained from Roche Applied Science, South Africa; whereas antibodies against human claudin-4 (3E2C1; catalogue #32-9400),

occludin (OC-3F10; catalogue #33-1500) and ZO-1 (ZO1-1A12; catalogue #33-9100) were obtained from Invitrogen, Thermo Fisher Scientific, USA. The following horseradish peroxidise-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, USA: anti-mouse (sc-2005) and anti-rabbit (sc-2313). Donkey anti-mouse IgG Cy3 conjugated antibody (catalogue #715-166-150) was obtained from Jackson ImmunoResearch Laboratories, USA.

2.3: Plasmids

The pMT-PR-B plasmid encoding human PR-B was a gift from Prof. S. Okret (Karolinska Institute, Sweden) and has been described previously (Cairns *et al*., 1993). The pcDNA3-hGR plasmid encoding human GR was a kind gift from Prof. D.W. Ray (University of Manchester, UK) and has been described previously (Ray *et al.*,1999). The pcDNA3 (empty vector, catalogue #A-150228) plasmid was obtained from Invitrogen, USA.

2.3.1: Transformation, preparation, purification and restriction enzyme digestion

Plasmids were transformed into competent *E. coli* DH5α cells as described previously (Sambrook *et al*., 1989). Briefly, 10 ng plasmid DNA was added to 100 µL competent cells and the mixture incubated on ice for 20 minutes, heat-shocked at 42°C for 2 minutes followed by 2 minutes of incubation on ice. Thereafter, the mixture was added into 900 µL Luria broth [LB: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride (NaCl)] and incubated at 37°C for 1 hour with shaking. The transformed cells were then plated onto LB-agar plates [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar] containing 100 µg/mL ampicillin and incubated overnight at 37˚C. Single colonies were selected and inoculated into 5 mL LB containing 100 µg/mL ampicillin and incubated at 37°C for 8 hours with shaking. Overnight cultures were made by adding 200 µL day cultures to 200 mL LB containing 100 µg/mL ampicillin and incubated at 37°C with shaking. Plasmid DNA was purified using the PureYield™ Plasmid Midi-prep system (Promega, USA, Catalogue #A2492) according to manufacturer's instruction. The yield and purity of the isolated DNA was assessed using the NanoDrop ND-100 spectrophotometer (Thermo Fisher, USA). The integrity of the plasmid preparation was analysed by restriction enzyme digestion and agarose gel electrophoresis.

2.4: Mammalian tissue culture

All cell lines were cultured in humidified (90%) 37°C incubators containing 5% CO_2 and were regularly tested for mycoplasma by means of Hoechst staining and fluorescent microscopy (Freshney, 1987). Only mycoplasma negative cells were used.

2.4.1: End1/E6E7 cells

The human endocervical epithelial cell line End1/E6E7 was a kind gift from Prof. R. Fichorova (Brigham & Women's Hospital, Boston, USA) and has been described previously (Fichorova *et al*., 1997). They were maintained in 75 cm^2 flasks (Greiner Bio-one International, Austria) in Stemline® Keratinocyte Medium II (SKM, Sigma Aldrich, South Africa, Catalogue #s0196) supplemented with 4 mM L-glutamine (Sigma Aldrich, South Africa, catalogue # G7513), Stemline® Keratinocyte Growth supplement (Sigma Aldrich, South Africa, catalogue #S9945), 100 units (U)/mL penicillin and 100 µg/mL streptomycin (Sigma Aldrich, South Africa, catalogue #P4333) and 0.5 µg/mL amphotericin B (Sigma Aldrich, catalogue # A2942), hence referred to as full SKM.

For TJ gene expression studies, End1/E6E7 cells are seeded at a density of 500,000 cells/mL/well in 12 well plates (Greiner Bio-One International, Austria) in full SKM and grown for 3 days until confluent. Thereafter, the cells were washed once with 1 X PBS and stimulated with 100 nM steroids or vehicle [0.1% (v/v) EtOH and 0.0001% (v/v) BSA] in the presence or absence of 1.15 nM TNF in serum-free SKM (without Stemline® Keratinocyte Growth supplement) for 24 hours. The cells were harvested in Tri-Reagent® (Sigma Aldrich, South Africa, catalogue #T9424) for total RNA isolation or in 50 µL 2 X sodium diodecyl sulphate (SDS) sample buffer for Western blotting to verify whether HA-Vpr was expressed.

For immune function gene expression studies, End1/E6E7 cells are seeded at a density of 100,000 cells/mL/well in 12 well plates in full SKM and grown for 2 days. Thereafter, cells were washed once with 1 X PBS and stimulated with 100 nM steroids or vehicle [0.1% (v/v) EtOH and 0.0001% (v/v) BSA or sterile Milli-Q water] in the presence or absence of 1.15 nM TNF or 5 µg/mL LPS in serum-free SKM for 24 hours or at different time points as indicated elsewhere. For some experiments, the cells were initially pre-treated with 100 nM DEX, MPA, CORT and NET prior to stimulation with immune activators. In others, the cells were treated with 100 nM DEX, MPA, CORT or NET alone or in combination 1.15 nM TNF and incubated at different time points.

Dose-responses were performed by treating the End1/E6E7 cells with concentrations of progestogens ranging between 10^{-9} and 10^{-6} M. For specific experiments, cells were treated with 10^{-9} to 10^{-6} M concentrations of MPA or CORT in the presence or absence of 1.15 nM. In another experiment (not parallel), cells were treated with increasing concentrations of TNF in the absence or presence of 100 nM MPA or CORT.

2.4.2: TZM-bl cells

The TZM-bl indicator cell line was obtained from the Nation Institute of Health AIDS Reagent Program (USA). They were maintained in 75 cm^2 flasks in phenol red Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, South Africa, catalogue #D5648) supplemented with 1 mM sodium pyruvate (Sigma Aldrich, South Africa, catalogue #S8636), 44 mM sodium bicarbonate (Sigma Aldrich, South Africa),10% (v/v) foetal calf serum (FCS, Thermo Scientific, South Africa), 100 U/mL penicillin and 100 µg/mL streptomycin.

2.4.3: COS-1 and HEK293T cells

The human embryonic kidney cells (HEK293T) and the African green monkey kidney fibroblast (COS-1) were purchased from America Type Culture Collection (ATCC, USA). They were maintained in 75 cm^2 flasks in phenol red Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, South Africa, catalogue #D5648) supplemented with 1 mM sodium pyruvate (Sigma Aldrich, South Africa, catalogue #S8636), 44 mM sodium bicarbonate (Sigma Aldrich, South Africa),10% (v/v) foetal calf serum (FCS, Thermo Scientific, South Africa), 100 U/mL penicillin and 100 µg/mL streptomycin.

2.4.5: Peripheral blood mononuclear cells

Blood was obtained from the Western Cape Province Blood Transfusion Services (Cape Town, South Africa). It was collected from women donating at the facility. Written informed consent was obtained from all donors. PBMCs was isolated from blood using the Histopaque® density gradient centrifugation method (Thorsby & Bratlie, 1970). Briefly, 15 mL Histopaque®-1077 reagent (Sigma Aldrich, South Africa) was placed in a 50 mL Leucosep tube (Greiner, Germany) and centrifuged in a swing-bucket centrifuge (Heraeus Megafuge 40, ThermoScientific, USA) at 2,500 rpm for 1 minute at room temperature. Whole blood was diluted 1:3 using a solution containing equal volumes of serum-free Roswell Park Memorial Institute medium (RPMI 1640, Lonza, Germany, catalogue #BE12-7012) and phosphate buffered saline (PBS, Sigma Aldrich, South Africa). Thirty (30) mL of the diluted blood was layered onto Histopaque in the 50 mL Leucosep tube and centrifuged at 2,500 rpm for 15 minutes at room temperature without brake. Thereafter, the buffy coat was aspirated using a serological pipette and washed twice by spinning at 1,200 rpm for 5 minutes, with each wash requiring 50 mL of PBS supplemented with 1% (v/v) charcoal-stripped (c-s) FCS. During the second wash, cell count and viability were determined by diluting the PBS-suspended PBMCs 1:10 with trypan blue dye (Lonza, Germany) and counting using a haemocytometer. After the second wash, isolated PBMCs were then resuspended and maintained in full RPMI 1640 media containing 10% (v/v) c-s FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37℃ with 90% humidity and 5% CO_2.

For gene expression studies, 2 million cells/mL PBMCs were seeded into 15 mL cell culture tubes in full RPMI I640 media and then stimulated with 100 nM steroids or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 5 µg/mL LPS for 24 hrs. Thereafter, supernatants were collected for downstream applications, cells washed once with 1 x cold PBS by centrifuging at 1,200 rpm for 5 minutes using a Heraeus Megafuge 40 centrifuge (ThermoScientific, USA) and then harvested in 800 µL of Tri-Reagent®

2.4.6: Ectocervical tissue explants

Ectocervical explants were obtained after informed consent from pre-menopausal women undergoing hysterectomy for benign conditions. The women tested negative for HIV-1 and had normal Pap smear; however, 100% tested for HSV-1, while 30% tested positive for HSV-2. Samples were collected from Groote Schuur and Tygerberg Hospitals in Cape Town and transported in full RMPI 1640 media containing 10 U/mL IL2 and 10 µg/mL fungizone (Sigma Aldrich, South Africa) to the Mammalian Tissue Culture facility at the Department of Molecular and Cell Biology, University of Cape Town, where they were processed within 4 hours after surgery. In brief, the tissue explants were rinsed in 1 X PBS and a customised stainless-steel punch was used to cut out 3 mm^3 tissue pieces. For gene expression studies, the pieces were randomly selected and transferred into round bottomed 96 well plate (Greiner Bio-One International, Germany). They were then treated with 100 nM DEX, MPA or vehicle containing 0.1% (v/v) EtOH and 0.0001% (v/v) BSA in the presence or absence of 1.15 nM TNF made up in full RPMI 1640 media containing 10 U/mL IL2, 100 U/mL penicillin and 10 µg/mL fungizone. Each ligand condition was at least done in triplicates. The tissue explants were then cultured for 24 hours at 37˚C with 90% humidity and 5% CO_2. At the end of the incubation period, supernatants were harvested and stored at -80˚C until use. Tissue pieces were harvested in Tri-Reagent® and stored at -80˚C until use.

2.4.7: Primary genital epithelial cells

PGECs were isolated from endocervical and endometrial tissue explants obtained from women undergoing hysterectomy for benign conditions at McMaster University Medical centre, Hamilton, Canada. Informed written consent was obtained in accordance with the approval of the Hamilton Health Sciences Research Ethics Board. The protocol for isolation and culture of PGECs has been described previously (Kaushic *et al*., 2011). Briefly, endometrial and endocervical tissues were cut into small pieces and digested in an enzyme mixture containing 0.1 mg/mL Hyaluronidase from sheep testes (Roche Diagnostics, Germany, catalogue #46254), 3.45 mg/mL pancreatic from porcine pancreas (Sigma Aldrich, Canada, catalogue #P3292), 1.5 mg/mL collagenase-D from *Clostridium histolyticum* (Roche Diagnostics, Germany, catalogue #11088882001) and 2 mg/mL D-glucose (EMD

Chemicals, USA, catalogue #DX01451) for 30 minutes at 37˚C with shaking. Thereafter, the digested tissue was passed through a series of nylon mesh filters of two different pores sizes: 250 µM and 20 µM (Component Supply Company, USA). The 250 µM filter retains tissue debris and the filtrate is passed through the 20 µM filter to separate PGEC sheets from single cell population. The PGEC sheets retained on the 20 µM filters were collected into a falcon tube and pelleted by centrifugation (Heraeus Megafuge 40, ThermoScientific, USA) at 2,500 rpm for 15 minutes at room temperature with no brake. The cells were counted and then resuspended in phenol red-free DMEM/F12 (Invitrogen, Canada, catalogue #11039021) containing 10 mM HEPES (Invitrogen, Canada, Catalogue #15630080), 2.5% Nu serum culture supplement (Becton Dickinson and Co, USA, Catalogue #355104), 2.5% Hyclone defined foetal bovine serum (Thermo Scientific, Canada, catalogue # SH3053102), 250 µg/mL fungizone (Invitrogen, Canada, catalogue #15290018), 4 mM L-glutamine (Invitrogen, Canada, catalogue #21051024) and 100 U/mL Penicillin-Streptomycin (Invitrogen, Canada, catalogue #P4338), hence referred to as full DMEM/F12.

2.5: Transepithelial electrical resistance (TER) measurements

TER measurements were used to assess the barrier function of End1/E6E7 cells and PGECs. One hundred thousand (100,000) End1E6E7 cells in 300 µL full SKM were seeded onto 0.4 µM pore-size polycarbonate Transwell® filters (Becton Dickinson and Co, USA, catalogue #353095) and 500 µL full SKM added to the basolateral chamber (see **Figure 2.1**). Similarly, 100,000 PGECs in 300 µL full DMEM/F12 were seeded onto Matrigel-coated (Becton Dickinson and Co, USA, catalogue #356235) 0.4 µM pore-size polycarbonate Transwell® filters and 500 µL full DMEN/F12 added to the basolateral chamber. The cells were then incubated at 37˚C for several days. Growth media in both chambers was changed every second day. Before this was done, the cells were allowed to rest at room temperature for 5 minutes and TER measurements were then taken using a Millicell ERS-2 Voltohmmeter with chopstick electrodes (Merck, USA). The chopstick electrodes were sterilised by rinsing with 70% ethanol followed by another rinse with sterile PBS. The resistance was calculated by subtracting blank resistance from the sample-well resistance. This value was then multiplied by the surface area of the filter. Confluency was defined as TER values higher than 1 KΩ/cm2. Confluent PGECs monolayers were treated with ligands and subsequently exposed to virus as described in **section 2.10.3**

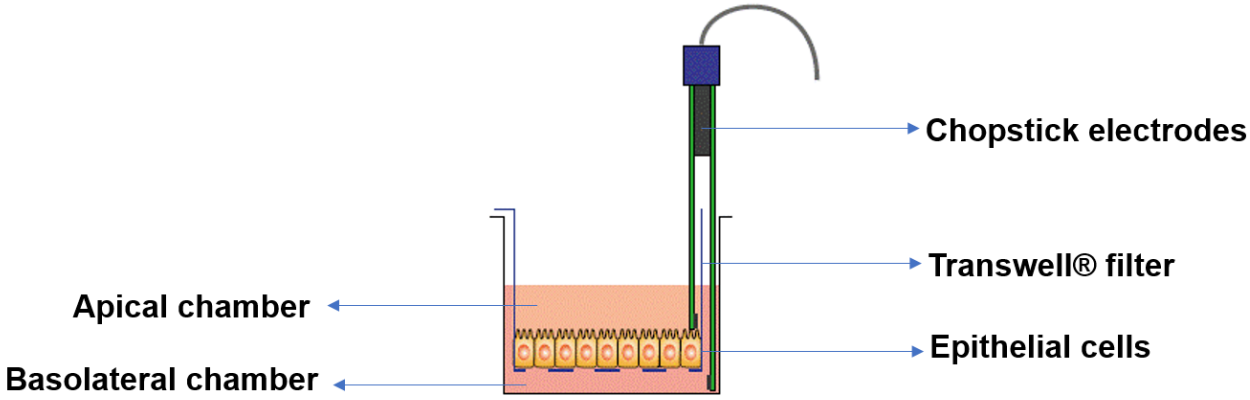

Figure 2.1: Transepithelial electrical resistance measurement with chopstick electrodes. Image source: https://ebrary.net/24380/health/measurement_transepithelial_electrical_resistance_teer

2.6: Generation of positive controls for western blotting

To generate GR and PR positive controls for western blotting, 250,000 COS-1 cells/mL were seeded in 6-well plates (Greiner Bio-One International, Austria) in full DMEM [10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin]. Twenty-four (24) hours later, they were transiently transfected with 1 µg pcDNA3-hGR, pMT-PR-B or empty vector using FuGENE™ 6 (Roche Applied Sciences, South Africa, catalogue #11814443001) according to manufacturer's instruction and incubated for another 24 hours. Thereafter, the cells were harvested in 50 µL 2 X SDS sample buffer, incubated for 10 minutes at 100°C and stored at -20°C until used.

2.7: GR Knockdown

End1/E6E7 cells at a density of 500,000 cells/mL in full SKM were seeded in 12-well plates and allowed to adhere for 24 hours. Thereafter, the media was changed, and the cells were transfected with 10 nM GR5 siRNA (HS_NR3C1_5, Qiagen, USA, catalogue #SI02654757) or non-silencing scrambled control (NSC) siRNA (Qiagen, USA, catalogue #1027310) using HiPerfect transfection reagent (Qiagen, USA, catalogue #301705). Briefly, a transfection mix was made by adding GR5 or NSC siRNAs diluted in Opti-MEM medium with GlutaMAX™ (Gibco, Thermo Fisher Scientific, USA, catalogue # 51985034) to 3.5 µL HiPerfect reagent. The mixture was incubated for 15 minutes at room temperature and thereafter added in a drop-wise manner to the cells. The cells were incubated for 48 hours after which they were washed with 1 X PBS and stimulated with ligands for 24 hours on serum-free media. Cells were then harvested in Tri-Reagent® for total RNA isolation or in 2 X SDS sample buffer (5X stock: 0.1 M Tris-Cl pH 6.8, 5% (v/v) SDS, 20% (v/v) glycerol, 5% β-mercaptoethanol and 0.1% (w/v) bromophenol blue) for Western blotting to verify GR knockdown.

2.8: Gene expression analysis

2.8.1: RNA isolation

Total RNA from End1/E6E7 cells, PBMCs or ectocervical tissue explants was isolated using Tri-Reagent® according to the manufacturer's instructions with slight modification. Briefly, Tri-Reagent® was added to End1/E6E7 cells or PBMCs pellets and incubated for 5 minutes at room temperature. Ectocervical tissue pieces were placed in cryovials and homogenised in Tri-Reagent® on ice using a hand-held homogeniser with disposable probes (TissueRuptor Probes, Qiagen, USA) according to the manufacturer's instructions and the homogenate incubated at room temperature for 5 minutes. The cell lysates or tissue homogenates were transferred into RNase-free micro-centrifuge tubes and centrifuged at 12,000 x g for 10 minutes at 4˚C to pellet cell debris. Supernatants were collected, transferred into new RNase-free micro-centrifuge tubes, 80 µL chloroform added and vortexed vigorously for 15 seconds at room temperature. The tubes were incubated for 2 minutes at room temperature and then centrifuged at 20,000 x g for 15 minutes at 4˚C. The aqueous phase was collected, transferred into new micro-centrifuge tubes, 200 µL isopropanol added and mixed gently by inverting several times. The tubes were incubated for 10 minutes at room temperature and then spun at 20,000 x g for 10 minutes at 4˚C to precipitate the RNA. Supernatants were discarded, and the precipitated RNA washed twice with 400 µL of 75% EtOH made in diethyl pyrocarbonate (DEPC)-treated water by centrifuging at 20,000 x g for 5 minutes at 4˚C. The resulting RNA pellets were air dried, resuspended in 10 µL of RNase-free water and incubated at 55˚C for 5 minutes. The isolated RNA was quantified using a NanoDrop®ND-1000 spectrophotometer (Thermo Fisher, USA) and its integrity assessed by running 250 ng on a 1% denaturing formaldehyde agarose gel (Sambrook *et al*., 1989). The RNA was then re-precipitated using 3 M sodium acetate (Ambion®, Thermo Fisher, USA) and stored as a pellet at -80˚C until use.

2.8.2: cDNA synthesis

cDNA was synthesized from 250 ng RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosciences™, Thermo Fisher, USA) according to manufacturer's instruction. cDNA samples were stored at -20 ˚C until use.

2.8.3: RT^2-PCR array profiling

For the specific purpose of RT^2-PCR array, total RNA was isolated from End1/E6E7 cells using the RNeasy® Plus Mini Kit (Qiagen, USA) according to manufacturer's instruction. RNA quantity and purity were determined using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was assessed using the RNA 6000 Nano Chip on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Four hundred (400) ng of RNA was converted to cDNA using

the RT^2 First Strand kit (Qiagen, USA. Catalogue #330401). The amplified cDNA was then diluted with nuclease-free water and added to the RT^{2}SYBR® Green ROX™ qPCR mastermix (Qiagen, USA, catalogue #330521) and 25 µL was then loaded onto the Human HIV Host Response PCR array (PAHS-051ZE-1, Qiagen, USA, catalogue #330231). Real-Time PCR was performed on the ABI 7900HT Fast Real-Time PCR System (Thermo Fisher, USA). The following thermal profile: stage 1 – 1 cycle at 95°C for 10 minutes; stage 2 – 95°C for 15 seconds followed by 60°C for 1 minute with 40 cycles; stage 3 (dissociation curve) – 95°C for 1 minute, 55°C for 30 seconds, and 95°C for 30 seconds. All data was acquired using using qbase$^+$ (BioGazelle, USA) and analysed using Qiagen Data Analysis Centre. Data were normalised to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA levels. Except for bioanalyzing and qRT-PCR that were performed at a commercial facility (Center for Proteomics and Genomic Research, Cape Town, South Africa), sample preparation and setting up PCR reaction was done by the candidate.

2.8.4: Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR) was performed using the Bioline SensiMix™ SYBR® no ROX kit (Bioline USA, catalogue # QT650-05) on a Corbett RotorGene 3000 qRT-PCR machine (Qiagen, USA) according to manufacturer's instructions. In brief, the reason mix contained; 10 µL SensiMix™, forward and reverse primers (see **Table 2.1**), 1 µL cDNA and PCR-grade water in a final volume of 20 µL. The reactions were amplified using the following protocol: 95˚C for 10 seconds followed by 40 cycles of 95°C for 10 seconds, annealing for 10 seconds (see **Table 2.1** for annealing temperatures) and 72˚C for 10 seconds. Data were acquired and analysed using the RotorGene software version 1.7 (Qiagen, USA). Melting curves analysis was performed to verify amplification and the PCR products were resolved on a 2% agarose gel to confirm product size. For each primer set, standard curves were generated using pooled cDNA and the primer efficiencies determined from these curves. The relative quantities of mRNAs were determined using the ''Fit Points'' method described by Pfaffl (2010) and were normalised using GAPDH as endogenous control.

Table 2.1: Primer details

Primer	Sequence 5'– 3;	Annealing ˚C	Product size Kb	Reference
GILZ	Quantitect Primer QT00091035	60	69	Qiagen, USA
IL6	F: TCTCCACAAGCGCCTTCG R: CTCAGGGCTGAGATGCCG	60	193	Wolf *et al*., 2002
IL8	F: TGCCAAGGAGTGCTAAAG R: CTCCACAACCCTCTGCAC	60	197	Wolf *et al.*, 2002
IL1β	F: CTGAAAGCTCTCCACCTC R: GATCTACACTCTCCAGCTG	57	184	Miao *et al.*, 2012
CCL20	F: GCAAGCAACTTTGACTGCTG R: CAAGTCCAGTGAGGCACAAA	58	342	Miao *et al*., 2012
CCL5	F: TACCATGAAGGTCTCCGC R: GACAAAGACGACTGCTGG	60	199	Wolf *et al.,* 2002
Claudin-4	F: CTGGGAAGTGCAGAGTGGAT R: AAGGAAGAGGAAAAAACCCCA	60	279	Varley *et al*., 2006
Occludin	F: CATTGCCATCTTTGCCTGTG R: AGCCATAACCATAGCCATAGC	55	149	Nazli *et al*., 2010
ZO-1	F: CGGTCCTCTGAGCCTGTAAG R: GGATCTACATGCGACGACAA	60	371	Yamaguchi *et al*., 2010
DSG-1	F: AATGGCTACATTTGCAGGACA R: ATCTCGGTCAGAGCCTCTTACA	60	256	This study
GAPDH	F: TGAACGGGAAGCTCACTGG R: TGTCAGTTGATAAAACCGCTGCC	55	307	Ishibashi *et al*., 2003
TNFRSF1B	F: GTC CAC ACG ATC CCA ACA C R: CACACCCACAATCAGTCCAA	58	145	This study
TNF	F: CAG AGG GCC TGT ACC TCA TC R: GGA AGA CCC CTC CCA GAT AG	60	219	This study
TLR2	F: GGCATGTGCTGTGCTCTGTT R: GCTTTCCTGGGCTTCCTTTT	60	125	Shan *et al*., 2011
TLR4	F: TTGAGCAGGTCTAGGGTGATTGAAC R: ATGCGGACACACACACTTTCAAATA	60	143	Shan *et al*., 2011
IRF1	F: AGATCCCATGGAAGCATGCTG R: CCTGGAACTGTGTAGCTGC	60	400	This study
IRF2	F: CAAGTGGCTTAACAAGG R: GGACCGCATACTCAGGAG	60	410	This study

2.9: Ligand combination (Chou) analysis

Chou analysis (Chou, 2010) was used to determine whether MPA or CORT synergises with TNF to upregulate gene expression. In brief, dose response experiments were performed as described in **section 2.4.1**. Cells were harvested, total RNA isolated, cDNA made, and real-time PCR performed as described in **section 2.8** for CCL20 and GAPDH. CCL20 fold changes in respond to single ligand or ligand combinations were calculated relative to GAPDH. Thereafter, the maximum fold change was set to 0.99 and the other responses calculated relative to this value. Each single or ligand combination dose and their respective responses were keyed into the Compusyn software (ComboSyn, Inc., USA) to determine the Combination indices (CI).

2.10: Protein Expression analysis

2.10.1: ELISA

Supernatants collected from End1/E6E7 cells, PBMCs, PGECs or ectocervical tissue explants were centrifuged at 1,500 rpm using a Microfuge® 20R Centrifuge (Beckman Coulter, USA) for 10 mins at 4°C to pellet cellular debris and supernatants were analysed for TNF, CCL20, IL6, CCL5 and IL1β protein levels by using DuoSet ELISA kits from R&D Systems (USA) according to manufacturer's instruction. However, a more sensitive kit was required to quantify CCL20 in ectocervical tissue explant supernatants (RayBio® Human MIP-3 alpha ELISA Kit, Raybiotech, USA) according to manufacturer's instructions.

2.10.2: Luminex

Aliquots of supernatants collected from ectocervical tissue explants (see section 2.9.1 for sample preparation) were shipped to Prof. Robbin Shattock's research group at Imperial College, London, UK where they were assessed for soluble immune proteins using an in-house Luminex panel. The following immune mediators were measured: TNF, IL8, CCL2, IFNβ, CCL5, IL1β, IL12, IL15, IL1α, IL4, IL2, CCL20, IL16, IL6, CXCL9, IFNy, TGFβ, G-CSF, CXCL12, CCL4, CXCL10, GM-CSF, IL7 and CCL8. This assay was performed by Dr. Julia Makinde.

2.10.3: Western blotting

Western blotting was performed according to the method described by Avenant *et al* (2010a). Equal amounts of End1/E6E7 or COS-1 whole-cell lysates (generated as described in **section 2.6**) were separated on 8% or 10% SDS-polyacrylamide gels at 120 V in 1 X running buffer [25 mM Tris-Cl, 250 mM glycine and 0.1% (w/v) SDS]. The separated proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences, South Africa, catalogue #RPN303D) for 1 hour at 180 mA in transfer buffer [25 mM Tris-Cl, 250 mM glycine and 20% (v/v) methanol]. The membranes were then blocked for 1 hour at room temperature in 4% (w/v) ECL blocking solution [4g ECL blocking powder in 100 mL Tris-buffered saline; 50 mM Tris-Cl, pH7.5 and 150 mM NaCl (TBS) containing 0.1% (v/v) Tween 20 (TBST)]. Thereafter, the membranes were incubated overnight with primary antibodies (see **Table 2.2** for dilutions) in 4% ECL blocking solution at 4°C. After washing three-times with 1 X TBST (each wash lasting for 5 minutes), the membranes were incubated with secondary antibodies made up in 5% non-fat dry milk–TBST solution at room temperature for 1 hour. The membranes were again washed 3 X with TBST and then rinsed in 1 X TBS. The proteins were visualised using Pierce® ECL Western blotting detection reagents (Thermo Scientific, USA, catalogue #32106) and Hyperfilm MP high performance autoradiography film (Amersham Biosciences, South Africa, catalogue #28906837) according to manufacturer's instructions. Bands on the X-ray film were

scanned, and quantification was performed using the AlphaEaseFC FluorChem 5500 software (Alpha Innotech, USA).

Table 2.2: Dilution of primary antibodies used in Western blotting analysis

Antibody	Dilution
GR	1:4,000
PR	1:1,000
Claudin-4	1:4,000
Occludin	1:2,000
HA	1:2,000
GAPDH	1:15,000

2.11: Preparation of HIV-1 infectious molecular clones and infection assay

HIV-1 M-tropic (or R5) infectious molecular clones were used in this study except indicated otherwise. $HIV_{BaL\text{-}Renilla}$ was a kind gift from Dr. Christina Ochsenbauer (University of Alabama, Birmingham, USA). It was made by inserting the Renilla luciferase gene next to the *env* in the HIV-1 NL4.3 backbone (Edmonds *et al*., 2010). HIV-$1_{BaL\text{-}Renilla}$ IMCs stocks were made as described previously (Pear *et al*., 1993). Briefly, 4 X 10^6 HEK293T cells were seeded in 75 cm^2 flasks and grown in phenol red DMEM containing 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin for 24 hours. Thereafter, culture media was changed to phenol red-free DMEM (Sigma Aldrich, South Africa, catalogue #D1145) supplemented with 10% c-s FCS, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were then transfected with 20 µg HIV-$1_{BaL\text{-}Renilla}$ plasmid DNA using X-tremeGENE™ 9 transfection reagent (Roche Applied Sciences, South Africa, catalogue #06366236 001) or control (DMEM) and incubated for 48 hours. Thereafter, supernatants were harvested, filtered through a 0.22 µM filter and cs-FCS added to final concentration of 40%. The stocks were aliquoted and stored at -80 ˚C until use.

HIV-1 IIIB (X4 tropic) were prepared from the chronically infected human $CD4^+$ T cell line H9 (AIDS Reagent Program, NIH, USA, catalogue #398). Cells were maintained in RPMI 1640 media containing 80% L-glutamine and 20% c-s FCS for 3 days. Supernatants were collected and concentrated using the Amicon Ultra-15 filtration system (Millipore, Billerica, US). The stocks were aliquoted and stored at -80 ˚C until use.

The TZM-bl TCID50 assay was used to determine viral titre of the stocks using the method previously described by Edmonds and colleagues (Edmonds *et al*., 2010). In brief, TZM-bl indicator cells were seeded at a density of 100,000 cells/well in 96-well plates in full phenol red DMEM [10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin] and incubated for 24 hours. The following day, an initial 1:5 dilution of the viral stocks was made in full phenol red-free DMEM [10% c-s FCS, 100 U/mL penicillin and 100 µg/mL streptomycin] and this was further diluted 8 more times, with each dilution done in quadruplicate in a separate 96-well plate. The media in the 96-well plate containing TZM-bl was removed and replaced with the diluted virus or no-virus control, with each dilution done in quadruplicate. The cells were then incubated for 72 hours after which they were harvested in 70 µL/well Bright-Glo™ Luciferase Assay System reagent (Promega, USA). Fifty (50) µL of the cell lysates was transferred onto white luminometer plates (Griener, Germany) and fluorescence [expressed as relative light units (RLU)] read on a Turner Biosystems Modulus Microplate reader (Promega, USA). Viral titres were determined as log infectious units (IU)/mL (Reed & Muench, 1938). The typical viral titre ranged from 1×10^4 - 4×10^5 IU/mL.

2.11.1: TZM-bl infection assay

The TZM-bl assay was used to investigate the effects of ligands on HIV-1 replication. In brief, TZM-bl indicator cells were seeded at a density of 10,000 cells/well in two 96-well plates in full phenol red DMEM [10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin] and incubated for 24 hours. Thereafter, the cells were washed one-time with PBS and exposed to 10 IU/mL HIV-$1_{BaL\text{-}Renilla}$ or virus control made up in full phenol red free DMEM [10% c-s FCS, 100 U/mL penicillin and 100 µg/mL streptomycin] for 24 hours. The following day, the cells were washed and stimulated with or without ligands in full phenol red-free DMEM. After 48 hours, cells in one plate were washed and lysed using 70 µL Bright-Glo™ Luciferase Assay System reagent (Promega, USA). Fifty (50) µL of the cell lysates was transferred onto white luminometer plates (Griener, Germany) and fluorescence [expressed as relative light units (RLU)] read on a Turner Biosystems Modulus Microplate reader (Promega, USA). Cells in the other plate were treated with 0.5 mg/mL MTT and incubated for another 2 hours. Thereafter, the media was aspirated and 100 µL acidified isopropanol to lyse the cells. The plate was read on a spectrophotometer (Thermo Scientific, USA) at 520 nm and 695 nm (background absorbance). Infectivity was expressed as RLU on MTT to account for differences in cellular viability over time.

2.11.2: Add back assay

The effects of supernatants from PBMCs treated with or without ligands on HIV-1 infection was assessed using TZM-bl indicator cells as described previously (Patel *et al*., 2014). Briefly, TZM-bl indicator cells were seeded at a density of 10,000 cells/mL in two 96-well plates in full phenol red

DMEM [10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin] and incubated for 24 hours. The following day, supernatants from PBMCs or control media with or without ligands stored at -80˚C were thawed and diluted 1:4 in full phenol red-free DMEM [10% c-s FCS, 100 U/mL penicillin and 100 µg/mL streptomycin] in a 24 well-plate. The cells were washed one-time with 1 X PBS and the culture media replaced with 100 µL of the diluted supernatants and control media and incubated for 1 hour at 37˚C. In parallel, 20 IU/mL HIV-$1_{BaL\text{-}Renilla}$ or virus control was added to 100 µL diluted supernatants and control media and incubated at 37˚C for 1 hour. The virus mixture was then added to the cells and incubated for another 48 hours. Thereafter, the cells were harvested as described in **section 2.11.1** above.

2.11.3: Exposure of genital epithelial to HIV-1 IMCs

2.11.3.1: PGECs

This experiment was performed in the laboratory of Prof. Charu Kaushic at McMaster University's Department of Pathology and Molecular Medicine, Hamilton, Canada. Confluent PGECs monolayers were pre-treated 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) in serum-free DMEM/F12 [100 U/mL Penicillin-Streptomycin, 10 mM HEPES, 250 µg/mL fungizone and 4 mM L-glutamine] for 24 hours. TER measurements were made before and after hormonal treatment. Thereafter, the monolayers were exposed to 10^5 IU/mL HIV-1 IIIB (X4 tropic) or virus control for 24 hours. Again, TER measurements were taken before and after HIV-1 exposure. Supernatants from the apical chamber were collected, inactivated with 5% (v/v) Empigen (Sigma-Aldrich, Canada) and centrifuged at 1,500 rpm for 10 minutes at 4°C using an Eppendorf 542R refrigerated microcentrifuge (Eppendorf, Canada). TNF-α protein levels determined by means of ELISA assays. For each ligand condition, post-exposure/treatment TER are normalised to pre-exposure/treatment TER and expressed as a percentage (% pre-exposure). The data presented in **Figure 4.3.7.2** was obtained by comparing TER post HIV-1 exposure to TER pre-hormonal treatment.

2.12: Confocal microscopy

Coverslips were prepared by washing twice in 3% (v/v) hydrochloric (HCl) acid with each wash lasting 30 minutes. This was followed by three 10-minute washes using Milli-Q water. The coverslips were then sterilised in absolute ethanol, rinsed several times with PBS and placed in 6-well plates. End1/E6E6 cells were seeded onto coverslips at a density 100,000 cells/mL in full SKM and grown for 10, 14, 16 and 18 days. Cells were harvested by washing with cold PBS, and then fixed and permeabilised using cold methanol for 10 minutes at -20˚C. The cells were washed three-times with PBS and then blocked with 5% (w/v) bovine serum albumin (BSA) in PBS for 1 hour at toom temperature. This was followed by staining with mouse monoclonal antibody raised against human

claudin-4 (1:100) made up in 5% (w/v) BSA in PBS for 1 hour at room temperature. The cells were washed three-times with 1% (w/v) BSA in PBS and stained with secondary anti-mouse-Cy3 conjugated antibody (diluted 1:1000, excitation maximal = 550 nm and emission maximal = 570 nm) in 5% BSA in PBS for 1 hour at room temperature in the dark. The cells were again washed three-times with 1% BSA and then counterstained with 100 µg/mL Hoechst in PBS for 5 minutes at room temperature. The coverslips were then mounted in Mowiol (Calbiochem, Merck, South Africa) containing the anti-fading agent n-propyl gallate (Sigma Aldrich, South Africa) and allowed to dry overnight at room temperature in the dark. The following day, the slides were rinsed with PBS, airdried and stored in the dark at 4˚C until visualisation. Images were acquired on a laser-scanning LSM 510 confocal microscope (Carl Zeiss, Germany) using the 40 X water-immersion objective, at the Confocal and Light Microscope Unit, University of Cape Town. Multiple z-stacks (0.1 µm) were acquired, exported as .tiff images and reconstructed using ImageJ (NIH). The representative middle slide of each stack is shown in XY (*en face*) projection. XZ projections were also generated to view the cell layer top to bottom.

2.13: Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 7 (GraphPad Software Inc, USA). Cell line, ectocervical tissues, PGECs, PBMCs or addback data were plotted as mean ± SEM. Dose response curses were plotted as Log [ligand] vs response with the maximal responses set to 100% and the Hill slope set to 1. Normality testing was performed on all data set using either Kolmogorov-Smirnov test for $n \geq 5$ or Shapiro-Wilk test for $n \geq 3$. This initial test was done to determine whether the data was geometrically distributed and hence the type of statistical analysis to be performed. Parametric data was analysed using One-Way ANOVA with post-hoc Tukey multiple comparison. Non-parametric data was analysed using Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison. When multiple comparisons failed to established statistical significance between treatments (as they are more stringent tests), less stringent pairwise comparisons was performed using either Mann-Whitney U test (when a ligand condition in the pair being compared was not set to 1 or 100%) or Kolmogorov-Smirnov test (when no condition in the pair being compared has been set to 1 or 100%) for non-parametric data, and the Student's t-test for parametric data if the overall ANOVA was significant. The following notations have been used in this thesis to differentiate between the various comparisons.

Table 2.3: Summary of statistical tests performed

Test	Notation	Explanation
One-Way ANOVA or Kruskal-Wallis	*	For multiple comparisons. Lines are used to indicate treatments being compared. Other comparison is between vehicle only and Steroids ± TNF/LPS
Unpaired Student's t-test or Mann-Whitney or Kolmogorov-Smirnov	#	For comparison between vehicle only and treatments –TNF/LPS
	$	For comparison between vehicle and treatments +TNF/LPS
	&	For comparisons between corresponding conditions in steroids - TNF/LPS and steroids +TNF/LPS

Chapter Three

Medroxyprogesterone acetate, like glucocorticoids but unlike other select progestins, regulates select tight junction and immune function target genes, including CCL20, alone and in combination with pro-inflammatory stimulators, in a gene-specific manner in an endocervical epithelial cell line

3.1: Aims

It has recently been reported that women with BV using DMPA-IM are more likely to acquire HIV-1 than their counterpart not usng hormonal contraception (Hadda *et al*., 2018). This suggests that DMPA-IM might cooperate with GTIs to further increase the risk of HIV-1 acquisition in women. The dysregulation of epithelial TJ and immune function genes is one of the several mechanisms by which HIV-1 infection can be established in the FGT (Nazli *et al*., 2010; Nazli *et al*., 2013; Quispe-Calla *et al*., 2016; Li *et al.*, 2009; Shang *et al*., 2017; Rollenhagen & Asin, 2011; Naranbhai *et al*., 2012; Kahle *et al*., 2015; Liebenberg *et al*., 2017). Therefore, the objectives of this chapter were to investigate aims **A** and **B** (**section 1.5**) with the following specific questions:

- Do GCs and progestogens regulate the expression of select TJ and immune function genes in female genital epithelial cells?
- Do GCs and progestogens cooperate with immune activators to regulate TJ and immune function gene expression in female genital epithelial cells?

The endocervical epithelial End1/E6E7 cell line was chosen because current evidence suggests that the endocervix is more readily infected with HIV-1 compared to mucosal surfaces of the lower FGT (Carias *et al*., 2016; Hladik & Hope, 2009). Unlike other endocervical epithelial cell lines, End1/E6E7 cells are non-cancerous and were generated by immortalising primary endocervical epithelial cells and so closely resemble their primary counterparts (Fichorova *et al*., 1997; Fichorova *et al*., 1999). The cells were treated with 100 nM GCs or select progestogens alone or in combination with 1.15 nM TNF or 5 µg/mL LPS. The concentrations of steroids used was chosen to reflect the highest peak serum concentration reported for MPA (Hapgood *et al*., 2018), although peak serum levels for DEX and endogenous CORT have been reported to be higher than 100 nM (Cho *et al*., 2017; Egerman *et al*., 1997). TNF and LPS, referred to as immune activators, were used to induce inflammatory responses like the type associated with microbial and non-HIV-1 viral GTIs, respectively. The concentration of TNF used has been shown to induce pro-inflammatory responses in End1/E6E7 cells (Verhoog et al., 2011) and most likely reflects the serum concentrations during infection. The concentration of LPS was chosen to reflect levels of LPS in CVL from women with bacterial vaginosis

((Aroutcheva *et al*., 2008). The cells were treated with ligands for 24 hours because they have been shown in previous studies to induce responses at this time point (Verhoog *et al*., 2011; Govender *et al*., 2014). Initial gene expression analysis was performed for the following TJ (claudin-4, occludin and ZO-1) and immune function genes (IL1β, IL6, IL8, CCL5, CCL20 and TLR2). These genes were selected because they have been shown to be relevant for HIV-1 infection (Nazli *et al*., 2010; Nazli *et al*., 2013, Bolduc *et al*., 2017; Cameron *et al*., 2010; Fichorova *et al*., 2015). Additional gene expression analysis was formed using the Human Host HIV-1 response RT^2 profiler to identify additional genes relevant for HIV-1 infection and pathogenesis that could be coregulates by progestogen and immune activators.

3.2: Results

3.2.1: DEX like MPA selectively represses basal and TNF-induced claudin-4, but not occludin and ZO-1 mRNA levels

First, the effects of DEX, MPA, P4, NET and LNG on claudin-4, occludin and ZO-1 mRNA levels were investigated. End1/E6E7 cells grown to confluence were treated with 100 nM of each hormone for 24 hours. As shown in **Figure 3.2.1.1**, treatment with DEX resulted in a significant reduction of claudin-4 (**Figure 3.2.1.1 A**) but not occludin (**Figure 3.2.1.1 B**) or ZO-1 (**Figure 3.2.1.1 C**) mRNA expression.

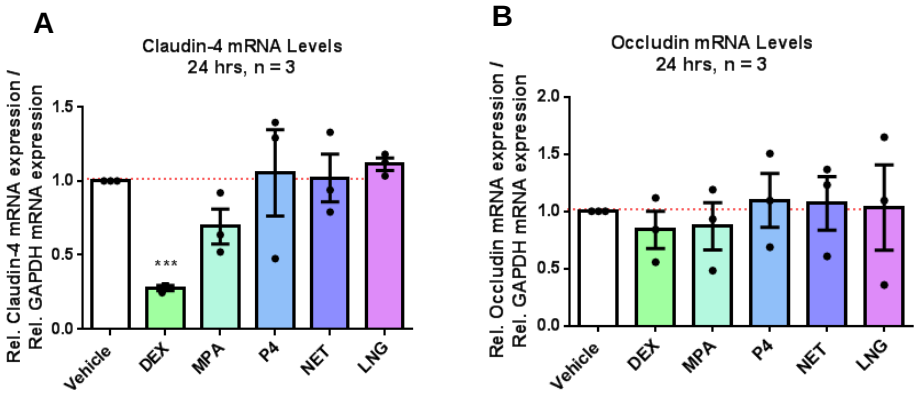

Figure 3.2.1.1: DEX differentially regulates claudin-4, occludin and ZO-1 mRNA expression in End1/E6E7 cells. (continued on the next page)

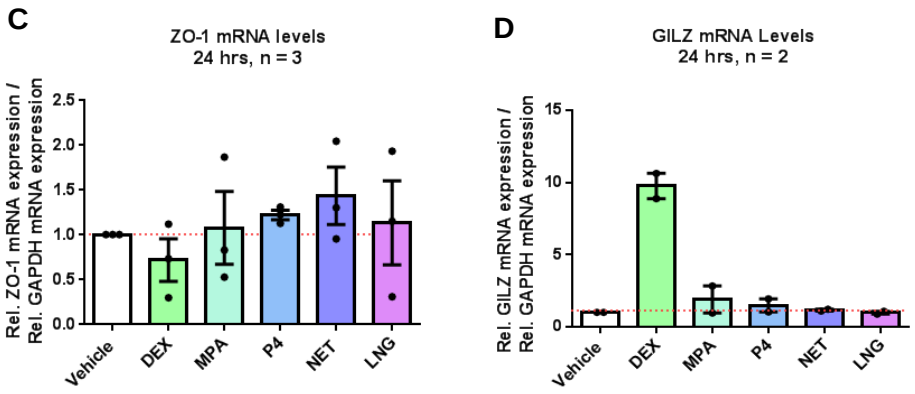

Figure 3.2.1.1: DEX differentially regulates claudin-4, occludin and ZO-1 mRNA expression in End1/E6E7 cells. End1/E6E7 cells grown to confluence were treated with 100 nM DEX, MPA, P4, NET LNG or 0.1% (v/v) EtOH (vehicle) for 24 hours. Cells were then harvested in Tri-Reagent®, total RNA isolated and 500 ng converted to cDNA. The relative mRNA levels of claudin-4 (**A**), occludin (**B**), ZO-1 (**C**) and GILZ (**D**) were determined by qRT-PCR performed using gene specific primers and normalised to GAPDH mRNA levels. The data include at least two independent biological repeats plotted as mean ± SEM in Graph Pad Prism 7 software. The data was plotted relative to vehicle only, which has been set to 1. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons and statistical significance denoted as *** to indicate $p<0.005$.

As expected, DEX also resulted in the induction of GILZ mRNA expression (**Figure 3.2.1.1 D**). Although not statistically significant, MPA appeared to suppress claudin-4, but not occludin or ZO-1 mRNA levels. The other ligands i.e. P4, NET and LNG did not alter mRNA levels of either claudin-4, occludin or ZO-1. Interesting, in this experiment neither MPA, P4, NET nor LNG significantly induced GILZ mRNA expression. Taken together, this result suggested only treatment with DEX and potentially MPA result in the selective downregulation of claudin-4 mRNA levels in End1/E6E7 cells. The other progestogens investigated i.e. P4, NET and LNG did not alter the expression of claudin-4, occludin and ZO-1 in End1/E6E7 cells.

The effects of DEX and MPA on the expression of TJ genes in presence of TNF were next investigated. In this experiment, confluent End1/E6E7 cells were treated with 100 nM DEX or MPA alone or in combination with 1.15 nM TNF for 24 hrs. As shown in **Figure 3.2.1.2**, both DEX and MPA significantly reduced claudin-4 mRNA levels but not occludin or ZO-1. TNF significantly upregulated claudin-4 and ZO-1, and also appeared to increase occludin mRNA levels although not significantly. While DEX and MPA significantly inhibited TNF-induced increase in claudin-4 mRNA expression, only DEX repressed TNF-induced increase in ZO-1 mRNA expression.

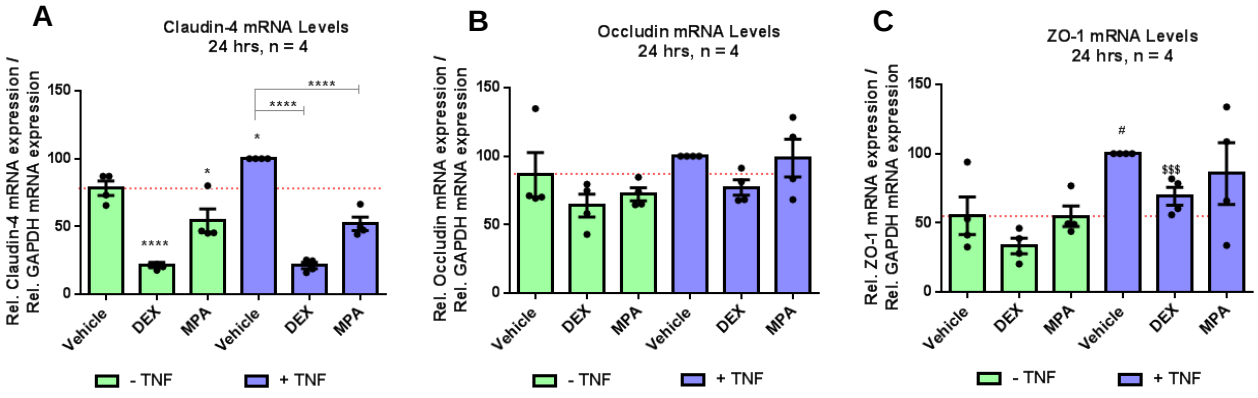

Figure 3.2.1.2.: MPA like DEX selectively suppress basal and TNF-induced claudin-4 mRNA expression in End1/E6E7 cells. Confluent End1/E6E7 cells were treated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF for 24 hrs. Cells were then harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of claudin-4 (**A**), occludin (**B**), ZO-1 (**C**) were measured by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. Fold changes in mRNA expression was determined by normalising the data relative to TNF only, which has been set to 100%. The data include four independent biological repeats plotted as mean ± SEM in Graph Pad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons. Statistical significance denoted as * and **** to indicate $p<0.05$ and $p<0.005$, respectively for comparisons between vehicle only and other treatments or between pairs of treatments. In some cases, the unpaired Student's t-test was used for comparisons between vehicle only and other treatments and statistical significance is denoted by $^{\#}$ to indicate. When the test compared TNF alone with TNF + steroids statistical significance is denoted by $^{\$\$\$}$ to indicate $p<0.005$.

3.2.2: DEX is more effective than MPA, NET, P4 and LNG in regulating claudin-4 and occludin protein levels in End1/E6E7 cells.

In the previous sections, it was established that DEX and MPA, unlike P4, NET and LNG, decreased claudin-4, but not occludin mRNA levels. It was next investigated whether the hormones also modulate claudin-4 and occludin protein levels.

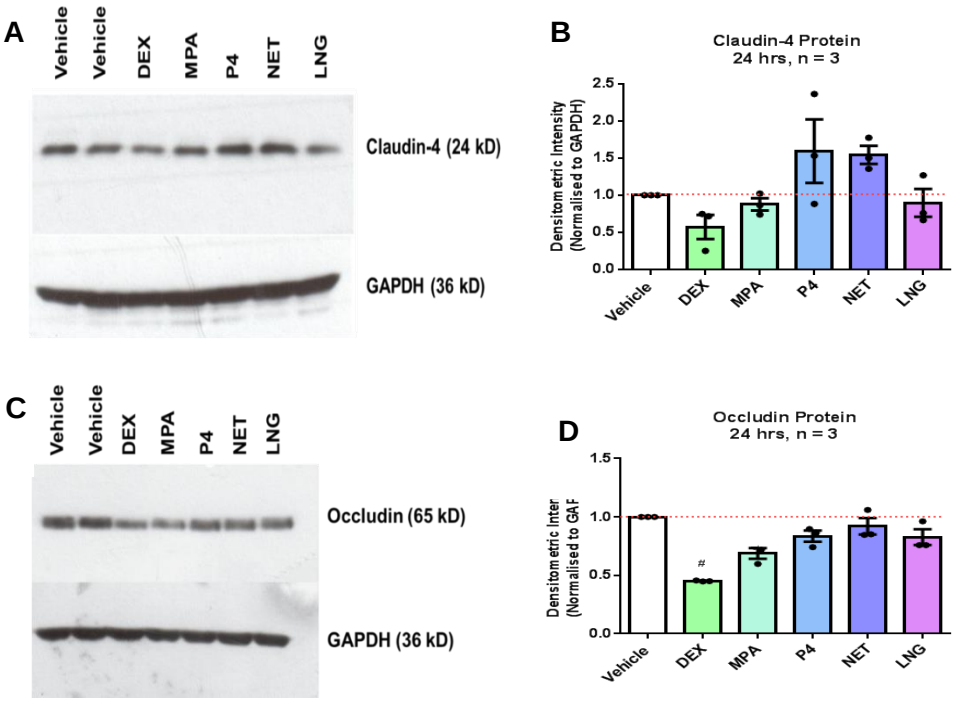

Figure 3.2.2.1: The effects of DEX and select progestogens on claudin-4 and occludin protein levels in End1/E6E7 cells. End1/E6E7 cells grown to confluence were treated with 100 nM DEX, MPA, P4, NET, LNG or 0.1% (v/v) EtOH (vehicle) for 24 hrs. Thereafter, cells were harvested in 2X SDS Loading Buffer, boiled for 10 minutes and resolved on a 10% SDS-PAGE. Thereafter western blot analysis was performed with antibodies specific for the claudin-4 (**A**, 24KD), occludin (**C**, 65KD) and GAPDH (36KD). The blots were then scanned, densitometrically quantified and expressed relative to vehicle control. (**A**, **C**): representative immunoblot from three biological repeats; (**B**, **D**): densitometry data plotted as mean ± SEM in Graph Pad Prism 7 software. **B** was analysed using one-way ANOVA with Tukey's multiple comparisons. **D** was analysed using the unpaired Kolmogorov-Smirnov test with statistical significance denoted as # to indicated $p<0.05$.

As shown in **Figure 3.2.2.1 A**, only treatment with DEX but not MPA, P4, NET or LNG appeared to decrease claudin-4 protein levels. Similarly, only DEX significantly reduced occludin protein levels (**Figure 3.2.2.1 B**). MPA appeared to reduce the occludin protein levels, although the effect was not statistically significant. Neither P4, NET nor LNG showed any significant effect on claudin-4 and occludin protein levels (**Figure 3.2.2.1 B**).

Next, it was also investigated whether DEX can alter claudin-4 and occludin protein levels in the presence of TNF. **Figure 3.2.2.2** shows that DEX significantly reduced claudin-4 (**Figure 3.2.2.2 A**) and occludin (**Figure 3.2.2.2 B**) protein levels.

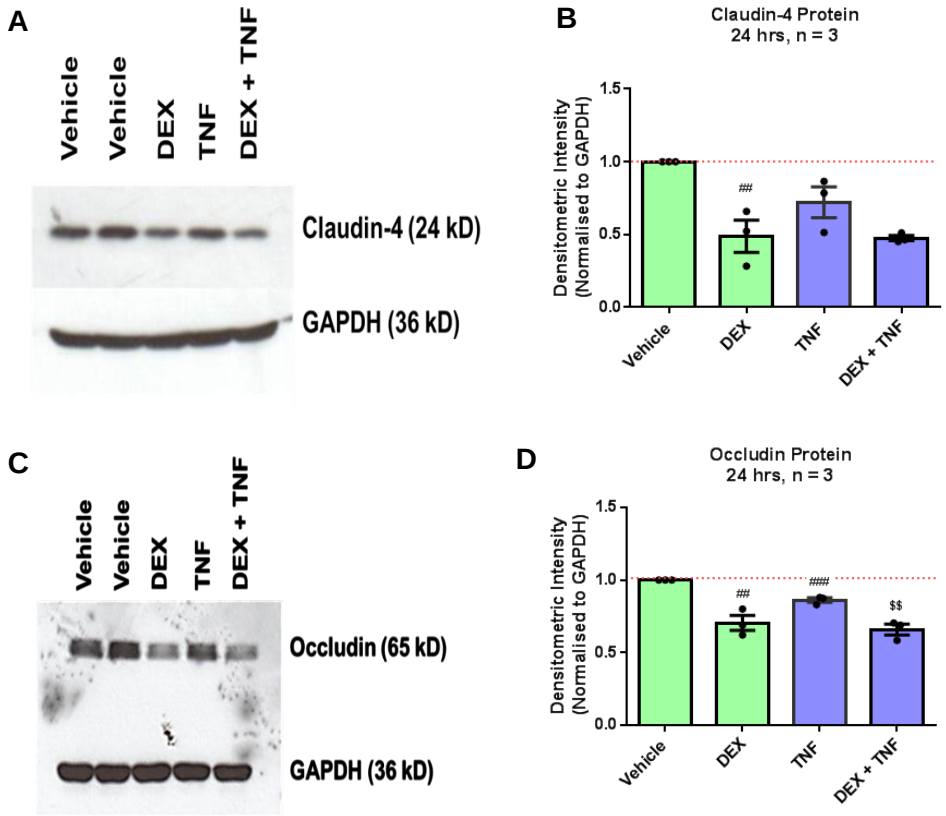

Figure 3.2.2.2: The effect of DEX on claudin-4 and occludin protein levels in End1/E6E7 cells in the absence and presence of TNF. End1/E6E7 cells grown to confluence were treated with 100 nM DEX or 0.1% (v/v) EtOH (vehicle) in the absence or presence of 1.15 nM TNF for 24 hrs after which cells were harvested and thereafter analysed by western blotting using antibodies specific for the claudin-4 (**A**, 24KD), occludin (**C**, 65KD) and GAPDH (36KD). The blots were then scanned, densitometrically quantified and expressed relative to vehicle control (NB: the average of the two vehicle control samples was used to normalise the data). (**A**, **C**): representative immunoblot from three biological repeats; (**B**, **D**): densitometry data plotted as mean ± SEM in Graph Pad Prism 7 software. Statistical analysis was performed using the unpaired Student's t-test and statistical significance denoted as $^{\#\#}$ or $^{\#\#\#}$ to indicate $p<0.01$ and $p<0.005$, respectively for comparison between vehicle and other treatments; or denoted as $^{\$\$}$ to indicate $p<0.01$ for comparison between TNF and TNF + steroids

TNF also appeared to reduce claudin-4, but significantly decreased occludin protein levels (**Figure 3.2.2.2**). This observation contrasted with the effect of TNF on claudin-4 and occludin mRNA levels, in which TNF increased claudin-4 mRNA levels and appeared to do the same with occludin mRNA levels (**Figure 3.2.1.2**). Co-treatment with DEX and TNF resulted in a decrease in claudin-4 and occludin protein levels, but the response was like that elicited by DEX alone.

Taken together, these results suggest DEX regulates mRNA and protein levels of select TJ genes. They also suggest that TNF increases mRNA levels of select TJ genes, but at the same time reduces

protein levels of the same genes. Moreover, the results suggest that MPA like DEX inhibits TNF-induced increase in TJ gene expression but has no effect on TNF-induced decrease in TJ protein levels.

3.2.3: MPA like GCs, but unlike NET, enhances basal and TNF-induced CCL20 mRNA expression, but supresses basal and TNF-induced IL6, IL1β, IL8 and CCL5 mRNA expression in End1/E6E7 cells.

Next, DEX, CORT, MPA and NET-mediated regulation of basal and induced CCL20, IL6, IL1β, IL8, CCL5 and GILZ mRNA expression in End1/E6E7 cells was investigated. To this end, End1/E6E7E6/E7 cells were treated with steroid hormones alone or in combination with TNF for 24 hours (co-stimulation – see **Figure 3.2.3.1**). TNF was used to induce inflammation. In the absence of TNF, treatment with DEX, CORT, MPA, but not NET resulted in a significant increase in CCL20 mRNA expression (**Figure 3.2.3.1 A**). In contrast, DEX, CORT and MPA, but not NET significantly inhibited basal IL6 mRNA expression (**Figure 3.2.3.1 B**). In addition, DEX, CORT and possibly MPA, but not NET reduced IL1β mRNA expression (**Figure 3.2.3.1 C)**. However, all four steroids did not induce any statistically significant changes in basal IL8 and CCL5 mRNA expression (**Figure 3.2.3.1 D, E**). It was also observed that DEX, CORT and potentially MPA, but not NET increased GILZ mRNA levels (**Figure 3.2.3.1 F**).

Treating End1/E6E7 cells with TNF resulted in increased CCL20, IL6, IL1β, IL8 and CCL5 mRNA levels (**Figure 3.2.3.1 A - E**). However, the effects of DEX, CORT, MPA and NET on TNF-induced gene expression were not always similar as some gene-specific differences were observed. For instance, DEX, CORT and potentially MPA, but not NET enhanced TNF-induced increase in CCL20 mRNA levels (**Figure 3.2.3.1 A**).

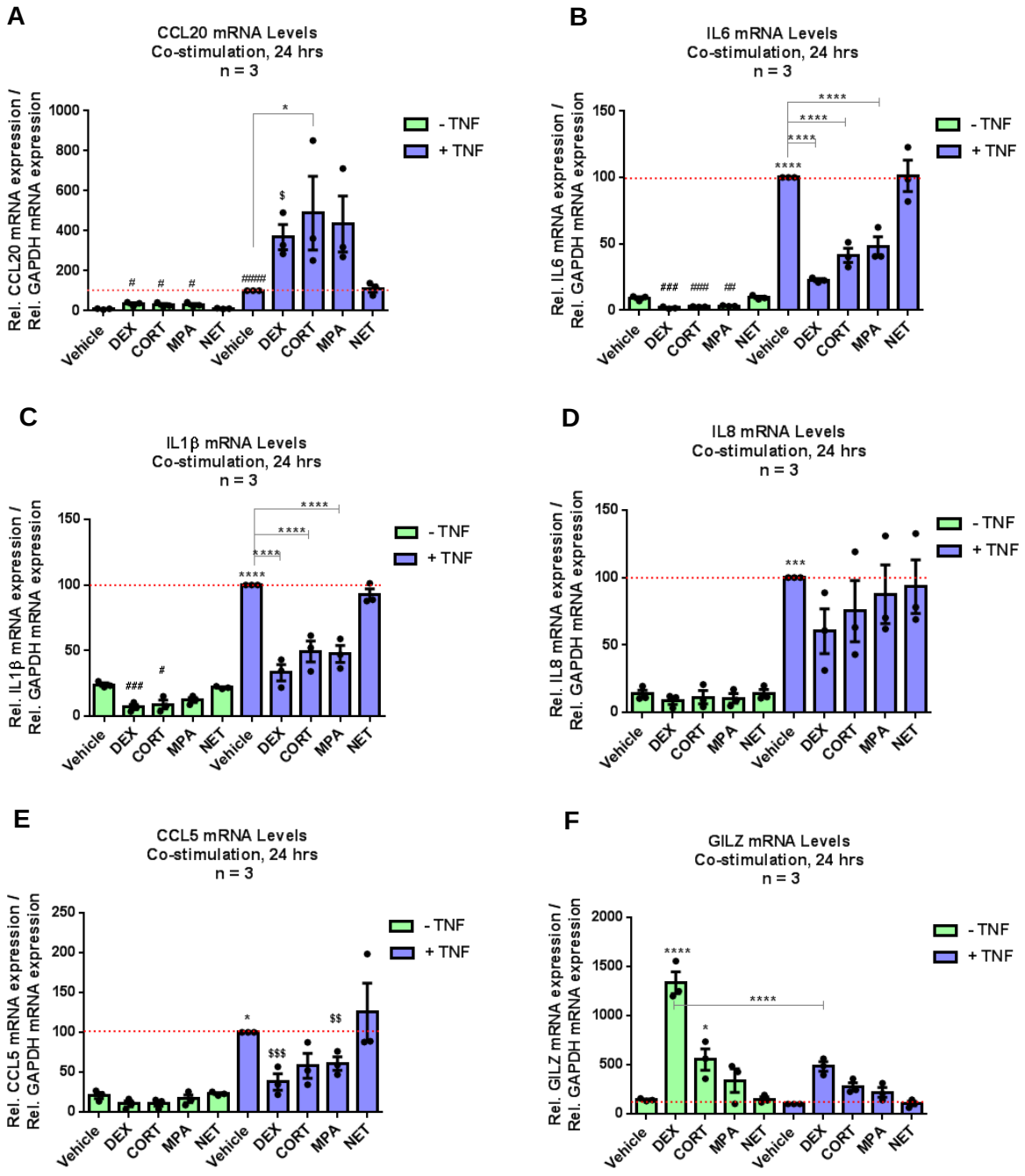

Figure 3.2.3.1: DEX, CORT and MPA, unlike NET, selectively upregulate CCL20 and GILZ, but repress IL6, IL1β, IL8 and CCL5 mRNA levels in End1/E6E7 cells in the presence of TNF. End1/E6E7 cells were stimulated with 100 nM DEX, CORT, MPA, NET or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM human TNF for 24 hours. Thereafter, cells were harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A**), IL6 (**B**), IL1β (**C**), IL8 (**D**), CCL5 (**E**) and GILZ (**F**) were then determined by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroids and TNF on mRNA levels of the selected genes were determined by normalising to the vehicle only control, which has been set to 100%. The data include three independent biological repeats plotted as mean ± SEM in Graph Pad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons. Statistical significance is denoted as *, *** or **** to indicate $p<0.05$, $p<0.005$ and $p<0.0001$, respectively for comparisons between vehicle only and

other treatments or between pair of treatments. In some cases, the unpaired Student's t-test was used for comparisons between vehicle only and other treatments and statistical significance is denoted as $^{\#}$, $^{\#\#\#}$, or $^{\#\#\#\#}$ to indicate $p<0.05$, $p<0.005$ and $p<0.0001$, respectively. When the comparison is between TNF and steroids + TNF statistical significance is denoted as $^{\$}$, $^{\$\$}$ or $^{\$\$\$}$ to indicate $p<0.05$, $p<0.01$ and $p<0.005$, respectively.

In contrast, DEX, CORT and MPA, but not NET inhibited IL6 and IL1β MRNA regulation by TNF (**Figure 3.2.3.1 B, C**). Similarly, TNF-induced CCL5 mRNA expression seemed to be inhibited by DEX, MPA and potentially CORT, but not NET; on the other hand, all four steroid hormones did not alter TNF-induced increase in IL8 mRNA levels (**Figure 3.2.3.1 D, E**). It was also observed that TNF significantly inhibited DEX- and CORT-induced increase in GILZ mRNA expression despite not showing a similar effect on basal GILZ expression (**Figure 3.2.3.1 F**).

Because treatment with DEX, CORT, MPA and NET did not alter TNF-induced expression of IL8, it was next investigated whether pre-treatment with hormones prior to TNF challenge would result in changes in findings reported in **Figure 3.2.3.1** above. In these experiments, End1/E6E7E6/E7 cells were initially pre-treated with steroid hormones for 2 hours before stimulating with TNF for another 24 hours (hormonal pre-treatment – see **Figure 3.2.3.2**).

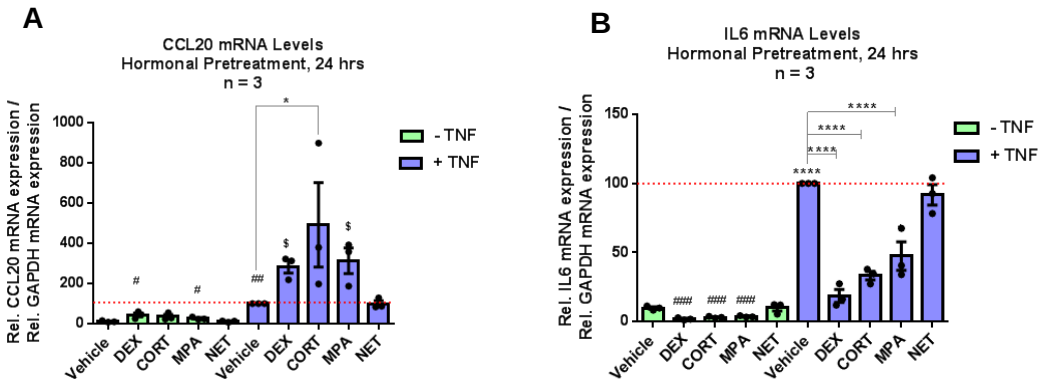

Figure 3.2.3.2: MPA like GCs, but not NET enhance TNF-induced CCL20 mRNA levels, but supress TNF-induced IL6, IL8 and IL1β mRNA levels in End1/E6E7E6/E7 cells. (continued on the next page)

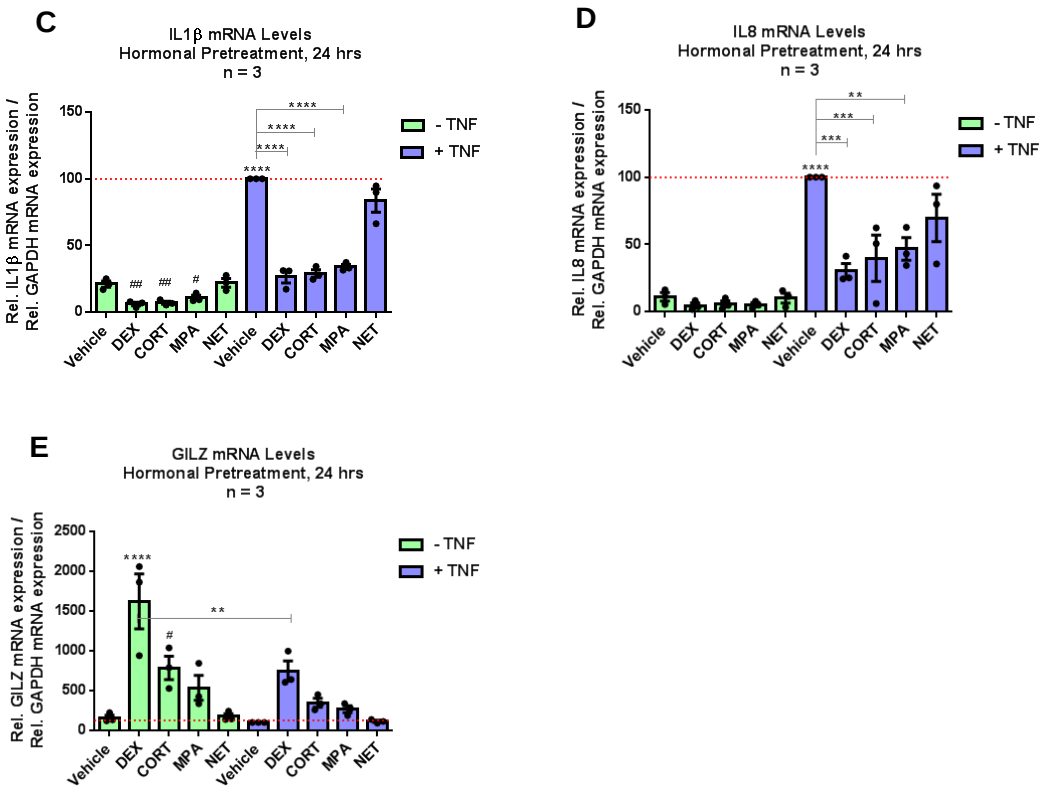

Figure 3.2.3.2: MPA like GCs, but not NET enhance TNF-induced CCL20 mRNA levels, but supress TNF-induced IL6, IL8 and IL1β mRNA levels in End1/E6E7E6/E7 cells. End1/E6E7 cells were pre-treated with 100 nM DEX, CORT, MPA, NET or 0.1% (v/v) EtOH (vehicle) for 2 hours and then stimulated with or without 1.15 nM human TNF for another 24 hours. Thereafter, cells were harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A**), IL6 (**B**), IL1β (**C**), IL8 (**D**) and GILZ (**E**) were then determined by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones and TNF on mRNA levels of the selected genes were determined by normalising to the vehicle only control, which has been set to 100%. The data include three independent biological repeats plotted as mean ± SEM in Graph Pad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons and statistical significance denoted as *, **, *** or **** to indicate $p<0.05$, $p<0.01$, $p<0.005$ and $p<0.0001$, respectively for comparisons between vehicle only and other treatments or between pair of treatments. In some cases, the unpaired Student's t-test was used for comparisons between vehicle only and other treatments, and statistical significance is denoted by $^{\#}$, $^{\#\#}$, or $^{\#\#\#}$ to indicate $p<0.05$, $p<0.01$ and $p<0.005$, respectively. When the comparison was between TNF and TNF + steroids, statistical significance is denoted by $^{\$}$ to indicate $p<0.05$.

It was observed that DEX, CORT and MPA, unlike NET, significantly enhanced TNF-induced CCL20, but suppressed TNF-induced IL6, IL1β and IL8 mRNA expression (**Figure 3.2.3.2 A – D**). As seen in **Figure 3.2.3.1 F** above, treatment with TNF repressed DEX- and CORT-, but not MPA-induced expression of GILZ (**Figure 3.2.3.2 E**).

Taken together, the results suggest that the effects of MPA like GCs, unlike NET, on mRNA expression in End1/E6E7 cells are gene-specific. They show that MPA like GCs, but unlike NET, increase basal as well as TNF-induced expression of a pro-inflammatory gene (CCL20). The results also suggest that MPA like GCs selectively suppressed basal as well as TNF-induced expression of some pro-inflammatory gene. Some gene could only be repressed by MPA and GCs in the presence of TNF. Moreover, some like IL8 was only repressed by MPA and GCs after the cells were pre-treated with the steroids prior to TNF stimulation. These results suggest that the regulation of pro-inflammatory gene expression by MPA is more complex them previously thought. Overall, there were no remarkable differences between the co-stimulation and hormonal pre-treatment protocols. Therefore, the co-stimulation method was used in subsequent experiments.

It was next investigated whether the regulation of gene expression by MPA, DEX and NET in End1/E6E7 cells was time-dependent. The cells were treated with steroids alone or in combination with TNF for 4, 24 and 48 hours. The results indicate that DEX- and MPA-induced enhancement of TNF-induced CCL20 mRNA expression might occur as early as 4 hours and is sustained 24 hours later; however, only MPA appeared to enhance TNF-induced increase in CCL20 expression at 48 hours (**Appendix A**, **Figure 1 A**). It was also observed that NET might not exhibit any time-dependent effects on TNF-induced CCL20 mRNA expression. Using IL6 as a model for transrepression, it was observed that DEX appeared to repress TNF-induced IL6 mRNA expression as early as 4 hours and this repression was sustained at 48 hours. However, neither MPA nor NET appeared to regulate IL6 mRNA expression in these experiments (**Appendix A**, **Figure 1 B**). However, because these effects were not statistically significance, caution should be taken in interpreting these results.

Next, the Human Host HIV-1 Response RT^2 Profiler PCR array (Qiagen, USA) was used to identify additional genes that are relevant to HIV-1 infection and pathogenesis coregulated by TNF and MPA in End1/E6E7 cells. Because CCL20 was not included in the panel, RNA samples used in the PCR array were assessed to verify that MPA and TNF coregulated CCL20 mRNA levels (**Appendix A**, **Figure 2**). As shown in **Appendix A** (**Table 1**) (which shows pooled data drawn from three independent experiments), most of the genes constituting the array appeared not to be regulated by MPA or TNF. However, 19 genes (22.62% of the array) appeared to show some form of regulation with either MPA or TNF. A gene was said to be regulated if it had a fold change ≥ 1.5 for any of the ligand treatment conditions.

As shown in **Appendix A** (**Figure 3 A – C**), MPA alone did not alter basal levels of most genes evaluated herein, but for CCL5 and IL1β that appeared to be suppressed. In contrast, TNF

significantly induced IL2, TNF, TNF receptor 2 (TNFRSF1B), interferon regulatory factor (IRF)1, IRF2, killer cell lectin-like receptor D (KLRD)1, NFKB inhibitor alpha (NFKB1A) and serine protease inhibitor alpha (SERPINA)1 expression. It was also observed that MPA significantly enhanced TNF-induced expression of IRF2. Although not statistically significant, MPA also appeared to enhance TNF-induced increase in IRF1, NFKBIA, TNFRSF1B, TNF, SERPINA1, IL1β, CCR4, CD69 and SLPI. Additionally, co-stimulation with MPA and TNF significantly upregulated STAT3 expression despite neither ligand altering its basal expression. Although MPA appeared to suppress basal IL1β and CCL5 expression, this apparent repressive effect was lost for IL1β, but retained for CCL5 in the presence of TNF (**Appendix A**, **Figure 3 A - C**). Hierarchical clustering was performed to assess the overall effect of MPA on TNF-induced activation of gene expression. This analysis revealed that in most cases MPA either enhanced or did not alter TNF-induced responses (**Appendix A**, **Figure 4 D**).

Furthermore, qRT-PCR was used to validate the PCR array data. Fresh RNA samples were isolated by Tri-Reagent® method for this analysis. By comparing the results obtained by qRT-PCR to those obtained in the array, some discrepancies were observed. For instance, the array predicted that MPA and TNF significantly coregulated IFR2 expression (**Appendix A, Figure 3 A**). In addition, the array predicted that MPA and TNF might coregulate IRF1, NFKBIA, SERPINAI, TNFRSF1B, IL1β, and TNF expression (**Appendix A**, **Figure 3 A – D**). However, the qRT-PCR validation showed that MPA repressed TNF-induced IL1β, IRF2 and potentially IRF1 expression (**Figure 3.2.3.3 A, E, F**). A combination of small responses induced by the ligands coupled with high technical error in the array experiment could explain these discrepancies.

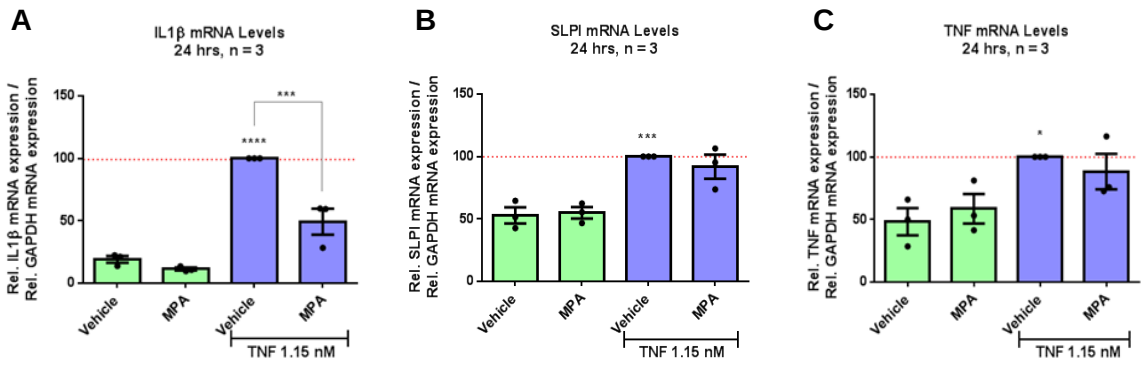

Figure 3.2.3.3: MPA selectively enhances TNF-induced TNFRSF1B expression in End1/E6E7 cells. (continued on the next page)

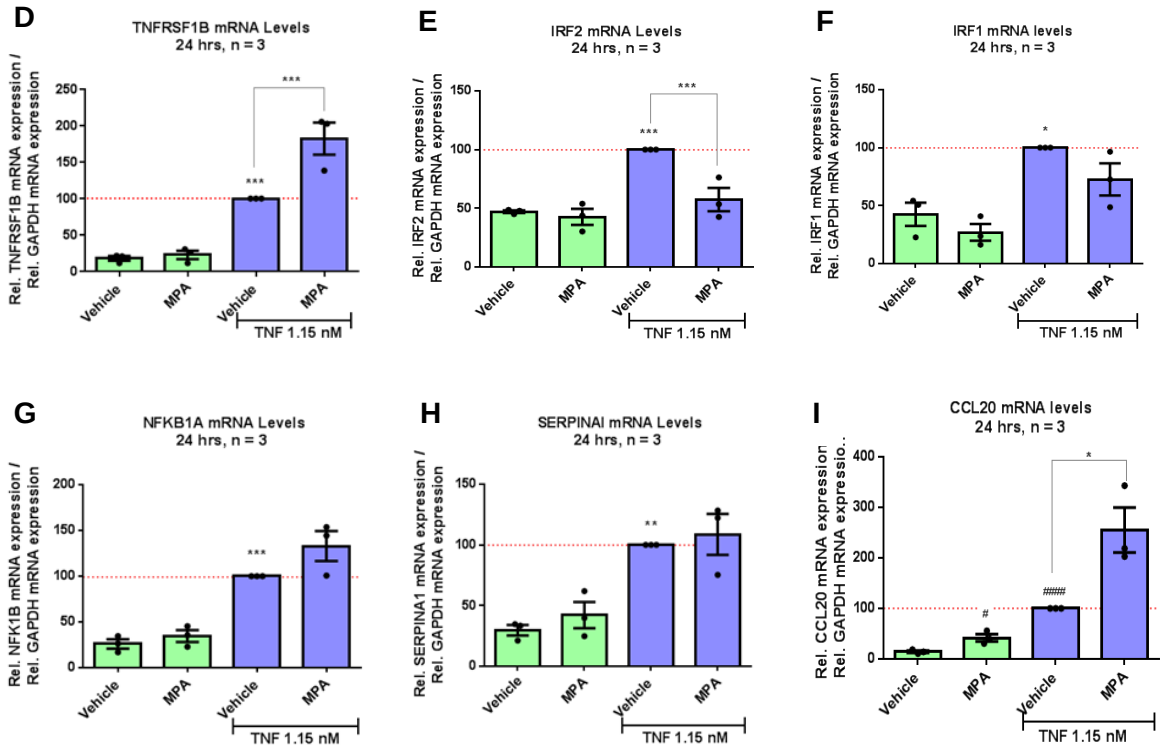

Figure 3.2.3.3: MPA selectively enhances TNF-induced TNFRSF1B expression in End1/E6E7 cells. End1/E6E7 cells were treated with 100 nM MPA or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF for 24 hours. Thereafter, total RNA was isolated using RNeasy Mini kit (Qiagen, USA), assessed for integrity by Bioanalysis and then converted. The relative mRNA levels of IL1β (**A**), SLPI (**B**), TNF (**C**), TNFRSF1B (**D**), IRF2 (**E**), IRF1 (**F**), NFKB1A (**G**), SERPINAI (**H**) and CCL20 (**I**) were determined by qRT-PCR performed using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of MPA, TNF or combo on mRNA levels of the selected genes were determined by normalising to vehicle only control, which has been set to 100%. The data include three independent biological repeats pooled and plotted as mean ± SEM in Graph Pad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons and statistical significance denoted as *, ** or *** to indicate $p<0.05$, $p<0.01$ and $p<0.005$, respectively for comparisons between vehicle only and other treatments or between pair of treatments. In some cases, the unpaired Student's t-test was used for comparisons between vehicle only and other treatments, and statistical significance is denoted as $^{\#}$ and $^{\#\#\#\#}$ to indicate $p<0.05$ and $p<0.0001$, respectively.

In addition, the validation showed that MPA did not regulate TNF-induced SLPI, TNF and SERPINAI expression (**Figure 3.2.3.3 B, C, H**). However, the validation established that MPA potentiates TNF-induced expression of TNFRSF1B and maybe NFKBIA (**Figure 3.2.3.3 D, G**). STAT3 could not be validated due to inability of primers to detect the gene. Because the samples used in this analysis were not the same as those used in the array, a CCL20 confirmatory run was performed. This showed that MPA and TNF upregulated CCL20 expression (**Figure 3.2.3.3 I**). Despite the discrepancies between the array and qPCR validation, this analysis could identify another gene co-regulated by

MPA and TNF in End1/E6E7 cells. Moreover, of all the genes tested, MPA only potentiates TNF-induced expression of TNFRSF1B and CCL20.

3.2.4: MPA like DEX, but unlike NET, selectively enhances basal TLR2, GILZ and LPS-induced CCL20, suppress LPS-induced IL6, IL8 and IL1β, but does not alter basal TLR4 mRNA expression in End1/E6E7 cells.

In **Section 3.2.3,** it was established that GCs and MPA, unlike NET, can positively cooperated with TNF to enhance CCL20 mRNA expression while retaining their ability to repress TNF-induced IL6, IL8 and IL1β mRNA expression in End1/E6E7 cells. Because TNF signals via NFκB activation, it was therefore investigated whether GC and MPA can also positively cooperate with other NFκB-activating ligands such as LPS to upregulate CCL20 and not IL6, IL8 and IL1β gene expression in End1/E6E7 cells. To this end, End1/E6E7 cells were treated with 100 nM DEX, MPA or NET alone or in combination with 5 µg/mL LPS for 24 hours.

As shown in **Figure 3.2.4.1 A - D**, LPS treatment significantly increased CCL20, IL6, IL1β and potentially IL8 mRNA levels. Concurrent treatment with DEX enhanced CCL20; suppressed IL6 and IL1β; and, appeared to inhibit IL8 expression in response to LPS (**Figure 3.2.4.1 A – D**). Although not statistically significant, MPA appeared to enhance CCL20 expression in response to LPS. In addition, MPA significantly suppressed LPS-induced IL6 and IL1β expression. Furthermore, MPA seemed to inhibit LPS-induced expression of IL8, but this effect was not statistically significant (**Figure 3.2.4.1 A – D**). Unlike DEX and MPA, NET did not affect LPS-induced induction of CCL20, IL6, IL8 or IL1β mRNA expression in End1/E6E7 cells. DEX and MPA, but not NET increased GILZ mRNA levels. While LPS inhibited the DEX-mediated increase in GILZ mRNA levels, it however did not alter MPA-induced GILZ mRNA expression (**Figure 3.2.4.1 E**).

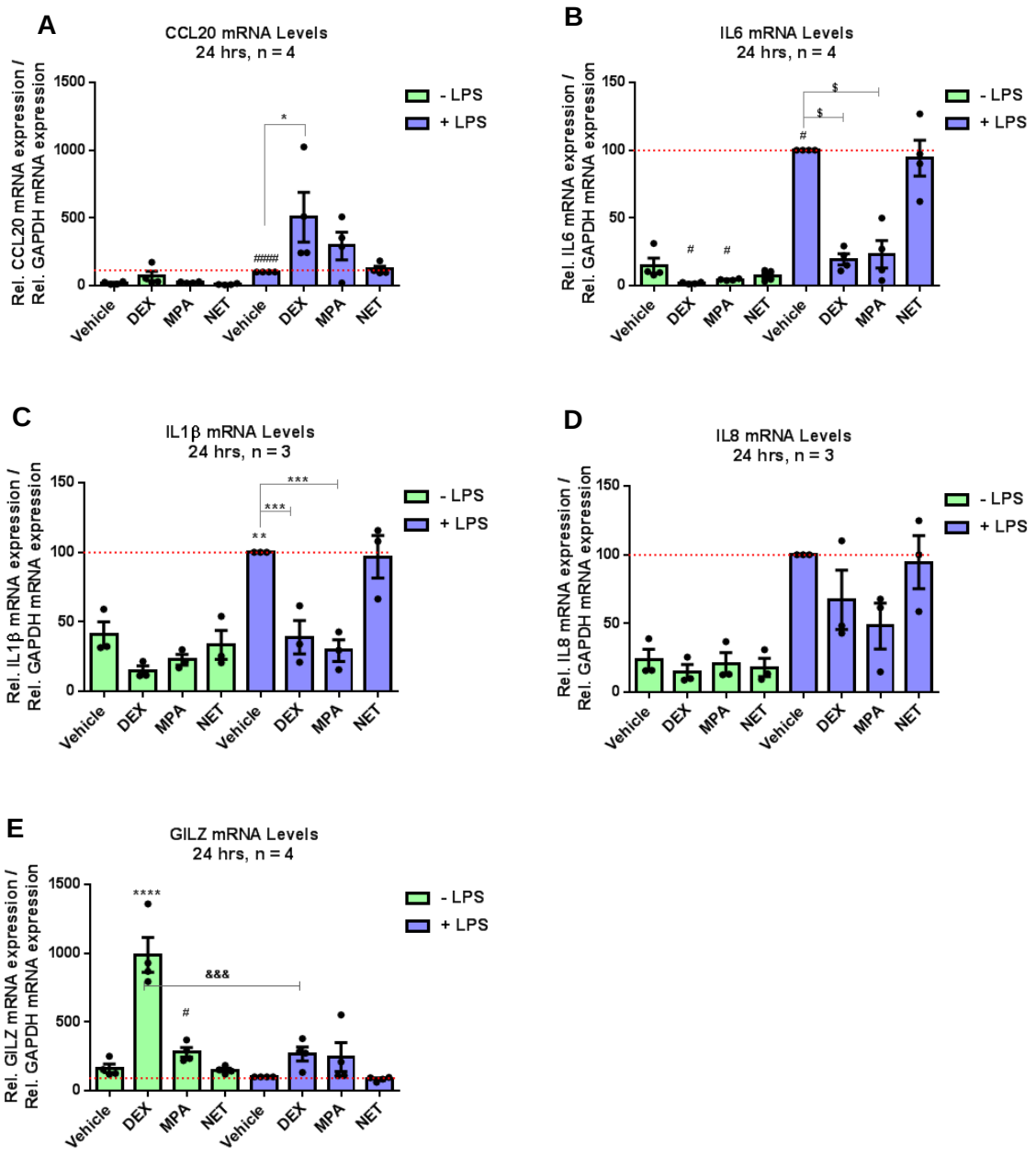

Figure 3.2.4.1: MPA like DEX, but not NET, regulates LPS-induced mRNA expression of select inflammatory genes in a ligand- and gene-specific manner in End1/E6E7 cells. End1/E6E7 cells were treated with 100 nM DEX, MPA, NET or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 5 µg/mL LPS for 24 hours**.** Thereafter, cells were harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A**), IL6 (**B**), IL1β (**C**), IL8 (**D**) and GILZ (**E**) was determined by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones and LPS on mRNA levels of the selected genes were determined by normalising to LPS, which has been set to 100%. The data represent at least three independent biological repeats plotted as mean ± SEM in Graph Pad Prism 7 software. For data that was parametric (**A, C, E**), statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons. Statistical significance denoted as *, **, *** and **** to indicate $p<0.05$, $p<0.01$, $p<0.005$ and $p<0.0001$, respectively for comparisons between vehicle only and other treatments or between other pair of treatments. In some cases, the unpaired Student's t-test was used for comparison between vehicle only and other treatments with statistical significance is denoted as $^{\#}$ and $^{\#\#\#}$ to indicate $p<0.05$

and p<0.005, respectively. When the comparison was between other pair of treatments statistical significance is denoted as $^{\&\&\&}$ to indicate p<0.005. Non-parametric data (**B**, **D**) were analysed using the Mann-Whitney U-test for comparisons between vehicle only and other treatments with statistical significance denoted as $^{\#}$ to indicate p<0.05, or the Kolmogorov-Smirnov test for comparison between LPS and LPS + steroids with statistical significance denoted as $^{\$}$ to indicate p<0.05.

Previous studies have shown that GCs can co-operate with immune activators to upregulate TLR2 and TLR4 mRNA levels (Shibata *et al*., 2009; Zhang *et al*., 2017; Su *et al.,* 2017), receptors via which LPS mediates it biological effects (Yang *et al*., 1998). LPS has been shown to induce the expression of these receptors in human monocytes (Ma & Yang, 2010), but it is currently unknown whether these receptors can coregulated by GCs and LPS. However, it is currently controversial whether endocervical epithelial cells express TRL4 and thus respond to LPS (Fichorova *et al*., 2002; Herbst-Kralovetz *et al*., 2008; Nold *et al*., 2012). Therefore, it was next investigated whether DEX, MPA or NET regulate TLR2 and TRL4 mRNA expression in the presence or absence of LPS in End1/E6E7 cells.

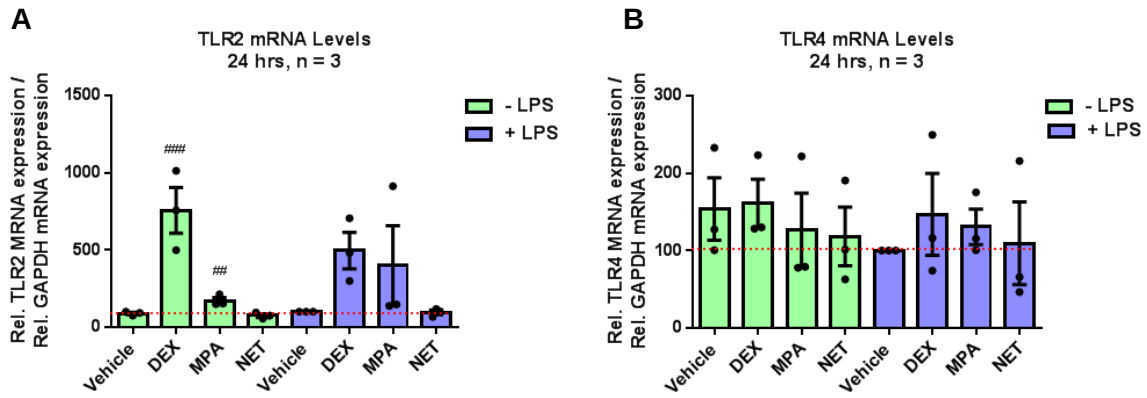

Figure 3.2.4.2: MPA and DEX unlike NET increases TRL2 but does not alter TRL4 mRNA levels in the absence or presence of LPS in End1/E6E7 cells. End1/E6E7 cells were treated with 100 nM DEX, MPA, NET or 0.1% (v/v) EtOH (vehicle) in the absence or presence of 5 µg/mL LPS for 24 hours. The cells were then harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of TRL2 (**A**) and TRL4 (**B**) were determined by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones and LPS on the mRNA levels of TLR2 and TLR4 were determined by normalising to LPS only, which was set to 100%. The data include three independent biological repeats plotted as mean ± SEM in Graph Pad Prism 7 software. Statistical analysis was performed using the unpaired Student's t-test with significance denoted as $^{\#\#}$ or $^{\#\#\#}$ to indicate p<0.01 and p<0.0001, respectively.

In the absence of LPS, MPA like DEX, but not NET, significantly increased TRL2 mRNA levels in End1/E6E7 cells after 24 hours (**Figure 3.2.4.2.A**). In contrast, neither DEX, MPA nor NET regulated TRL4 mRNA levels (**Figure 3.2.4.2 B).** This result suggests that the MPA- or DEX-induced increase in TRL2 levels might be involved in the upregulation of CCL20 mRNA expression in End1/E6E7 cells in the presence of LPS. Unlike MPA and DEX, LPS did not regulate TRL2 and TLR4 mRNA levels in End1/E6E7 cells. The results suggest that LPS does not need to upregulate the expression of TLR2, TLR4 or both receptors in order to enhance its biological effects in End1/E8E7 cells. They also suggest that End1/E6E7 cells express TLR4 and respond to treatment with LPS. Differences in primers, concentration of LPS, and laboratory-specific changes in the properties of End1/E6E7 cells could explain the inconsistency between this study and Fichorova *et al* (2002).

Taken together, these results suggest that MPA like DEX, but not NET, can modulate LPS-mediated transcription of immune mediators in End1/E6E7 cells; positively cooperating with LPS to upregulate CCL20 mRNA expression, but antagonising LPS-induced IL6, IL1β and potentially IL8 mRNA expression. These results also suggest that LPS inhibits GILZ expression in response to DEX and MPA.

3.2.5: MPA increases TNF- and LPS-induced CCL20 protein secretion by End1/E6E7 cells.

Considering that DEX and MPA, unlike NET, enhanced TNF- and LPS-induced CCL20 mRNA expression, it was next determined whether similar changes could be observed in CCL20 protein levels. CCL20 ELISA assays were performed using supernatants collected from experiments described in **Figures 3.2.3.1** and **3.2.4.1**.

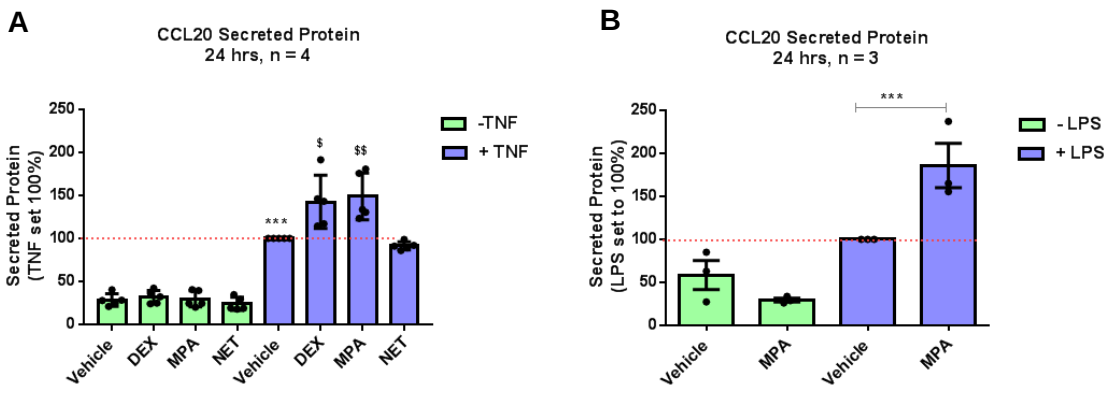

Figure 3.2.5: MPA potentiates both TNF- and LPS-mediated secretion of CCL20 protein by End1/E6E7 cells. End1/E6E7 cells were treated with 100 nM DEX, MPA, NET or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF (**A**) or 5 µg/mL LPS (**B**) for 24 hrs. Thereafter, supernatants were

collected and CCL20 ELISA assay was performed to quantify the concentration of secreted CCL20. The data include at least three independent biological repeats pooled and plotted as mean ± SEM in Graph Pad Prism 7 software. For comparisons, the data has been plotted relative to TNF (**A**) or LPS (**B**), which are both set to 100%. Statistical analysis was performed using either one-way ANOVA with Tukey's multiple comparisons. Statistical significance is denoted as *** to indicate $p<0.005$ for comparisons between vehicle only and other treatments or between pair of treatments. In some cases, the unpaired Student's t-test for comparison between TNF and TNF + steroids with statistical significance denoted as $^{\$}$ or $^{\$\$}$ to indicate $p<0.05$ and $p<0.01$, respectively.

As shown in **Figure 3.2.5 A**, treatment with TNF resulted in a significant increase in CCL20 secreted protein. In contrast, neither DEX, MPA nor NET altered basal CCL20 protein secretion. This was a surprising observation as DEX and MPA caused an increase in CCL20 mRNA expression. Interestingly, co-treatment of cells with DEX or MPA and TNF induced a stronger secretion of CCL20 protein than TNF alone. Although not statistically significant, treating End1/E6E7 cells with LPS resulted in an increase in CCL20 protein secretion (**Figure 3.2.5 B**). MPA again significantly enhanced LPS-induced CCL20 protein secretion (**Figure 3.2.5 B**).

Taken together, these results suggest DEX and MPA can potentiate TNF or LPS-mediated secretion of CCL20 protein in End1/E6E7 cells as found for mRNA. However, the protein expression data contrasted with the mRNA data in that neither DEX nor MPA induced basal CCL20 protein secretion.

3.2.7: Summary of Findings

In this chapter, incubations with 100 nM steroids were performed, and steroid dose-dependency was not investigated. The results show that MPA like DEX, but unlike NET, LNG or P4, downregulates claudin-4 mRNA levels, but not occludin and ZO-1 mRNA levels in End1/E6E7 cells. However, only DEX significantly downregulated occludin protein levels. TNF was found to induce claudin-4, ZO-1 and potentially occludin mRNA expression and DEX more so than MPA inhibited the TNF-induced response. Despite increasing their mRNA levels, TNF was found to reduce occludin and potentially claudin-4 protein levels. DEX did not regulate TNF-induced reduction of occludin and claudin-4 protein levels.

Results in this chapter also established that MPA like GCs, but unlike NET, differentially regulates basal and TNF- or LPS-stimulated expression of CCL20, IL6, IL1β, IL8 and CCL5 mRNA in End1/E6E7 cells. More specifically, it was established that in the absence of immune activators MPA

like GCs, but not NET, enhanced CCL20 mRNA expression, but suppressed basal IL6 and IL1β mRNA expression, and did not alter basal IL8 and CCL5 mRNA expression. In addition, it was shown that MPA like GCs, but unlike NET, selectively enhanced CCL20 mRNA expression, but suppressed IL6, IL1β and IL8 expression in the presence of TNF or LPS. The results further show that MPA like DEX can enhance TNF- or LPS-induced secretion of CCL20 protein secretion by End1/E6E7 cells. In addition, it was observed that MPA like DEX, but unlike NET, induced TLR2 mRNA expression in End1/E6E7 cells. While TLR2 was not co-regulated by MPA and LPS, it was found that MPA can enhance TNF-induced expression of TNF receptor type 2 (TNFRSF1B).

Taken together, MPA like GCs regulates the basal expression of specific tight junction and immune function genes in End1/E6E7 cells, but only cooperate with immune activators to upregulate the expression of select immune function but not tight junction genes.

Chapter Four

The glucocorticoid receptor mediates the potent partial agonistic MPA-induced downregulation of claudin-4 gene expression as well as the gene-specific and synergistic interactions between MPA and immune activators that upregulate CCL20 gene expression in the endocervical epithelial cell line

4.1: Aims

In the previous chapter, it was established that MPA, unlike other progestins, downregulates select TJ genes, and positively cooperates with immune activators to upregulate CCL20, but not IL6 expression in the endocervical epithelial End1/E6E7 cell line. An intriguing question is whether this can occur at physiologically relevant concentrations and what steroid receptor is involved. Therefore, the objectives of this chapter were to investigate aims **C** and **D** (**section 1.5**) with the following specific questions:

- What are the efficacies, potencies and biocharacters of GCs and progestogens for regulation of gene expression?
- Do GCs and progestogens act synergistically with immune activators to regulate gene expression?
- Is the observed regulation dependent on the GR?

These questions were investigated in End1/E6E7 cells because they have been shown to express the GR but not the PR (Govender *et al*., 2014). The role of the GR in the regulation of gene expression by GCs and progestogens was investigated using 1 µM RU486. Because the test steroids were used at a concentration of 100 nM, it was predicted that about a ten-fold higher concentration of RU486 to out-compete the test steroids for receptor binding, given that the affinity is similar or greater than DEX for the GR. GR knockdown by siRNA was used in some specific experiments to obtain further proof. Dose response analysis was performed to determine the efficacies, potencies and biocharacter of progestogens relative to DEX, a synthetic GR ligand on the expression of claudin-4, occludin and GILZ. This was done in the absence of TNF as neither DEX nor progestogens coregulated TJ gene expression in End1/E6E7 cells. Additional dose-response assessment was performed to determine the efficacies, potencies and biocharacter of MPA relative to CORT in the absence or presence of TNF on the expression of CCL20, IL6 and GILZ. To determine whether MPA like CORT synergised with TNF to upregulate CCL20, End1/E6E7 cells were treated as described in **section 4.2.3**. The biocharacters of MPA were initially compared to DEX, but subsequent comparisons were done

relative to CORT as the latter is more physiologically relevant and women using DMPA have been shown to have high serum CORT levels (Virutamasen *et al*., 1986).

4.2: Results

4.2.1: MPA is a partial agonist in repressing claudin-4 and occludin and upregulating GILZ mRNA levels in End1/E6E7 cells

Dose-response analyses were performed to determine the potency (EC_{50}), efficacy (maximal response) and biocharacter of DEX and MPA in regulating claudin-4, occludin and GILZ mRNA levels in End1/E6E7 cells. These analyses revealed that the potencies of DEX and MPA in repressing claudin-4 mRNA levels were 4.35 nM and 14.27 nM, respectively (**Figure 4.2.1 A, B**). It was also observed that MPA like DEX inhibited occludin mRNA expression in a dose-dependent manner with potencies of 131.2 nM and 8.56 nM, respectively (**Figure 4.2.1 C, D**). Furthermore, MPA like DEX increased GILZ mRNA levels in a dose-dependent manner with potencies of 36.07 nM and 12.16 nM, respectively. Compared to DEX, the efficacies of MPA for repressing claudin-4 and occludin mRNA expression were 53.83% and 29.01%, respectively; while its efficacy for inducing GILZ mRNA expression was 33.12%. This suggest that MPA is partial agonist for transrepression and transactivation in End1/E6E7 cells (Ronacher *et al*., 2009). Neither NET nor P4 regulated claudin-4 and occludin mRNA levels even at higher doses (**Appendix B, Figure 1**). Taken together, these results demonstrate that DEX is more potent and efficacious than MPA in suppressing claudin-4 and occludin mRNA expression, and in inducing GILZ expression in End1/E6E7 cells. They also show that claudin-4 is much more sensitive than occludin to repression by DEX or MPA.

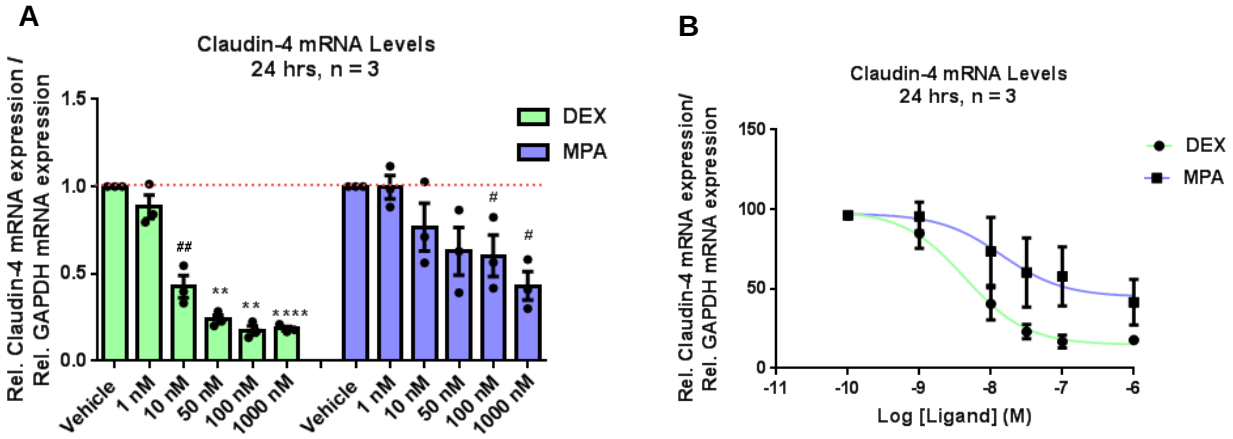

Figure 4.2.1: The regulation of claudin-4 and occludin mRNA levels by DEX and MPA in End1/E6E7 cells occurs in a dose-dependent manner. (continued on the next page)

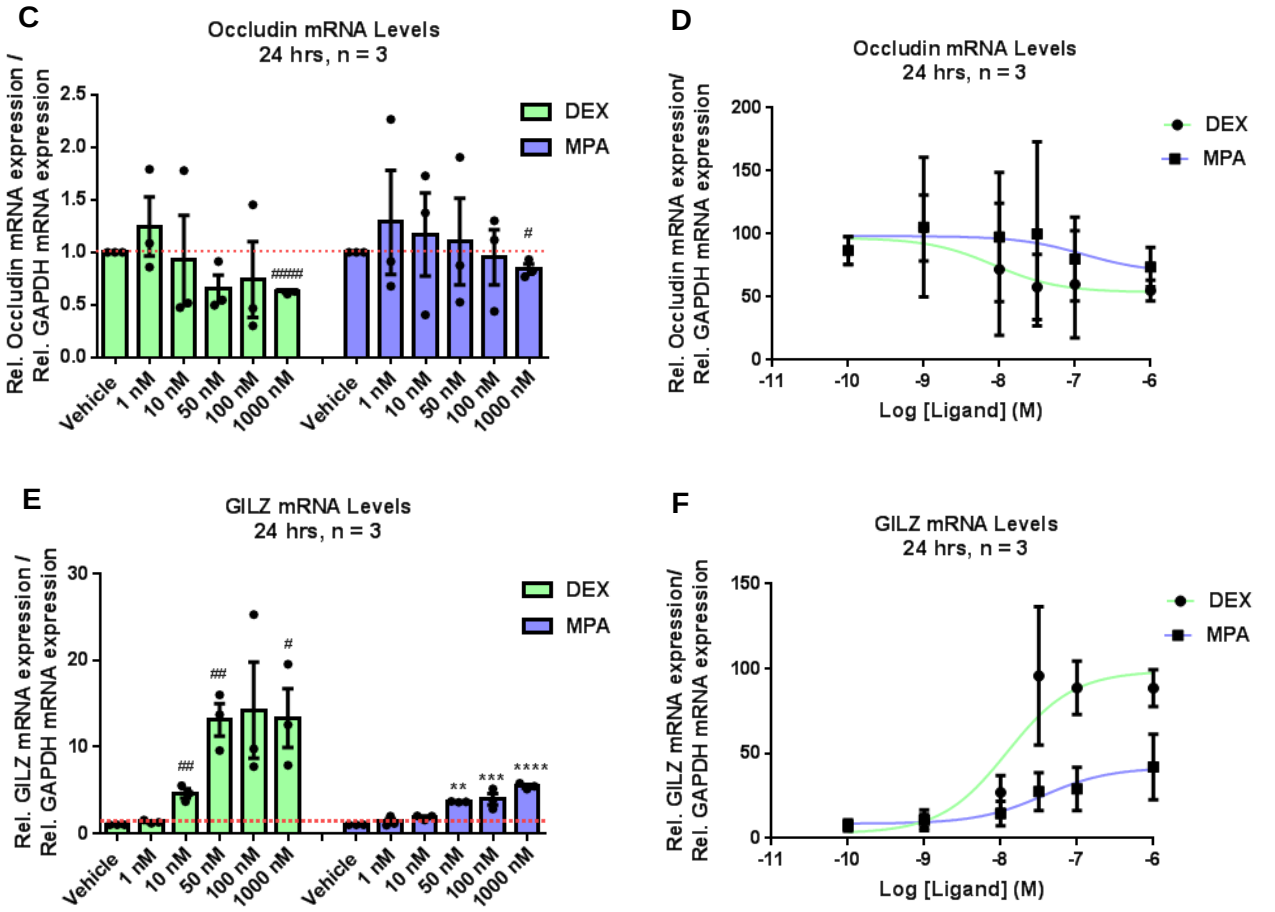

Figure 4.2.1: The regulation of claudin-4 and occludin mRNA levels by DEX and MPA in End1/E6E7 cells occurs in a dose-dependent manner. Confluent End1/E6E7 cells were stimulated with increasing concentrations of DEX or MPA for 24 hours. Thereafter, the cells were harvested in Tri-Reagent®, total RNA isolated and converted to cDNA and relative mRNA levels of claudin-4 (**A**), occludin (**C**), GILZ (**E**) were determined by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones on mRNA levels of the selected genes were determined by normalising to own vehicle, which is set to 1. For dose-response curve generation, data shown in (**A**), (**C**) and (**E**) was re-normalised by setting maximal response generated by DEX to 100%. Curves for claudin-4 (**B**), occludin (**D**) and GILZ (**F**) were plotted using a non-linear regression model, plotting log agonist vs response, with the Hill slope set to 1. The data include at least three independent biological repeats plotted as mean ± SEM in GraphPad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons and statistical significance denoted as **, ***, or **** to indicate p<0.01, p<0.005 and p<0.0001, respectively. In some cases, the unpaired Student's t-test was used for comparisons and statistical significance was denoted as #, ## and #### to indicate p<0.05, p<0.01 and p<0.0001, respectively.

4.2.2: The repression of claudin-4 mRNA expression by DEX and MPA occurs via the GR

The steroid receptor antagonist RU486 was used to verify the involvement of the GR in the repression of claudin-4 mRNA levels by DEX and MPA in End1/E6E7 cells**.** Previous studies have shown that

RU486 is not a GR-specific antagonist as it can also antagonise the PR (Beck *et al*., 1993). In this regard, Western blot analysis was performed to check for the expression of the GR and PR in End1/E6E7 cells. As shown in **Appendix B Figure 2**, End1/E6E7 cells expressed detectable levels of the GR, but not the PR. This result confirmed previous findings by Govender *et al* that End1/E6E7 cells do not express the PR (Govender *et al*., 2014). Govender and colleagues further showed that End1/E6E7 cells also do not express detectable levels of the MR, AR and ER protein (Govender *et al*., 2014). Taken together, this result implied treating End1/E6E7 cells with RU486 would result in the inhibition of only GR-regulated pathways.

Next, the involvement of the GR in the DEX- and MPA-induced reduction of claudin-4 mRNA levels in End1/E6E7 cells was investigated. Confluent End1/E6E7 cells were pre-treated for 2 hours with 1 µM RU486 and then exposed to 100 nM DEX or MPA for a further 24 hours. As shown in **Figure 4.2.2**, DEX- and MPA-induced reduction in claudin-4 mRNA levels were significantly inhibited in the presence of RU486. This result shows that the GR is involved in the reduction of claudin-4 mRNA levels in End1/E6E7 cells by DEX and MPA.

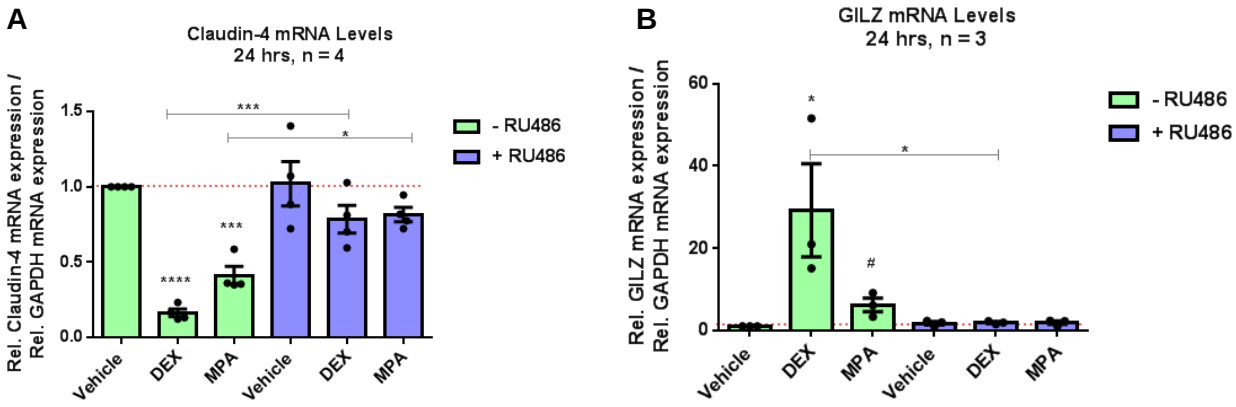

Figure 4.2.2: The glucocorticoid receptor is required for DEX- and MPA-induced reduction in claudin-4 mRNA levels in End1/E6E7 cells. Confluent End1/E6E7 cells were pre-treated with 1 µM RU486 or 0.1% (v/v) EtOH (vehicle) for 2 hours and thereafter stimulated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) for another 24 hours. Cells were then harvested in TriReagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of claudin-4 (**A**), GILZ (**B**) were determined by qPCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones in the presence or absence of RU486 were determined by normalising to the vehicle only control, which has been set to 1. The data includes at least three independent biological repeats plotted as mean ± SEM in GraphPad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons and statistical significance denoted as *, *** or **** to indicate $p<0.05$, $p<0.005$ and $p<0.0001$, respectively. In some cases, the unpaired Student's t-test was used to compare vehicle only with other ligand conditions and statistical significance is denoted as $^{\#}$ to indicate $p<0.05$.

Further experiments to verify the requirement of the GR for DEX- and MPA-induced repression of claudin-4 mRNA production were performed. In these experiments, End1/E6E7 cells were transiently transfected with 20 nM scramble or GR5 siRNA for 48 hours and then treated with 100 nM DEX or MPA for another 24 hours. As shown in **Appendix B Figure 3**, GR knockdown only slightly lifted the DEX- and not the MPA-induced repression of claudin-4 mRNA production. The overall percentage of knockdown achieved was approximately about 50%. Under this condition, it is possible that there was still enough GR in the cells to mediate the repressive effects of DEX and MPA on claudin-4 mRNA expression. However, it remains unclear why DEX-induced repression of claudin-4 and not MPA is sensitive to reduced GR levels, given that both DEX- and MPA-induced expression of GILZ was partially inhibited. Taken together, these results suggest that GR is required for the DEX- and MPA-induced repression of claudin-4 mRNA levels in End1/E6E7 cells. In addition, the GR also mediates DEX- and MPA-induced expression of GILZ in End1/E6E7 cells.

4.2.3: MPA like CORT dose-dependently regulates CCL20, IL6 and GILZ mRNA levels in End1/E6E7 cells

In **Chapter 3**, it was established that GCs and MPA enhanced CCL20, but repressed IL6 mRNA expression in the absence or presence of TNF in End1/E6E7 cells. It was also observed that TNF antagonised GC- and MPA-induced expression of GILZ. Because these effects were observed at 100 nM GC or MPA and 1.15 nM TNF, it was investigated whether lower doses of the ligands could differentially regulate CCL20, IL6 and GILZ. To this end, End1/E6E7 cells were treated for 24 hours with;

1. Increasing concentrations of TNF in the absence or presence of 100 nM MPA or CORT
2. Increasing concentrations of MPA or CORT in the absence or presence of 1.15 nM TNF

The panel of genes were reduced to GILZ, IL6 and CCL20 to represent transactivation, transrepression and transactivation with positive cooperativity models, respectively. Furthermore, the steroid hormones were also reduced to MPA and CORT in order to compare MPA with a natural GC. NET was excluded because if failed to induce any noticeable responses at 100 nM, a concentration that is higher than serum concentrations in women using NET as hormonal contraceptive.

4.2.3.1: Increasing TNF and constant MPA or CORT

Compared with vehicle control, TNF appeared to increase CCL20 mRNA levels in a dose-dependent manner (**Figure 4.2.3.1 A**). Doses as low as 0.057 nM appeared to induce CCL20 mRNA expression. The addition of MPA appeared to enhance TNF-induced increase in CCL20 mRNA levels. For

instance, CCL20 mRNA levels induced by 0.057 nM TNF rose from 18.81 in the absence of MPA to 59.56-fold in the presence of MPA (**Figure 4.2.3.1 A**). In addition, TNF significantly induced IL6 mRNA expression in a dose-dependent manner – an effect blocked by MPA (**Figure 4.2.3.1 B**). Although not statistically significant, it was observed that TNF suppressed basal and MPA-induced increase in GILZ mRNA expression (**Figure 4.2.3.1 C**). Concentrations of TNF as low as 0.057 nM might reduce basal GILZ and MPA-induced mRNA expression.

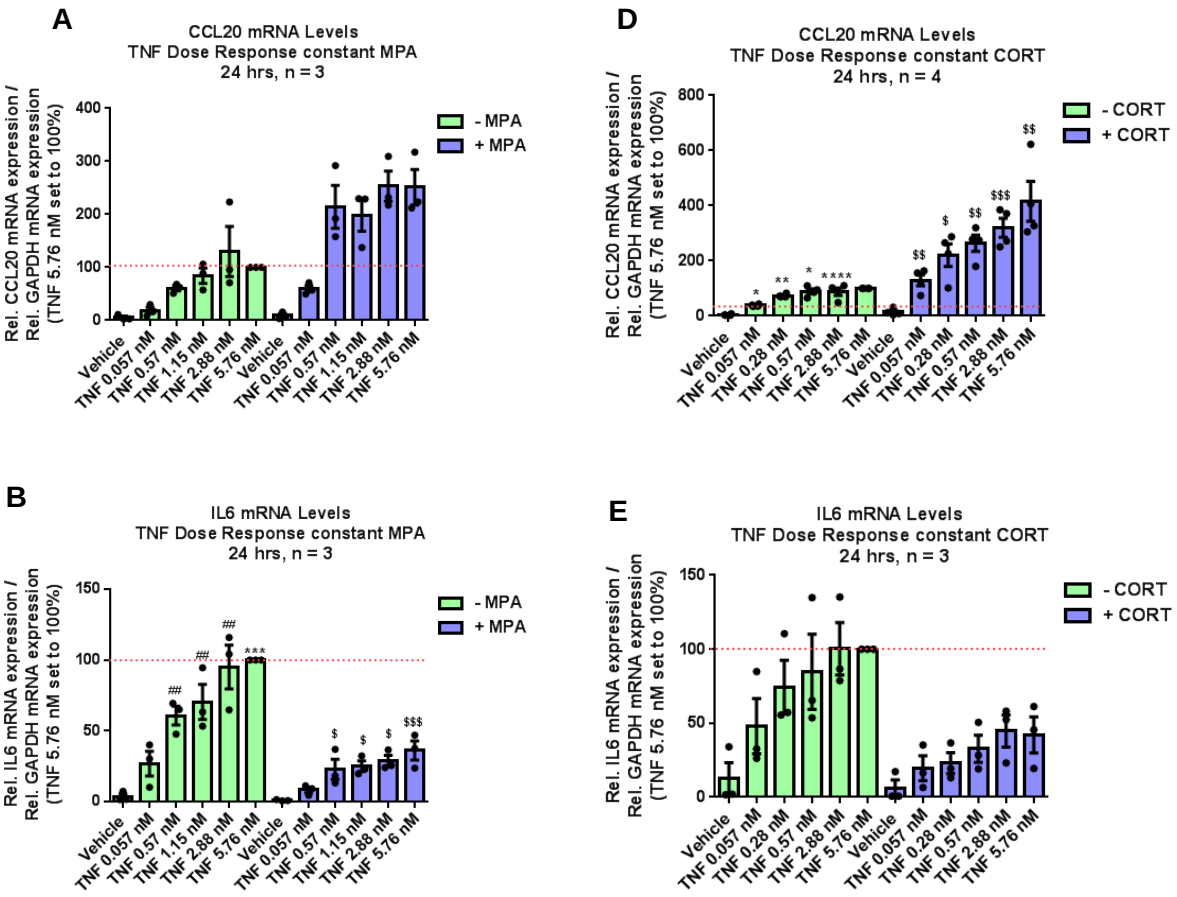

Figure 4.2.3.1: The regulation of CCL20, IL6 and GILZ mRNA levels by TNF in the absence or presence of MPA or CORT in End1/E6E7 cells is dose-dependent. (continued on the next page)

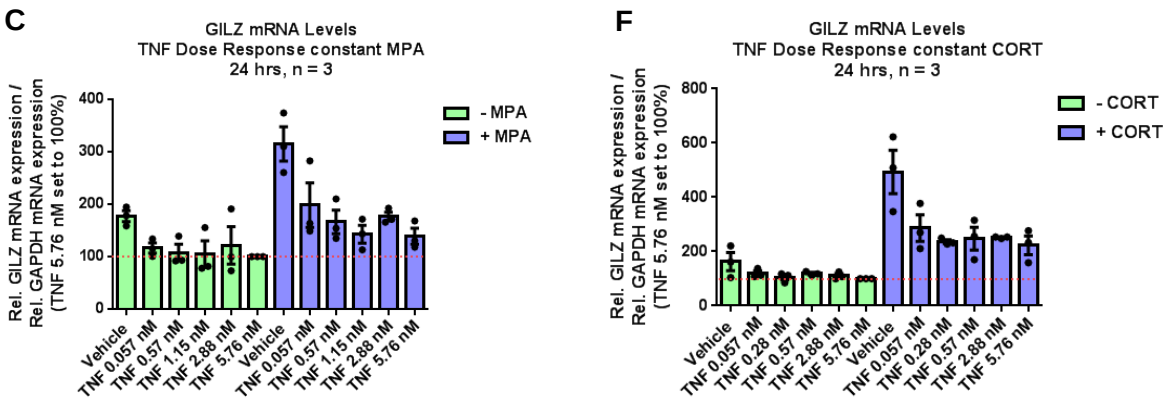

Figure 4.2.3.1: The regulation of CCL20, IL6 and GILZ mRNA levels by TNF in the absence or presence of MPA or CORT in End1/E6E7 cells is dose-dependent. End1/E6E7 cells were stimulated with increasing concentrations of human TNF in the presence or absence of 100 nM MPA for 24 hours. In another non-parallel experiment, the cells were stimulated with increasing concentrations of human TNF in the presence or absence of 100 nM CORT for 24 hours the Thereafter, cells were harvested in Tri-Reagent®, total RNA was isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A, D**), IL6 (**B, E**), GILZ (**C, F**) were determined by qPCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of each ligand and ligand combinations on mRNA levels of the selected genes were determined relative to TNF 5.76 nM, which was set to 100%. The data include at least three independent biological repeats pooled and plotted as mean ± SEM in GraphPad Prism 7 software. (**B, D**) was analysed using one-way ANOVA with Tukey's multiple comparison. Statistical significance is denoted as *, ** or *** to indicate $p<0.05$, $p<0.01$ and $p<0.005$, respectively for comparison between vehicle only and other treatments. In some cases, the unpaired Student's t-test was used for comparisons between vehicle only and other treatments and statistical significance denoted as $^{\#}$, $^{\#\#}$ or $^{\#\#\#\#}$ to indicate $p<0.05$, $p<0.01$ and $p<0.0001$, respectively. When the comparison is between corresponding TNF doses in the presence or absence of MPA statistical significance is denoted as $^{\$}$, $^{\$\$}$ or $^{\$\$\$}$ to indicate $p<0.05$, $p<0.01$ and $p<0.005$, respectively. (**A**, **C, E, F**) was analysed using Kruskal-Wallis with Dunn's multiple comparison and in some cases, the Mann-Whitney U-test for pairwise comparisons.

It was also observed that the effects of MPA on TNF-induced gene expression were like those of CORT. As shown in **Figure 4.2.3.1 D**, CORT at 100 nM significantly enhanced TNF-induced CCL20 mRNA expression even at TNF doses as low as 0.057 nM. In contrast to CCL20, CORT suppressed TNF-induced IL6 mRNA expression and retained this ability even at higher doses of TNF (**Figure 4.2.3.1 E**). On the other hand, TNF at doses as low as 0.057 nM appeared to suppress CORT-induced GILZ mRNA expression (**Figure 4.2.3.1 F**).

4.2.3.2: Increasing MPA or CORT and constant TNF

Compared to vehicle control, MPA increased CCL20 in a dose-dependent manner, with significant expression occurring at 100 nM (**Figure 4.2.3.2 A**). The addition of TNF at 1.15 nM enhanced MPA-mediated increase in CCL20 mRNA expression. It was observed that TNF was able to enhance MPA-induced response at concentrations as low as 1 nM. In contrast, MPA inhibited basal as well as TNF-induced expression of IL6 in a dose-dependent manner, with significant inhibition occurring at 100 nM and 1 nM, respectively. It was also observed that MPA induced GILZ transcription in a dose-dependent manner. MPA-induced increase of GILZ mRNA levels was inhibited by TNF (**Figure 4.2.3.2 A, B**).

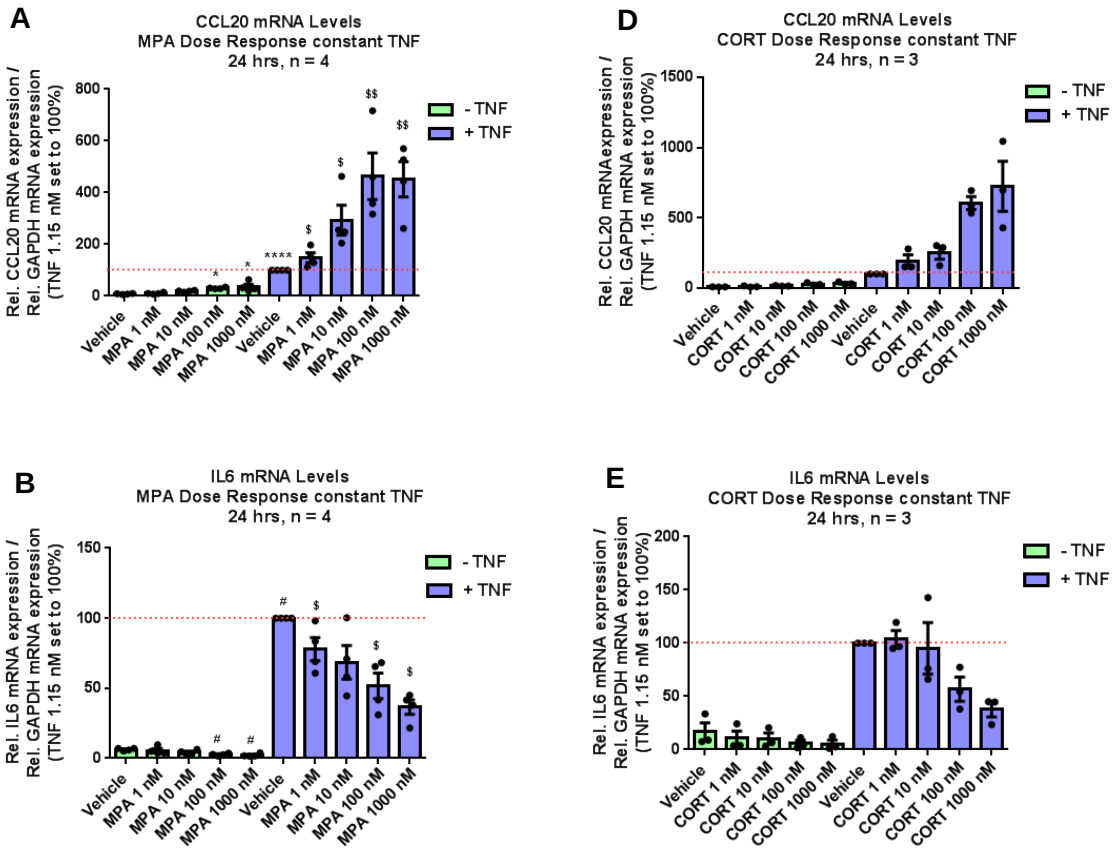

Figure 4.2.3.2: The regulation of CCL20, IL6 and GILZ mRNA levels by MPA or CORT in the absence or presence of TNF in End1/E6E7 cells is dose-dependent. (continued on the next page)

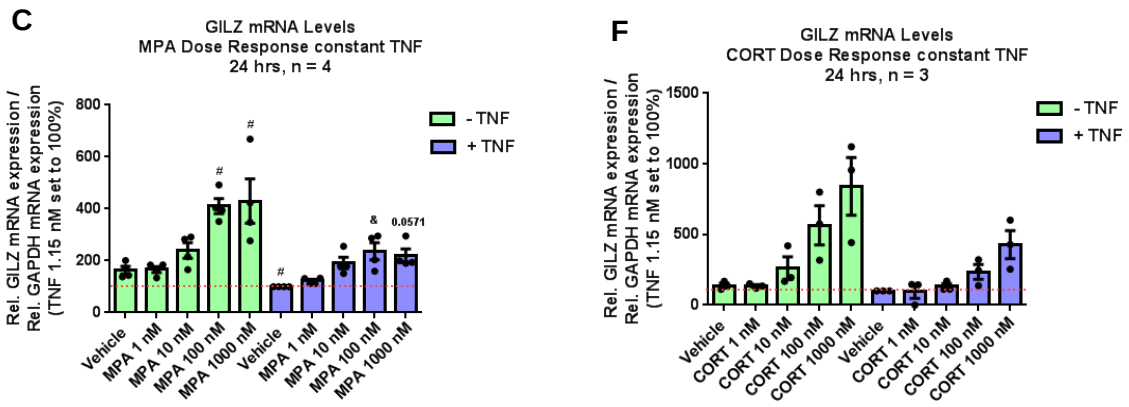

Figure 4.2.3.2: The regulation of CCL20, IL6 and GILZ mRNA levels by MPA or CORT in the absence or presence of TNF in End1/E6E7 cells is dose-dependent. End1/E6E7 cells were treated with increasing concentrations of MPA in the presence or absence of 1.15 nM human TNF for 24 hours. In another non-parallel experiment, cells were treated with increasing concentrations of CORT in the presence or absence of 1.15 nM human TNF for 24 hours. Thereafter, the cells were harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A**, **D**), IL6 (**B**, **E**), GILZ (**C**, **F**) were determined by qPCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of various MPA and TNF combinations on mRNA levels of the selected genes were determined by normalising the date to TNF 1.15 nM (**A**, **B**, **C**), which is set to 100%. The data include at least three independent biological repeats pooled and plotted as mean ± SEM in GraphPad Prism 7 software. (**A**) was analysed using one-way ANOVA with Tukey's multiple comparisons and statistical significance denoted as * or **** to indicate $p<0.05$ and $p<0.0001$, respectively for comparison between vehicle only and other treatments. In some cases, the unpaired Student's t-test was used for comparisons between TNF only and other treatments with statistical significance denoted as $ or $$ to indicate $p<0.05$ and $p<0.01$, respectively. (**B**, **C**) was analysed using the Mann-Whitney U-test (or Kolmogorov-Smirnov test when TNF is set to 100%). Statistical significance is denoted as # to indicate $p<0.05$ for pairwise comparison between vehicle only and other treatments. For pairwise comparisons between TNF only and other treatments, significance is denoted as $ to indicate $p<0.05$. When the comparison was between corresponding MPA doses ±TNF, significance is denoted as & to indicate $p<0.05$.

The effects of MPA on basal and TNF-induced gene expression mirrored those of CORT (**Figure 4.2.3.2 D – F)**. CORT appeared to increase basal and TNF-induced CCL20 mRNA expression in a dose-dependent manner. CORT doses as low as 1 nM might enhanced TNF-induced but not basal CCL20 mRNA expression; CORT appeared to increase basal expression of CCL20 mRNA at 10nM and not 1nM (**4.2.3.2 D**). It was also observed that only higher doses of CORT (>100 nM) may block basal and TNF-induced IL6 mRNA expression (**Figure 4.2.3.2 E**). Lastly, CORT appeared to induce GILZ mRNA expression in a dose-dependent manner and TNF at 1.15 nM might inhibit the CORT-mediated effect (**Figure 4.2.3.2 F**).

In summary, these results demonstrate a reciprocal regulation of gene expression by MPA (as well as CORT) and TNF. They also demonstrate that TNF may increase or decrease the sensitivity of a gene to MPA or CORT.

4.2.3.3: MPA/CORT and TNF mutually alter each other's efficacies and potencies as they coregulate CCL20, IL6 and GILZ mRNA levels in End1/E6E7 cells.

In **Figure 4.2.3.1** and **Figure 4.2.3.2**, it is seen that the transactivation of CCL20 and GILZ by MPA and CORT can either be increased (CCL20) or antagonised (GILZ) by TNF. Likewise, MPA and CORT increased TNF-induced CCL20 mRNA expression, but inhibited TNF-induced IL6 mRNA expression. These observations suggest that in some cases MPA or CORT and TNF positively cooperate to enhance gene expression (CCL20) and in others they antagonise each other (GILZ and IL6). It was next investigated whether this reciprocal regulation is achieved by altering each other's potencies for activating or inhibiting gene expression.

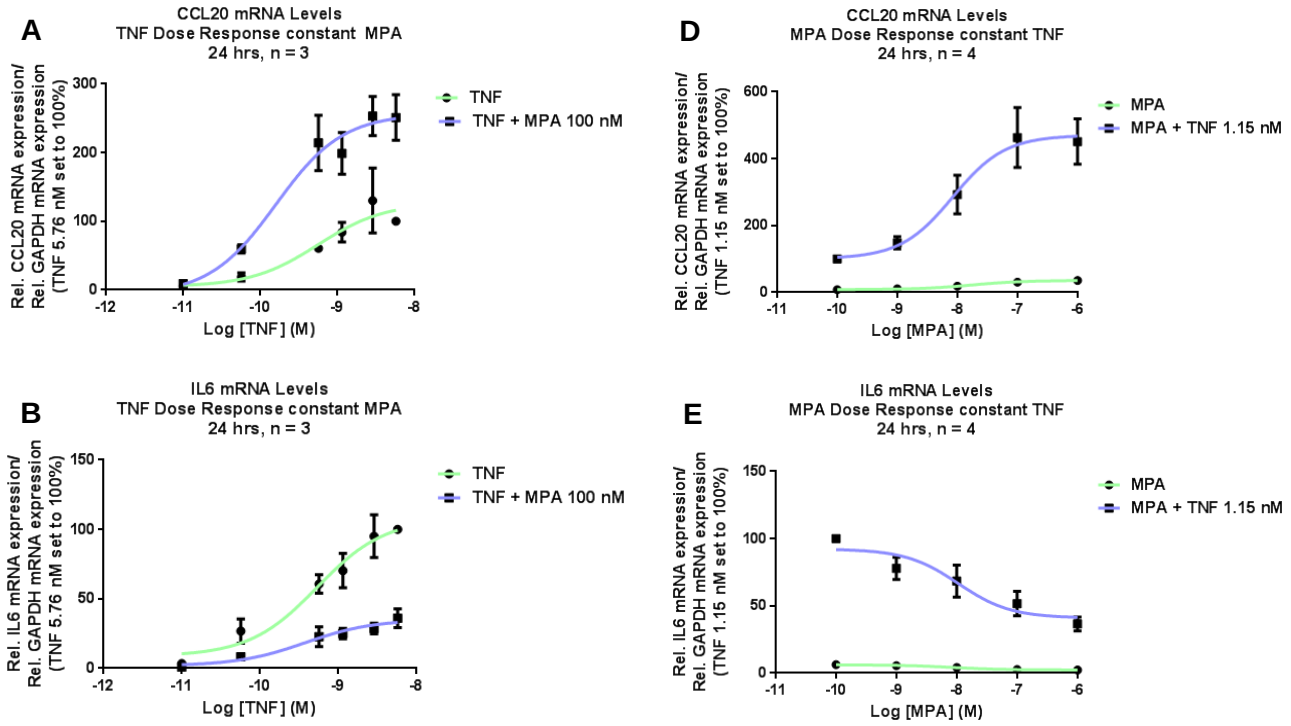

Figure 4.2.3.3.1: The co-regulation of CCL20, IL6 and GILZ mRNA levels by MPA and TNF in End1/E6E7 cells involves the ligands affecting each other's efficacies and maybe potencies.
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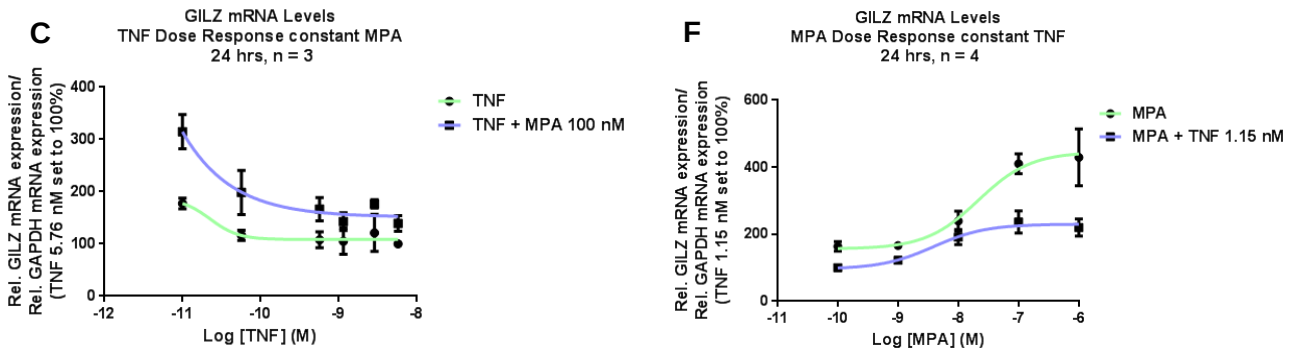


Figure 4.2.3.3.1: The co-regulation of CCL20, IL6 and GILZ mRNA levels by MPA and TNF in End1/E6E7 cells involves the ligands affecting each other's efficacies and maybe potencies. In (**A**, **B** and **C**), End1/E6E7 cells were stimulated with increasing concentrations of human TNF in the presence or absence of 100 nM MPA for 24 hours. In (**D**, **E** and **F**), End1/E6E7 cells were treated with increasing concentrations of MPA in the presence or absence of 1.15 nM human TNF for 24 hours. Cells were then harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A, D**), IL6 (**B, E**), GILZ (**C, F**), were determined by qPCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones and TNF on mRNA levels of the selected genes were determined by either normalising to TNF5.76 nM or TNF1.15 nM, with both TNFs set to 100%. The data include at least three independent biological repeats pooled and plotted as mean ± SEM in GraphPad Prism 7 software.

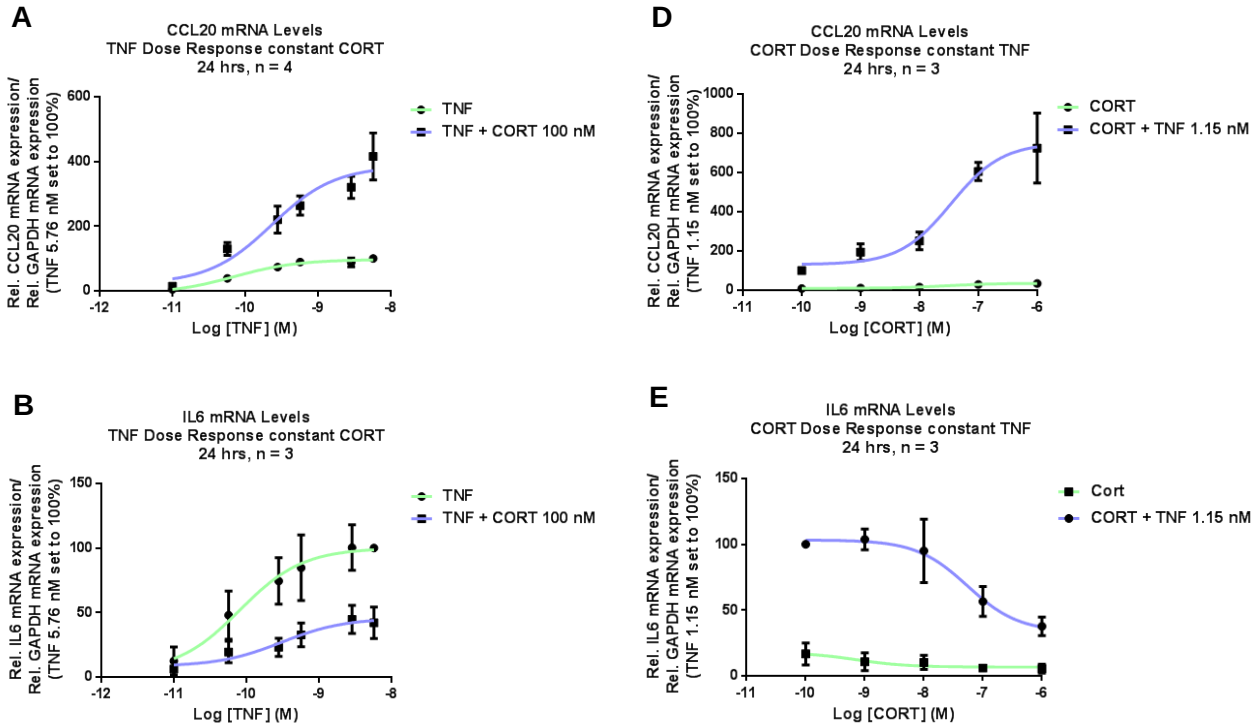


Figure 4.2.3.3.2: The co-regulation of CCL20, IL6 and GILZ mRNA levels by CORT and TNF in End1/E6E7 cells involves the ligands affecting each other's efficacies and maybe potencies. (continued on the next page)

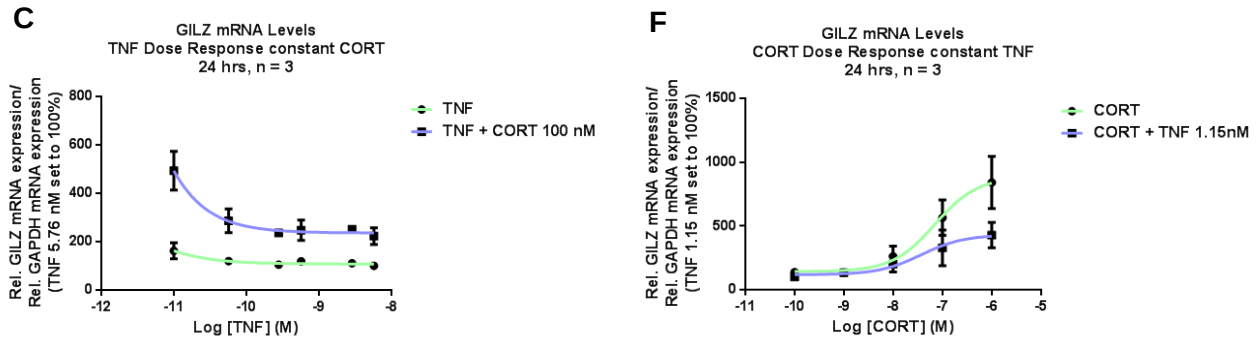


Figure 4.2.3.3.2: The co-regulation of CCL20, IL6 and GILZ mRNA levels by CORT and TNF in End1/E6E7 cells involves the ligands affecting each other's efficacies and maybe potencies. In (**A**, **B** and **C**), End1/E6E7 cells were stimulated with increasing concentrations of human TNF in the presence or absence of 100 nM CORT for 24 hours. In (**D**, **E** and **F)**, End1/E6E7 cells were treated with increasing concentrations of CORT in the presence or absence of 1.15 nM human TNF for 24 hours. Cells were then harvested in TriReagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A, D**), IL6 (**B, E**), GILZ (**C, F**), were determined by qPCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones and TNF on mRNA levels of the selected genes were determined by either normalising to TNF5.76 nM or TNF1.15 nM, with both TNFs set to 100%. The data include at least three independent biological repeats pooled and plotted as mean ± SEM in GraphPad Prism 7 software.

Data presented in **Figures 4.2.3.1** and **Figure 4.2.3.2** were used to generate dose-response curves, which are now shown in **Figure 4.2.3.3.1** and **Figure 4.2.3.3.2**. The maximum responses (efficacies) and potencies (EC_{50}) were deduced from these curves for each gene and have been summarised in **Tables 4.2.1.1.** As shown in **Table 4.2.1.1**, GILZ was induced by MPA, while co-treatment with TNF significantly reduced GILZ maximum mRNA expression from 459.2 ± 33.30 to 231.1 ± 53.59. IL6 was induced by TNF and the presence of MPA significantly reduced the efficacy of TNF from 113.6 ± 8.839 to 42.16 ± 19.32. When CCL20 was induced by MPA, co-treatment with constant TNF increased the maximum response from 40.92 ± 23.12 to 481.2 ± 148. When CCL20 was induced by TNF, co-treatment with 100 nM MPA enhanced the maximum response from 127.0 ± 40.76 to 260.0 ± 50.56. Given the error in the EC_{50} value, and few points on the slope, it could not be determined whether the potencies of each ligand for transactivation and transrepression were affected in the presence of the other. Further experiments to include more doses are needed to provide clarity on the matter.

CORT also increased the maximum response of TNF for transactivating CCL20 from 97.18 ± 10.77 to 361.4 ± 43.15. CORT also reduced TNF's efficacy for inducing IL6 from 104.9 ± 16.56 to 46.15 ± 21.16. On the other hand, TNF increased the efficacy of CORT for CCL20 gene expression from 35.79 ± 9.368 to 803.0 ± 332.8. No major differences in potencies were recorded for similar reasons mentioned above.

Table 4.2.1.1: The changes in efficacies (maximum responses) and potencies (EC_{50}) of selected inflammatory genes coregulated by MPA/CORT and TNF in End1/E6E7 cells

	CCL20		IL6		GILZ	
	Efficacy (%TNF[ab])	**EC_{50} (nM)**	**Efficacy (%TNF[ab])**	**EC_{50} (nM)**	**Efficacy (%TNF[ab])**	**EC_{50} (nM)**
MPA ↑	40.92 ± 23.12	65.92 ± 111.7	1.988 ± 0.866	26.12 ± 31.65	459.2 ± 175.5	25.54 ± 33.30
MPA ↑ + constant TNF[a]	481.2 ± 148.8[#]	14.87 ± 14.00	36.48 ± 12.86[#]	45.82 ± 80.87	231.1 ± 53.59[#]	4.891 ± 4.007
TNF ↑	127.0 ± 40.79	0.545 ± 0.150	113.6 ± 8.839	0.901 ± 0.935	~ 89543 ± 154789	~0.023 ± 0.017
TNF ↑ + constant MPA[b]	260.0 ± 50.56[***]	0.199 ± 0.094	42.16 ± 19.32[***]	1.208 ± 1.559	~ 194.5 ± 146.8	~0.0194 ± 0.314
CORT ↑	35.79 ± 9.368	31.92 ± 12.76	5.217 ± 5.823	68.90 ± 115.1	901.0 ± 416.1	82.33 ± 73.53
CORT ↑ + constant TNF[a]	803.0 ± 332.8	59.97 ± 68.88	32.87 ± 11.84	75.08 ± 103.5	557.3 ± 71.92	801.9 ± 1209
TNF ↑	97.18 ± 10.77	0.087 ± 0.047	104.9 ± 16.56	0.171 ± 0.129	~ 105.8 ± 9.967	~0.035 ± 0.035
TNF ↑ + constant CORT[b]	361.4 ± 43.15[***]	0.266 ± 0.286	46.15 ± 21.16[#]	0.308 ± 0.200	~ 247.4 ± 24.40	~0.008 ± 0.014

[a] For MPA dose, constant 1.15 nM human TNF was used, and this was set to 100%

[b] For TNF dose the highest concentration i.e. 5.76 nM was set to 100%

Statistics: One-way ANOVA with Tukey's multiple correction and statistical significance is denoted as *** to indicate $p<0.005$.

Mann-Whitney U-test and statistical significance denoted as [#] to indicate $p<0.05$

NB: Data indicates mean ± SD

4.2.3.4: The cooperative upregulation of CCL20 mRNA levels in End1/E6E7 cells by MPA or CORT and TNF is synergistic

To determine whether the coregulation of CCL20 mRNA production by TNF and MPA or CORT in End1/E6E7 cells is synergistic, the data presented in **Figures 4.2.3.1,2** was subjected to Chou Analysis. This method was introduced by Chou and Talalay (Chou, 2010) and was designed to determine whether a combination of drugs can elicit a response that is synergistic. This is achieved by computing a combination index (CI) for each drug combination using the Compusyn software. An CI less than 1 is defined as synergism. Using this method, CI values for each MPA or CORT: TNF combination were determined and are shown in **Figure 4.2.3.4**. It was observed that MPA or CORT doses ranging from 10 nM to 1000 nM strongly synergised with 1.15 nM TNF to upregulate CCL20 transcription in End1/E6E7 cells. Similarly, TNF doses ranging from 0.057 nM to 5.76 nM strongly synergised with 100 nM MPA or CORT to upregulate CCL20 mRNA levels in this cell line.

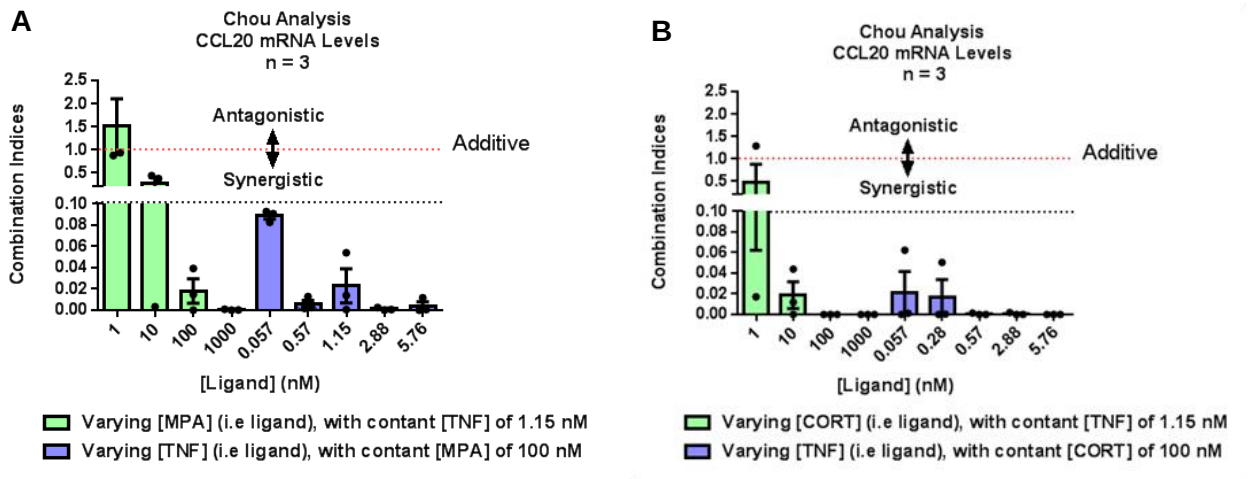


Figure 4.2.3.4: MPA like CORT synergises with TNF to upregulate CCL20 mRNA levels in End1/E6E7 cells. In one experiment, End1/E6E7 cells were treated with varying concentrations of MPA in the absence or presence of constants 1.15 nM TNF. In another, the cells were treated with increasing concentration of TNF in the absence or presence of 100 nM MPA or CORT for 24 hours. Thereafter, the cells were harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 and GAPDH were determined by qPCR using gene specific primers. The average GAPDH Ct values for one experiment was used to normalised GAPDH Ct values in the other and fold changes in CCL20 expression in were determined by normalising to adjusted GAPDH levels. The relative effects of various MPA (**A**) or CORT (**B**) and TNF combinations on CCL20 mRNA expression was determined by normalising vehicle only control. Thereafter, highest fold change for each biological repeat was set to 0.99 and others calculated relative to this value. Each single or ligand combination dose and their respective response were keyed into the Compusyn software to determine the Combination indices (CI). The CI were then plotted in GraphPad Prism 7 software as mean ± SEM. CI values below the red dotted line indicate synergism (CV < 1). CI below the black dotted line indicates very strong synergism.

4.2.4: MPA like GCs acts via the GR to synergise with immune activators to upregulate CCL20 mRNA levels, repress IL6 mRNA levels and increase GILZ mRNA levels in End1/E6E7 cells.

To examine the role of the GR in the differential regulation of CCL20, IL6 and GILZ, End1/E6E7 cells were pre-treated with 1 µM RU486 for 2 hours before concurrent stimulation with hormones and TNF. As shown in **Figure 4.2.4.1 A**, DEX, CORT and MPA, but not NET appeared to increase basal and TNF-induced production of CCL20 mRNA. These effects were completely prevented in the presence of RU486, showing that the GR is involved in the enhancement of basal and TNF-induction of CCL20 by DEX, CORT and MPA. In addition, both basal and TNF-induced IL6 expression was significantly suppressed by DEX, CORT and MPA, but not NET. This suppression was, however, lifted in the presence of RU486, again showing that the GR was required for the DEX-, CORT- and MPA-induced repression of IL6 expression (**Figure 4.2.4.1 B**). Furthermore, RU486 prevented the induction of GILZ by DEX, CORT and MPA in the absence or presence of TNF (**Figure 4.2.4.1 C**).

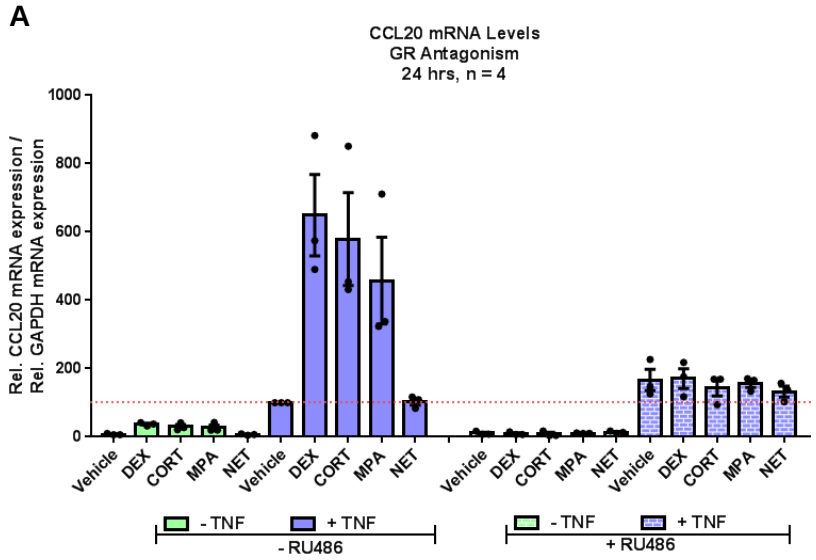


Figure 4.2.4.1: The GR is required for DEX, CORT and MPA upregulation of TNF-induced CCL20 mRNA levels, suppression of TNF-induced IL6 mRNA levels, and upregulation of GILZ mRNA levels in End1/E6E7 cells. (continued on the next page)

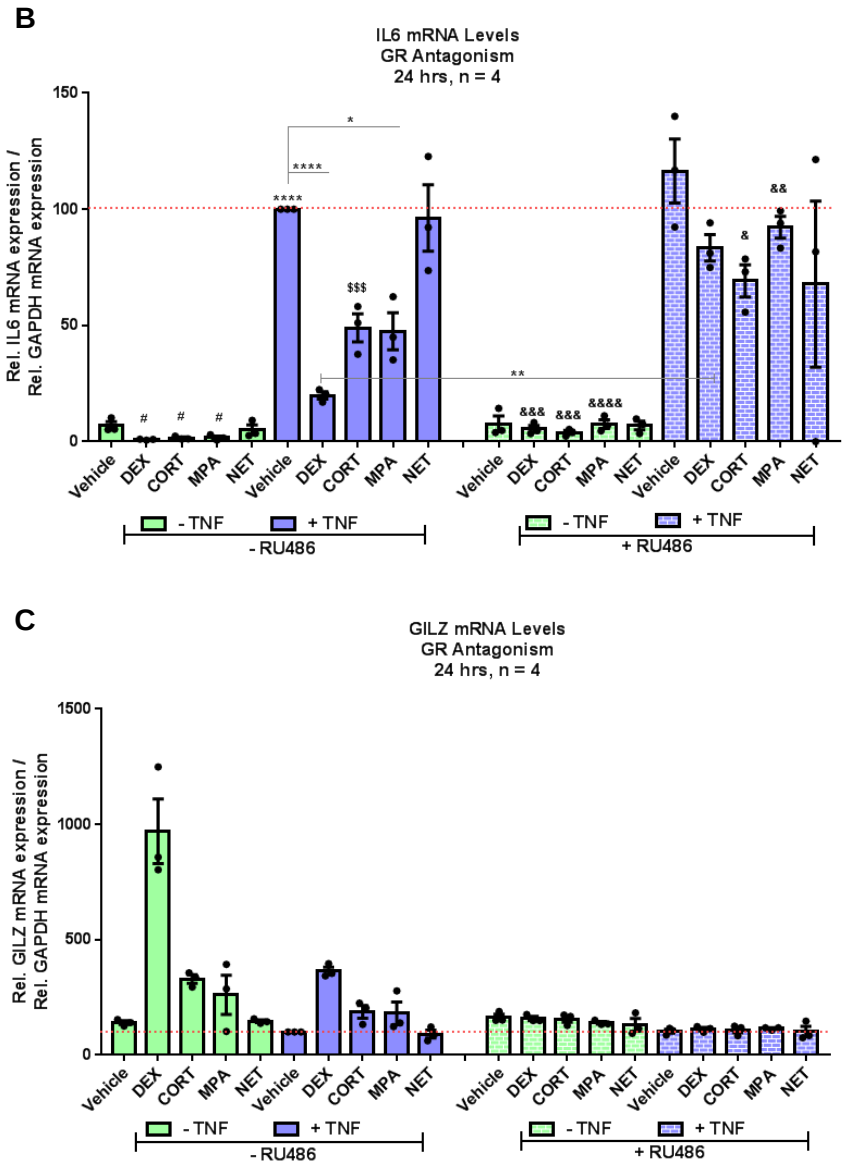


Figure 4.2.4.1: The GR is required for DEX, CORT and MPA upregulation of TNF-induced CCL20 mRNA levels, suppression of TNF-induced IL6 mRNA levels, and upregulation of GILZ mRNA levels in End1/E6E7 cells. End1/E6E7 cells were pre-treated for 2 hours with 1 µM RU486 or 0.1% (v/v) EtOH (vehicle) and thereafter treated with 100 nM DEX, CORT, MPA, NET or 0.1% (v/v) EtOH in the absence or presence of 1.15 nM TNF for another 24 hours. The cells were the harvested in Tri-Reagent®, total RNA isolated and cDNA made. The relative mRNA levels of CCL20 (**A**), IL6 (**B**) and GILZ (**C**) were determined by qPCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones and TNF on mRNA levels of the selected genes were determined by normalising the data to TNF only, which is set to 100%. The data include at least three independent biological repeats was plotted as mean ± SEM in GraphPad Prism 7 software. (**B**) was analysed using one-way ANOVA with Tukey's multiple comparisons and statistical significance denoted as *, ** or **** to indicate $p<0.05$, $p<0.01$ and $p<0.0001$, respectively for comparisons between vehicle only and other treatments or between other pair of treatments. In some cases, the unpaired Student's t-test was used for comparisons between vehicle and other treatments and statistical significance denoted as $^{\#}$ to indicate $p<0.05$. When the comparison was between TNF and other treatments, statistical significance is denoted as $^{\$\$\$}$ to indicate $p<0.005$. When the comparison was between treatments in control and their corresponding treatments in

the presence of RU486, significance is denoted as &, &&, &&& or &&&& to indicate p<0.05, p<0.01, p<0.005 and p<0.0001, respectively. (**A**, **C**) were analysed using Kruskal-Wallis with Dunn's multiple comparison and in some cases, the Mann-Whitney U-test for pairwise comparisons.

Next, it was investigated whether the GR mediates the upregulation of LPS-induced CCL20 expression by DEX or MPA in End1/E6E7 cells. As shown in **Figure 4.2.4.2 A**, RU486 strongly inhibited upregulation of LPS-induced CCL20 mRNA expression that occurs in the presence of DEX or MPA. In addition, RU486 significantly inhibited the suppression of LPS-induced IL6 mRNA expression by DEX and MPA (**Figure 4.2.4.2 B**). Furthermore, RU486 inhibited the DEX and MPA-induction of GILZ mRNA expression in the absence or presence of LPS (**Figure 4.2.4.2 C**).

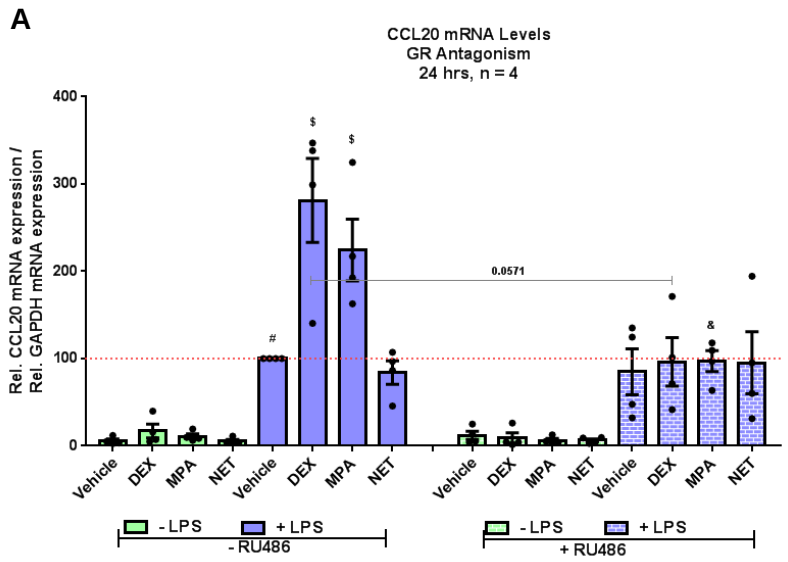


Figure 4.2.4.2: The GR is required for DEX and MPA enhancement of LPS-induced CCL20 mRNA levels, suppression of LPS-induced IL6 mRNA levels, and upregulation of GILZ mRNA levels in End1/E6E7 cells. (continued on the next page)

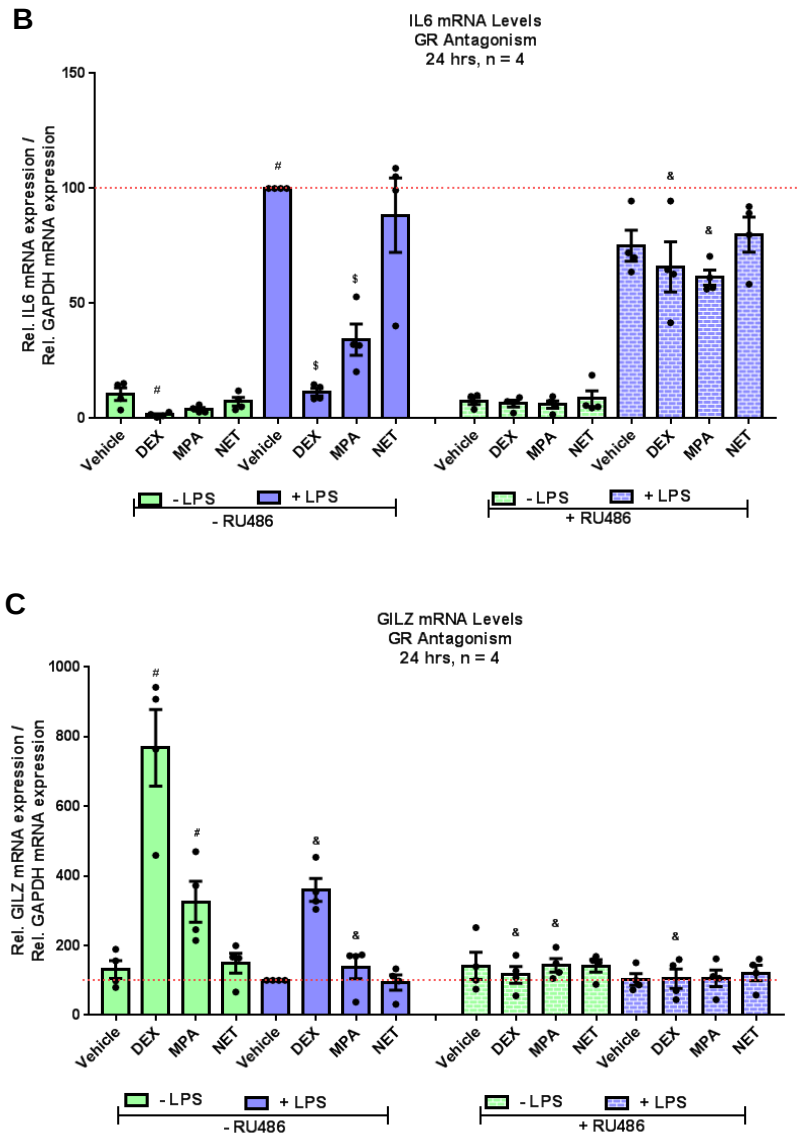


Figure 4.2.4.2: The GR is required for DEX and MPA enhancement of LPS-induced CCL20 mRNA levels, suppression of LPS-induced IL6 mRNA levels, and upregulation of GILZ mRNA levels in End1/E6E7 cells. End1/E6E7 cells were pre-treated for 2 hours with 1 µM RU486 or 0.1% (v/v) EtOH (vehicle) and thereafter treated with100 nM DEX, MPA, NET or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 5 µg/mL LPS for another 24 hours. The cells were then harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A**), IL6 (**B**) and GILZ (**C**) were determined by qRT-PCR performed using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones and LPS on mRNA levels of the selected genes were determined by normalising to LPS only, which has been set to 100%. The data include at least three independent biological repeats plotted as mean ± SEM in GraphPad Prism 7 software. The data was then tested for normality using the Shapiro-Wilk test and found to be non-parametric. Statistical analysis was performed using the Mann-Whitney U-test (or Kolmogorov-Smirnov test when the control was set to 100%). Statistical significance is denoted as $^{\#}$ to indicate $p<0.05$ for pairwise comparison between vehicle only and other treatments. For pairwise comparisons between TNF and other treatments, significance is denoted as $^{\$}$ to indicate $p<0.05$. When the comparison was between conditions in control and their corresponding treatments in the presence of LPS or RU486, significance is denoted as $^{\&}$ to indicate $p<0.05$.

Taken together, these results show that MPA like GCs, but unlike NET, acts via the GR to upregulate basal GILZ and CCL20 mRNA levels, while repressing IL6 mRNA levels in End1/E6E7 cells. In addition, these results show that the GR is involved in the expression of GILZ by MPA in the presence of immune activators. Because immune activators only partially repressed GILZ expression, these results suggest some components of GC- and MPA-induced pathways are insensitive to inhibition by LPS and TNF.

4.2.5: The potentiation of TNF-induced CCL20 mRNA production by DEX and MPA in End1/E6E7 cells does not require changes in GR protein levels

In the previous section, it was shown that the GR was required for the regulation of basal and TNF-induced CCL20 by DEX and MPA (**Figure 4.2.4.1**). However, it does not indicate the features of the GR that are involved in the DEX or MPA enhancement of TNF-induced expression of CCL20. GCs are known to induce rapid turnover of GR protein levels. In contrast, previous studies have shown that TNF can induce nascent and/or stabilise pre-existing GR cellular levels. It was next investigated whether the synergistic upregulation of CCL20 mRNA levels by DEX or MPA and TNF occurs because TNF prevents DEX or MPA-induced GR turnover.

To verify these hypotheses, two parallel experiments were setup in which End1/E6E7 cells were treated with either 100 nM DEX or MPA in the absence or presence of 1.15 nM TNF for 24 hours. One experiment was harvested for RNA preparation to verify CCL20 mRNA levels and the other for protein to asses GR protein levels.

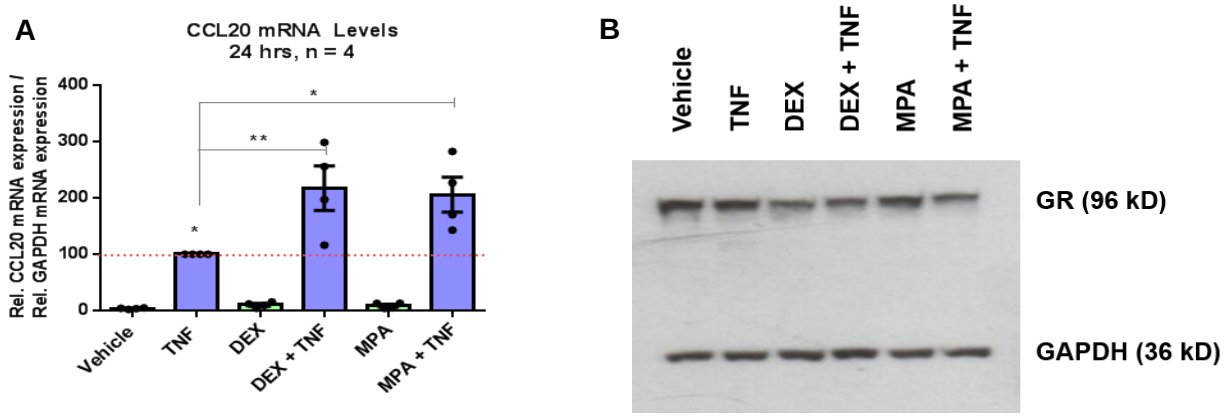


Figure 4.2.5: The synergistic regulation of CCL20 mRNA levels by DEX or MPA and TNF in End1/E6E7 cells does not involve changes in GR protein levels. (Continued on the next page)

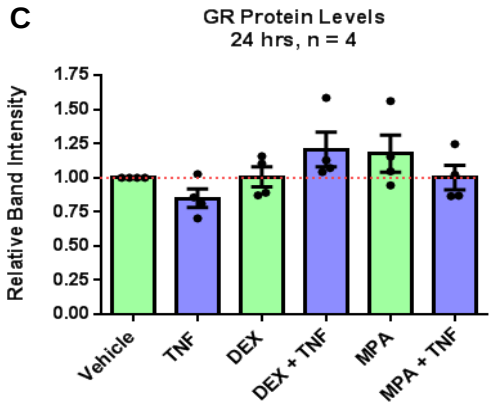


Figure 4.2.5: The synergistic regulation of CCL20 mRNA levels by DEX or MPA and TNF in End1/E6E7 cells does not involve changes in GR protein levels. Parallel experiments were set up in which End1/E6E7 cells were treated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (Vehicle) in the presence or absence of 1.15 nM TNF for 24 hours. In (**A**), cells were then harvested in Tri-Reagent®, total RNA isolated and converted to cDNA and relative mRNA levels of CCL20 (**A**), were determined by qRT-PCR performed using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones only or in combination with TNF on mRNA levels of the selected genes were determined by normalising to TNF, which has been set to 100%. The data include at least three independent biological repeats plotted as mean ± SEM in Graph Pad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons and statistical significance denoted as *, or ** to indicate $p<0.05$ and $p<0.01$, respectively. In (**B**)**,** cells were harvested in 2X SDS Loading Buffer and equal amounts were resolved on an 8% SDS-PAGE. Thereafter, western blot analysis was performed with antibodies specific for GR and GAPDH. **B** is a representative blot and **C** is the densitometric analysis of blots from four independent biological repeats. The data is plotted relative to vehicle control, which has been set to 1.

As shown in **Figure 4.2.5 A**, both DEX and MPA significantly enhanced the TNF-induced increase in CCL20 mRNA levels. However, neither DEX nor MPA altered steady state GR protein levels after 24 hours (**Figures 4.2.5 B, C**). In addition, TNF did not alter GR protein levels both in the absence or presence of DEX/MPA (**Figures 4.2.5 B, C**). This result suggests that DEX, MPA or TNF alone or in combination does not induce changes in the synthesis or degradation of GR protein in End1/E6E7 cells. This implies that changes in GR protein levels might not be required for the upregulation of TNF-induced CCL20 mRNA expression by DEX and MPA.

4.2.6: Summary of Findings

The results demonstrated that MPA like DEX, but unlike NET or P4, represses claudin-4 and occludin mRNA levels in End1/E6E7 cells in a dose-dependent manner. When compared to DEX, MPA was found to act as a partial GR agonist in repressing claudin-4 and occludin mRNA expression and inducing GILZ mRNA expression. Like CORT, MPA may act like a full GR agonist in inducing CCL20 and

repressing IL6 mRNA expression, but unlike CORT acts like a partial GR agonist in inducing GILZ mRNA expression. It was also established that MPA like CORT exhibited true synergism with TNF to increase CCL20 mRNA expression in a gene-specific manner. Interestingly, TNF was found to enhance the efficacy of MPA from 40.92% to 481.2 % or that of CORT from 35.79% to 803.0% for upregulating CCL20 mRNA expression in End1/E6E7 cells. MPA like CORT was found to enhance the efficacy of TNF for upregulating CCL20 mRNA expression but reduces its efficacy for upregulating IL6 mRNA expression in End1/E6E7 cells. These processes were found to be mutual. Finally, it was established that all these observed effects of MPA occurred via the GR.

Chapter Five

MPA unlike NET downregulates desmoglein-1 mRNA levels in ectocervical tissue explants, positively cooperate with immune activators to increase HIV-1 infection in TZM-bl cells and augments CCL20 expression in PBMCs

5.1: Aims

The previous chapters established that MPA unlke NET downregulates TJ genes in an endocervical epithelial cell line. In addition, it was established that MPA unlike NET cooperatess with immune activators to upregulate the expression of select pro-inflammatory genes (CCL20 and TNFRSF1B) in End1/E6E7 cells. Therefore, the objectives of this chapter were to investigate aims **E** and **F** (**section 1.5**) with the following specific questions:

- Does regulation of TJ and immune function genes by MPA and NET occur in physiologically relevant primary human tissue and cell models from the FGT and blood?

More specifically, under this aim, it will be determined for MPA and NET whether:

a. they cooperate with immune activators to regulate the expression of select immune function genes in ectocervical tissue explants.

b. they regulate mucosal barrier function and the expression of select TJ genes in primary epithelial cells or tissue explants from the FGT.

c. they coregulate with immune activators the expression of select immune function genes in PBMCs

- What are the effects of progestins alone or in combination with immune activators on mucosal barrier function and HIV-1 infection?

More specifically, under this aim, it will be determined for select progestins whether:

a. the progestins alone or in combination with immune activators increase HIV-1 infection and replication in TZM-bl indicator cells.
b. the changes in gene expression in PBMCs affects HIV-1 infection and replication in TZM-bl indicator cells.

This section of the thesis intends to answer the question whether the results reported in the previous chapters can occur in a physiologically relevant model. Because of constraints on obtaining endocervical tissue explants, ectocervical tissue explants were used instead. The tissue explants were treated with 100 nM DEX, MPA, NET and P4, alone or in combination with TNF. The levels of soluble immune

mediators secreted in respond to MPA alone or in combination with TNF were assessed using a customised 22-plex Luminex assay. The following 22 proteins were selected to include the following: i) cytokines (TNF, IL6, IL1β, IL7, IL15, IL2, IL16, IL1α, G-CSF, GM-CSF), ii) chemokines (CCL2, IL8, CCL5, CCL4, CXCL10, CCL8, CCL20), iii) antimicrobial and antiviral factors (secretory leukocyte protease inhibitor [SLPI], CXCL12) and iv) growth factor (TGFβ). Gene expression analysis for all sample sets was also investigated by real-time PCR. This sections also intends to provide answers to the question whether MPA and DEX can coregulate mucosal permeability with HIV-1 IMCs. In this regard, PGECs were exposed to HIV-1 IIIB IMCs to assess whether DEX and MPA enhance HIV-1-mediated disruption of genital epithelial mucosal barrier. HIV-1 IIIB like other HIV-1 IMCs have been shown to disrupt mucosal barriers formed by PGECs in vitro (Nazli *et al*., 2010). HIV-1 infection assays were performed in TZM-bl cells, using HIV-$1_{BaL\text{-}Renilla}$ IMCs. LPS was used to stimulate a pro-inflammatory response in PBMCs as TNF based on the protocol used in this study could induce such a response. It was also investigated whether secretions from PBMCs treated with GCs and progestogens alone or in combination with LPS affect HIV-1 replication in TZM-bl cells.

5.2: Results

5.2.1: MPA like DEX suppresses basal secretion of select soluble immune mediators, appears to increase CCL20 expression in a gene-specific manner, while TNF does not appear to modulate these responses in ectocervical tissue explants

In the previous sections, treating End1/E6E7 cells with DEX or MPA resulted in the differential regulation of basal and TNF-induced expression of immune mediators. Of interest was the observation that DEX or MPA synergised with TNF to upregulate CCL20 mRNA expression and protein secretion. It was next investigated whether similar regulation could be seen in a more physiologically relevant model such as ectocervical tissue explants. Ectocervical tissue explants from pre-menopausal women were treated with 100 nM DEX or MPA in the presence or absence of 1.15 nM TNF for 24 hours. Thereafter supernatants were collected and subsequently analysed by Luminex assay for secreted immune mediators. A 22-plex panel was selected comprising TNF, IL8, CCL2, IFNβ, IFNγ, TGFβ, G-CSF, IL1β, CCL5, CXCL12, CCL4, CXCL10, IL6, GM-CSF, IL12, CCL8, IL7, IL15, IL1α, IL2, CCL20 and IL16. Of these, 11 cytokines fell below the limit of detection and thus could not be quantified: TNF, IFNβ, IFNy, CXCL10, GM-CSF, IL12, CCL8, IL7, IL15, IL1α and IL2.

In the absence of TNF, MPA like DEX significantly reduced GCSF and IL8 protein levels when compared with vehicle only control (**Figure 5.2.1.1 A**, **B**). Although not statistically significant, DEX and MPA also appeared to decrease IL6, IL16 and CXCL9 protein levels (**Figure 5.2.1.1 C – E**). In contrast, MPA appeared to increase CCL5 and CCL20 protein secretion by ectocervical tissue explants (**Figure 5.2.1.1**

H, I). In the presence of TNF, GCSF protein level was significantly increased when compared with vehicle only control (**Figure 5.2.1.1 A**). TNF also appeared to increase IL6, CXCL9 and TGFβ protein secretion by ectocervical tissue explants, but these increases were not statistically significant (**Figure 5.2.1.1 C, E, J**). MPA like DEX appeared to inhibit IL6 secretion in the presence of TNF (**Figure 5.2.1.1 C**). MPA also appeared to block CCL2 secretion, but it may increase IL1β and CCL20 secretion in the presence of TNF (**Figure 5.2.1.1 F, G, I**).

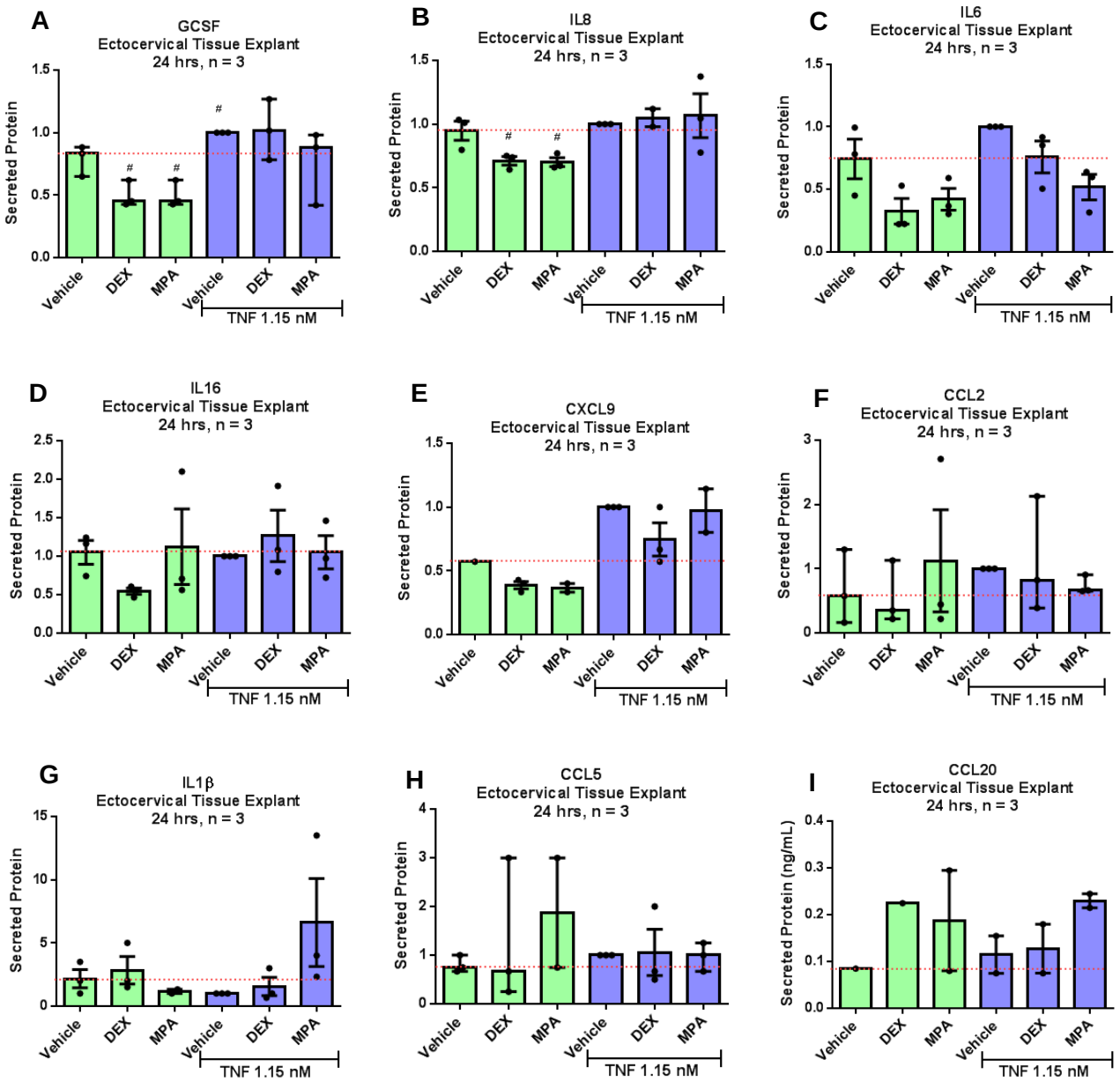


Figure 5.2.1.1: The MPA like DEX inhibits basal secretion of select soluble immune mediators but does not appear to regulate their secretion in the presence of TNF in ectocervical tissue explants. (continued on the next page)

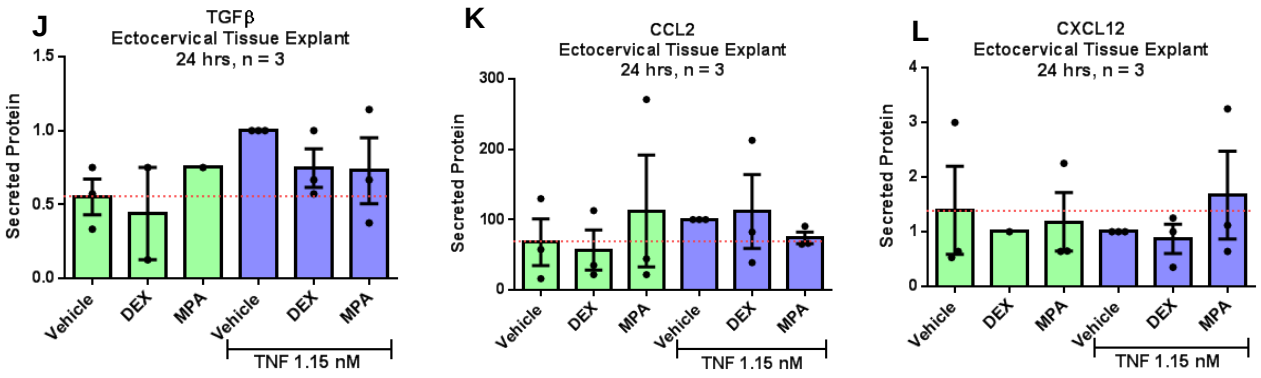


Figure 5.2.1.1: The MPA like DEX inhibits basal secretion of select soluble immune mediators but does not appear to regulate their secretion in the presence of TNF in ectocervical tissue explants. Ectocervical tissue explant samples from pre-menopausal women were co-treated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF for 24 hours. Thereafter, supernatants were collected and stored at -80°C until used. Aliquots of these were sent to Professor R. Shattock's lab at Imperial College London (UK) where cytokine levels were analysed by Luminex assay. The graphs are plotted in Graph Pad Prism 7 software as mean ± SEM and include data from three donors. (**A** - **K**) are plotted relative to TNF (which has been set to 1), whereas in (**L**) only absolute values have been plotted because of missing values in the TNF control. Statistical analysis was performed using the unpaired Student's t-test for comparison between vehicle only and other treatments and statistical significance is denoted as $^{\#}$ to indicate $p<0.05$. This test was used only when the data was parametric; for data that was non-parametric the Kruskal-Wallis with Dunn's multiple comparisons test was used.

As indicated in the legend of **Figure 5.2.1.1**, the Luminex analysis was performed in Professor Robin Shattock's lab at Imperial College (London, UK). Because samples had to be shipped overseas for analysis, it is possible that some analytes degraded during the process. To make sure this was not the case, aliquots of these samples and those collected from fresh experiments were (re-)analysed by ELISA for IL6, CCL5 and CCL20 protein levels. In parallel, total RNA was isolated from cultured tissue explant pieces and used to quantify IL6, CCL20 and GILZ (positive control) expression by qRT-PCR. Prominent biomarkers for FGT inflammation include IL1β, IL6, IL8, CCL5 and CCL20 (Morrison *et al*., 2014; Fichorova *et al*., 2015; Mauck *et al*., 2016). From this group, IL6, CCL5 and CCL20 were selected and their expression was quantified using ELISA and/or qRT-PCR.

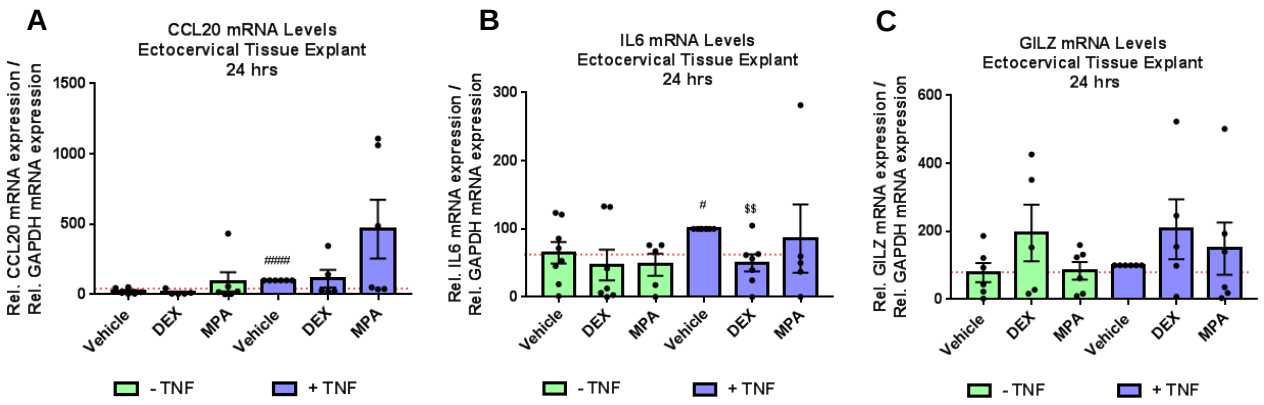


Figure 5.2.1.2: The effects of DEX and MPA on CL20, IL6 and GILZ mRNA levels in human ectocervical tissue explants in the absence and presence of TNF. Ectocervical tissue explants from pre-menopausal women were treated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF for 24 hours. Thereafter, supernatants were collected and stored at -80°C until used and the rest of the tissue pieces were homogenised in Tri-Reagent®, total RNA isolated and converted to cDNA. In (**A – C)**, CCL20 (**A**), IL6 (**B**) and GILZ (**C**) mRNA levels were quantified by qRT-PCR and normalised to GAPDH mRNA levels. The relative effects of the ligands on mRNA levels of the selected genes were determined by normalising to TNF only, which is set to 100%. The data was plotted in Graph Pad Prism 7 software as mean ± SEM. Sample size: [CCL20; Vehicle (n = 10), DEX (n = 9), MPA (n = 6)], [IL6: Vehicle (n = 8), DEX (n = 7), MPA (n = 5)], [GILZ; Vehicle (n = 6), DEX (n = 5), MPA (n = 6)]. Statistical analysis was performed using the Kolmogorov-Smirnov test and statistical significance is denoted as $^{\#}$ or $^{\#\#\#\#}$ to indicate $p<0.05$ and $p<0.0001$, respectively for comparisons between vehicle only and TNF only; or as $^{\$\$}$ to indicate $p<0.05$ for comparison between TNF and other treatments.

As shown in **Figure 5.2.1.2 A**, MPA but not DEX appeared to enhance TNF-induced CCL20 mRNA expression in ectocervical tissue explants. Although the effect of MPA on CCL20 mRNA levels in the presence of TNF was not statistically significant, it appeared to be ligand specific as neither P4 nor NET could generate a similar effect (**Appendix C, Figure 1A**). In addition, it was observed that this occurs in some but all not donor samples, suggesting Interdonor variation in the response (**Appendix C, Figure 2**). Basal IL6 mRNA levels appeared to be downregulated by DEX but not MPA (**Figure 5.2.1.2 B**). In addition, DEX unlike MPA significantly inhibited TNF-induced increase in IL6 mRNA levels. In contrast, P4 was found to suppress IL6 mRNA levels both in the absence and presence of TNF. NET did not regulate basal CCL20 and IL6 mRNA levels but was found to suppress IL6 and not CCL20 mRNA levels in the presence of TNF (**Appendix C, Figure 1 B**). Neither DEX, MPA nor NET significantly regulated GILZ mRNA levels (**Figure 5.2.1.2 C** and **Appendix C Figure 1 C**)

At the protein level, it was observed that MPA appeared to induce CCL20 secretion in the absence TNF in some (2 out of 5), but not all donor samples, suggesting inter-donor variations in the response. It was

also observed that TNF failed to induce CCL20 secretion, which could explain the apparent lack of cooperativity between the ligands to upregulate CCL20 secretion (**Figure 5.2.1.3 A**). TNF appeared to induce IL6 and CCL5 protein secretion in ectocervical tissue explants. In contrast, MPA seemed to suppress basal IL6 and not CCL5 secretion. Additionally, MPA appeared to block TNF-induced secretion of IL6 and CCL5. Due to the small sample size, statistical analysis could not be performed to determine whether the putative effects of TNF and MPA on IL6 and CCL5 secretion were significantly different compared with vehicle control (**Figure 5.2.1.3 B**, **C**). More experiments are needed to build on the sample size and hence improve the power of the statistical analysis.

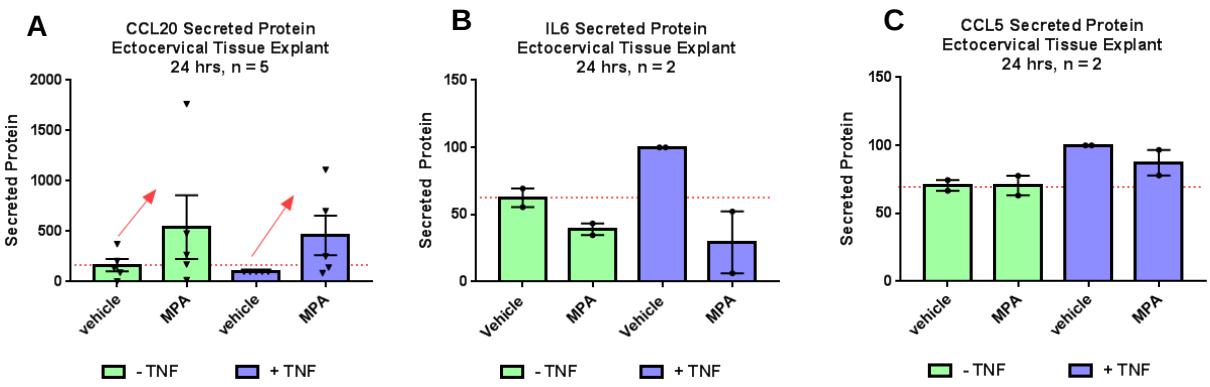


Figure 5.2.1.3: The effects of MPA on CCL20, IL6 and CCL5 protein secretion in ectocervical tissue explants in the absence and presence of TNF. Ectocervical tissue explants from pre-menopausal women were treated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF for 24 hours. Thereafter, supernatants were collected and stored at -80°C until used. ELISA assays were then performed to determine the levels of CCL20 (**A**), IL6 (**B**) and CCL5 (**C**) secreted in supernatants. The relative effects of the ligands on the levels of the selected cytokines were determined by normalising to TNF, which is set to 100%. The data was plotted in Graph Pad Prism 7 software as mean ± SEM. (**A**) was analysed using one-way Kruskal-Wallis test with Dunn's multiple comparisons.

Taken together, these results suggest that MPA can suppress GSCF, IL8 and potentially IL6 protein secretion by ectocervical tissues. However, MPA might enhance CCL20 mRNA expression and CCL20 protein secretion in ectocervical tissue explant in the presence of TNF. This might occur against the backdrop of MPA suppressing TNF-induced secretion of IL6. The results further suggest that the potential enhancement of CCL20 expression in ectocervical tissues by MPA is ligand-specific as neither DEX, P4 nor NET could induce a similar effect.

5.2.2: MPA supresses desmoglein-1 mRNA in ectocervical tissue explants, but fails to disrupt tight junction formation in endometrial and endocervical primary epithelial cell cultures even in the presence of HIV-1

Next, it was investigated whether DEX, MPA and NET regulate tight junction gene expression in ectocervical tissue explants. For these assessments, cDNA generated from experiments described in **Figure 5.2.2.1** and **Appendix C** (**Figure 1**) were used to quantify by qPCR mRNA levels of claudin-4, occludin and the adherens junction gene desmoglein-1. Desmoglein-1 was included in this analysis to verify a report by Quispe-Calla *et al* (2016) that suggested a decrease in its expression in ectocervical biopsy tissues from women on MPA. As shown in **Figure 5.2.2.1 A, B**, treatment of ectocervical tissue explants with DEX, but not MPA or NET resulted in a significant reduction in claudin-4 mRNA levels. Neither DEX, MPA nor NET reduced occludin mRNA expression. It was also observed that MPA, unlike DEX and NET, significantly suppressed desmoglein-1 mRNA production (**Figure 5.2.2.1 C**).

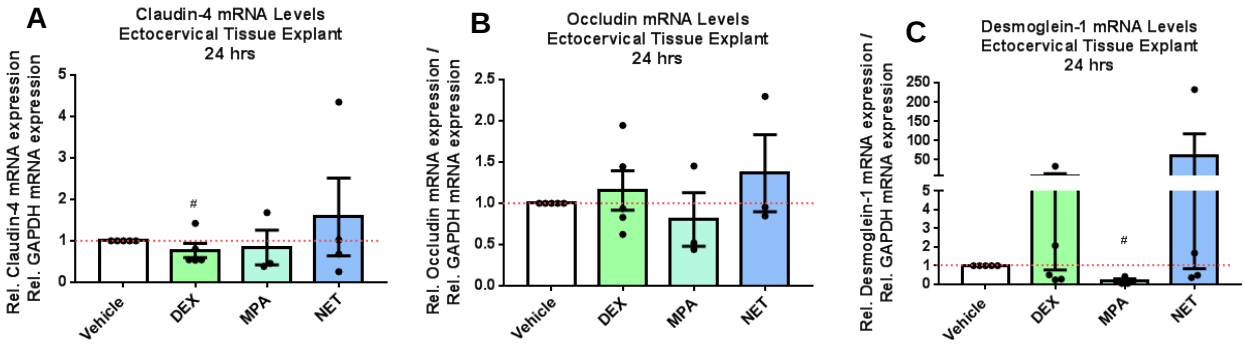


Figure 5.2.2.1: The effects of DEX, MPA and NET on claudin-4, occludin and desmoglein-1 mRNA expression in ectocervical tissue explants. Ectocervical tissue explant samples from pre-menopausal women were treated with 100 nM DEX, MPA, NET or 0.1% (v/v) EtOH (vehicle) for 24 hours. Thereafter, tissue pieces were homogenised, total RNA isolated and converted to cDNA. The relative mRNA levels of claudin-4 (**A**), occludin (**B**) and desmoglein-1 (**C**) were determined by qRT-PCR performed using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones were determined by further normalising by setting vehicle to 1. The data includes at least three independent biological repeats (DEX, n = 5; MPA, n = 3; NET, n = 4) plotted as mean ± SEM. Statistical analysis was performed using the Kolmogorov-Smirnov test and statistical significance denoted as $^{\#}$ to indicate $p<0.05$.

The inability of MPA to suppress claudin-4 and occludin mRNA expression contrasted with previous findings in End1/E6E7 cells. However, by suppressing desmoglein-1 mRNA production, MPA might disrupt the mucosal surface of the ectocervix. It should be recalled that TJs are needed to maintain the integrity of mucosal surfaces. It is currently not known whether DEX or MPA can disrupt mucosal barrier functions in the upper FGT by altering TJ gene expression. One way to determine this is by growing

epithelial cells from the FGT on permeable Transwell filters and using transepithelial electrical resistance (TER) measurements to evaluate barrier function after exposing the monolayers to MPA or DEX in the presence or absence of HIV-1.

These experiments were conducted in the laboratory of Prof. Charu Kaushic at McMaster University, Hamilton (Ontario, Canada) and had as aim to verify whether MPA like DEX cooperates with HIV-1 to disrupt the mucosal integrity of confluent PGECs. PGECS were isolated from the endometrium and endocervix using the protocol established by Kaushic *et al* (2011). Confluent epithelial monolayers were pre-treated for 24 hours with 100 nM DEX, MPA or 0.1% vehicle in serum-free media. Thereafter, the monolayers were exposure to 10^5 IU/mL HIV-1 IIIB (X4 tropic) for 24 hours. Pre- and post-exposure TER measurements were recorded. Supernatants from the apical chamber were collected for TNF-alpha ELISA.

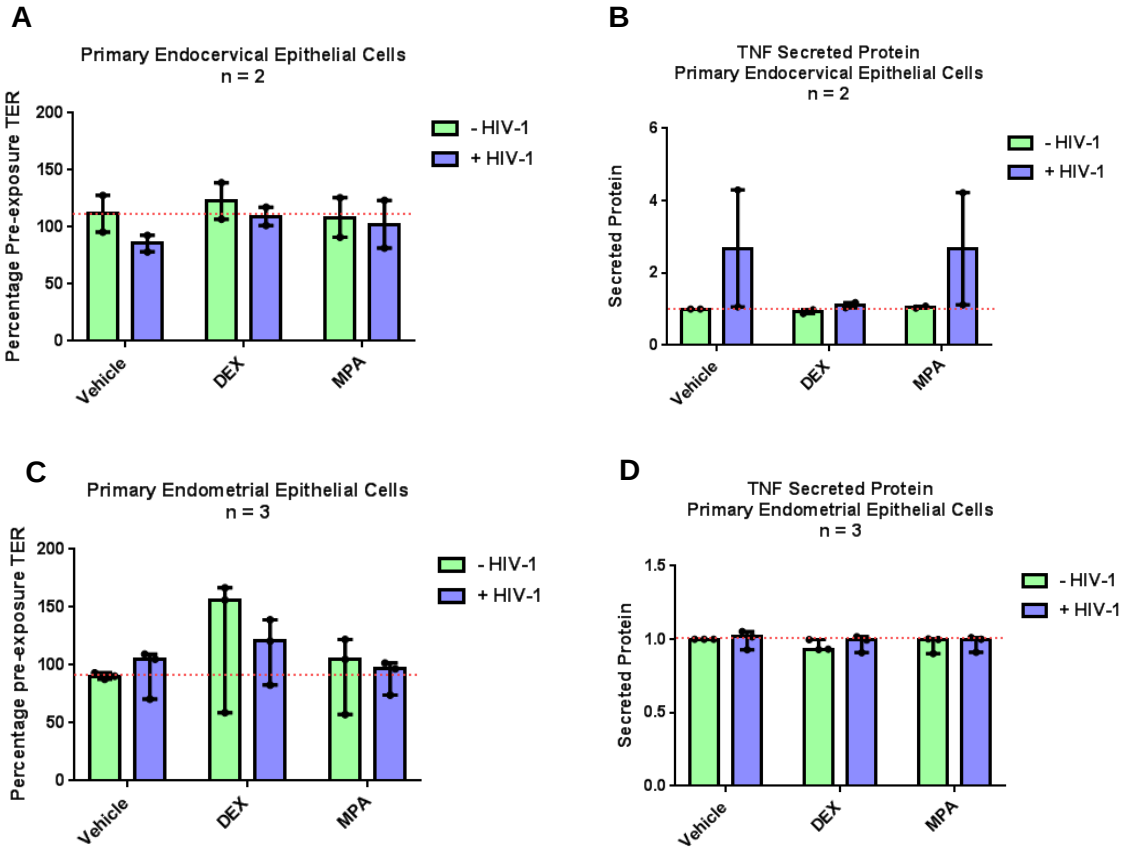


Figure 5.2.2.2: DEX, MPA or HIV-1 IMCs did not alter barrier function of primary genital epithelial monolayers. In **(A**, **C**), confluent primary epithelial cells from the FGT where pre-treated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) for 24 hours, and thereafter exposed to 10^5 IU/mL HIV-1 IIIB (X4-Tropic,) or virus control made up in fresh serum-free media containing 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) for another 24 hours. NB: pre-virus supernatants were not washed away. TER measurements were taken before and after exposure to hormones and HIV-1 and expressed as percentage pre-exposure [i.e. (TER after HIV-1 exposure/TER

before hormonal pre-treatment) X 100]. In (**B**, **D**), Supernatants collected after HIV-1 exposure were used to determine by ELISA the level of secreted TNF. The data includes at least two independent biological repeats (endocervical, n = 2; and endometrial, n = 3) was plotted as median with 95% confidence interval in GraphPad Prism 7 software.

As shown in **Figure 5.2.2.2 A** and **C**, neither DEX nor MPA reduced the TER of confluent primary endometrial and endocervical epithelial cells. Similarly (and contrary to expectations), HIV-1 exposure failed to decrease the TER of confluent cultures both in the absence and presence of DEX or MPA. HIV-1 exposure also failed to induce TNF production in all but in one endocervical sample (**Figure 5.2.2.2 B**, **D**). It is not known whether the failure of HIV-1 to induce a pro-inflammatory response resulted in its inability to disrupt the mucosal barriers evaluated herein. It should be noted that no significant reduction in TER was recorded for the endocervical experiment that saw TNF induced by HIV-1 exposure.

Taken together, these results show that MPA like NET does not regulate the expression of TJ genes in the ectocervix in vitro, but unlike NET downregulates the expression of desmoglein-1. Considering that MPA downregulated select TJ genes in End1/E6E7, it might be that MPA targets these genes to disrupt mucosal barrier function in the endocervix, but not ectocervix. However, MPA did not alter mucosal barrier function of primary endometrial and endocervical epithelial cells. Thus, it remains to be established whether MPA can increase the permeability of upper FGT mucosal tissues.

Attempts were made to investigate this question using End1/E6E7 cells. It was investigated whether this endocervical epithelial cell line form confluent monolayers and thus be used as an in vitro model to study mucosal barrier functions of the endocervix. Using confocal microscopy, it was observed that End1/E6E7 cells (grown on cover-slips) form discontinuous claudin-4 TJs (**Appendix C, Figure 3 A**). Continuous TJs are formed when the TJ proteins are expressed around the apical perimeter of neighbouring epithelial cells creating a structure that resembles honey-comb lattices in the field of view. The formation of discontinuous TJs are a result of the cells growing on top of each other forming a multi- rather than a monolayer (**Appendix C, Figure 3 B**). This suggests that End1/E6E7 cells are not a suitable in vitro model to study the mucosal barrier properties of the endocervical epithelial cells

5.2.3: MPA like DEX, but not NET, upregulates TNF- or LPS-induced increase in HIV-1 replication in TZM-bl Cells.

Next, it was investigated whether DEX, MPA and NET regulate HIV-1 replication in TZM-bl indicator cells in the presence of immune activators. In this regard, TZM-bl cells were exposed to 10 IU/mL $HIV_{BaL\text{-}Renilla}$ for 24 hours and subsequently treated with 100 nM DEX, MPA or NET in the presence or absence of 1.15 nM TNF for another 48 hours before measuring replication. At the end of this incubation period, cells were harvested in the Bright-Glo™ Luciferase Assay System reagent and luminescence quantified using the luminometer. As shown in **Figure 5.2.3.1 A**, DEX and maybe MPA increased HIV-I replication in TZM-bl cells when compared with virus only control. TNF also significantly increased HIV-1 replication, and together with DEX or MPA induced an even bigger increase in replication. In contrast, NET did not alter basal nor TNF-induced increase in HIV-1 infection and replication. This result suggests that DEX and MPA positively cooperative with TNF to enhance HIV-1 infection of TZM-bl cells.

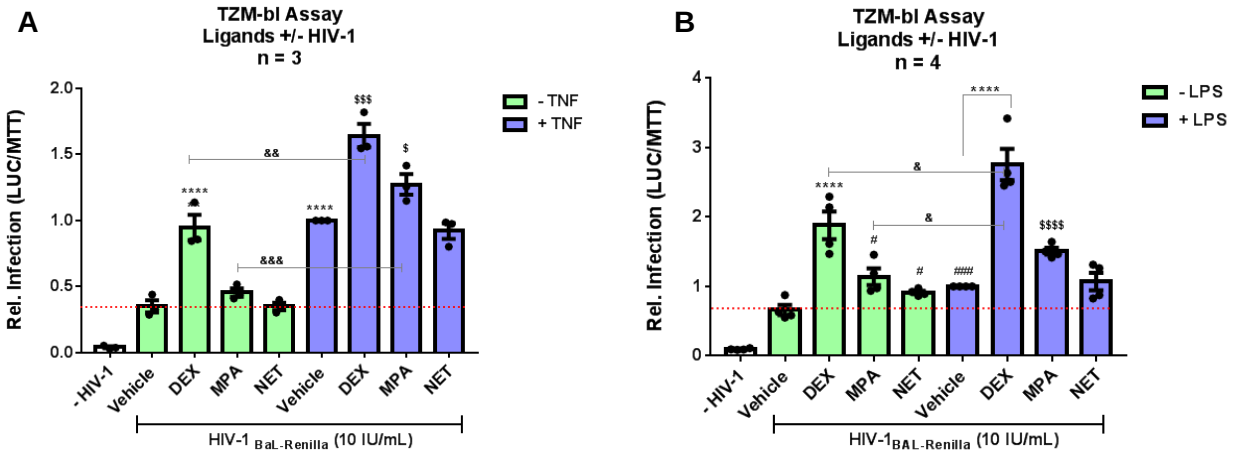


Figure 5.2.3.1: DEX and MPA, unlike NET, enhance HIV-1 replication in TZM-bl cells in the absence and presence of TNF or LPS. TZM-bl cells were treated with 10 IU/mL of HIV-$_{1BaL\_Renilla}$ IMCs or Mock control for 24 hours. Thereafter, the cells were co-stimulated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) and 1.15 nM TNF (**A**) or 5 µg/mL LPS (**B**) for another 48 hours. At the end of the 72 hours incubation period with HIV, luciferase activity was quantified using the Bright-Glo™ Luciferase Assay System reagent (Promega, USA) on the Turner Biosystems Modulus Microplate reader (Promega, USA). Relative light units (RLU) were normalised relative to MTT optical density (OD) scores from a parallel experiment. The data represent at least three independent biological repeats plotted as mean ± SEM in GraphPad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons. Statistical significance is denoted as **** to indicate p<0.0001 for comparisons between vehicle only and other treatments or between pair of treatments. In some cases, the unpaired Student's t-test was used to compare pair of treatments; when comparison was between vehicle and other treatments, statistical significance is denoted as $^{\#}$ to indicate p<0.05; when comparison was between TNF or LPS and other treatments significance is denoted as $^{\$}$, $^{\$\$\$}$ or $^{\$\$\$\$}$ to indicate

p<0.05, p<0.005 and p<0.0001, respectively; when comparison was between other pair of treatments significance is denoted as $^{\&}$, $^{\&\&}$ or $^{\&\&\&}$ to indicate p<0.05, p<0.01 and p<0.005, respectively.

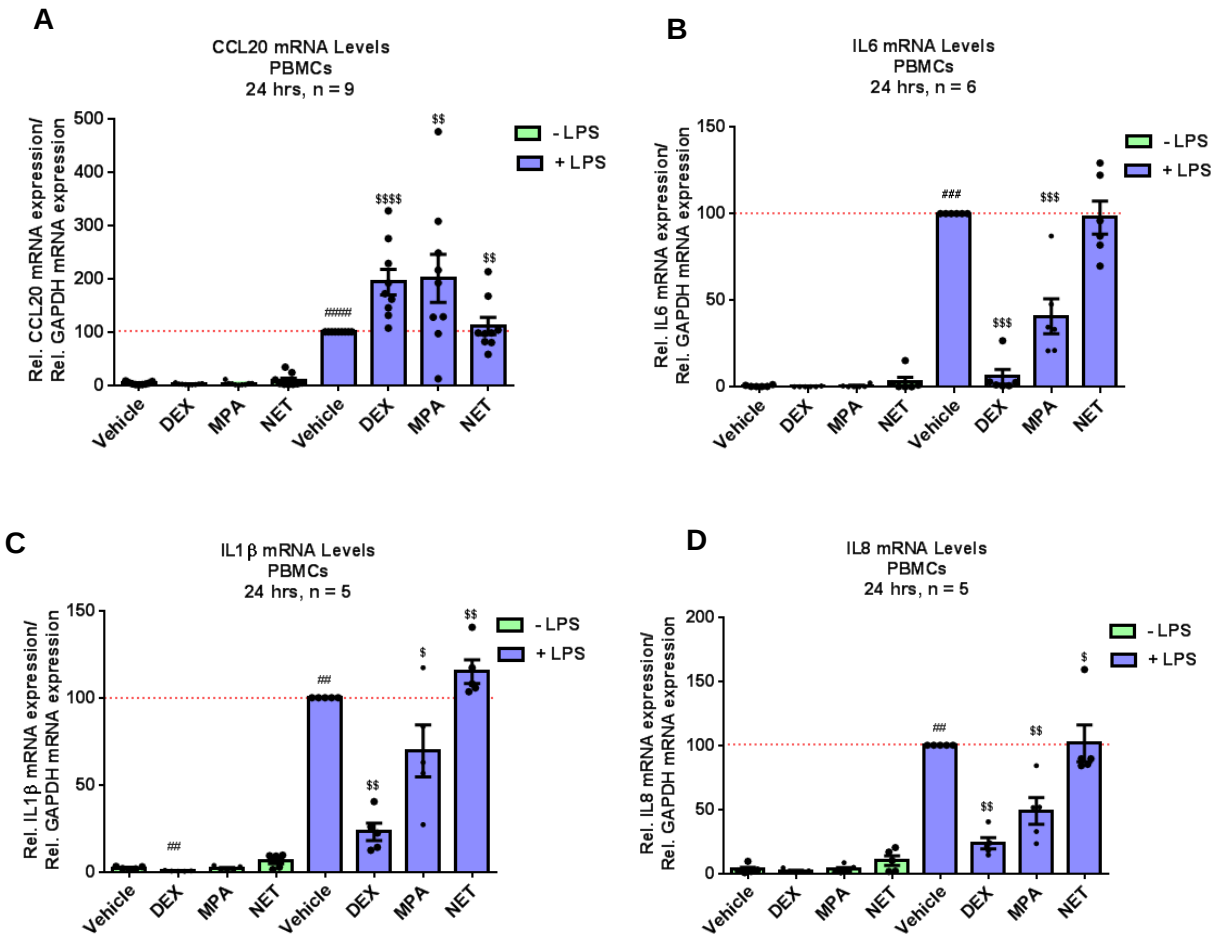
Next, it was investigated whether DEX and MPA can cooperate with LPS to enhance HIV-1 infection and replication. In this regard, TZM-bl cells were exposed to 10 IU/mL $HIV_{BaL\text{-}Renilla}$ IMCs or no virus control for 24 hours and then treated with 100 nM DEX, MPA or NET in the presence or absence of 5 µg/mL LPS for another 48 hours before measuring replication. As shown in **Figure 5.2.3.1 B**, DEX, MPA and NET significantly increased HIV infection when compared to vehicle control. The ability of NET to slightly enhance infection and replication in this experiment was unexpected as it did not induce a similar effect in **Figure 5.2.3.1 A**. Treating TZM-bl cells exposed to HIV-1 with LPS resulted in an increase in infection and replication. DEX and MPA enhanced HIV replication in the presence of LPS when compared with LPS only or hormones only. Although NET increased infection and replication in the absence of LPS in this experiment, it did not enhance LPS-induced increase in infection and replication. Moreover, when data from experiments in **Figure 5.2.3.1** were pooled (experiments done in parallel), MPA but not NET enhanced HIV-1 infection and replication (**Appendix C, Figure 4**).

5.2.4: MPA and NET differentially and selectively regulate pro-inflammatory gene expression in PBMCs.

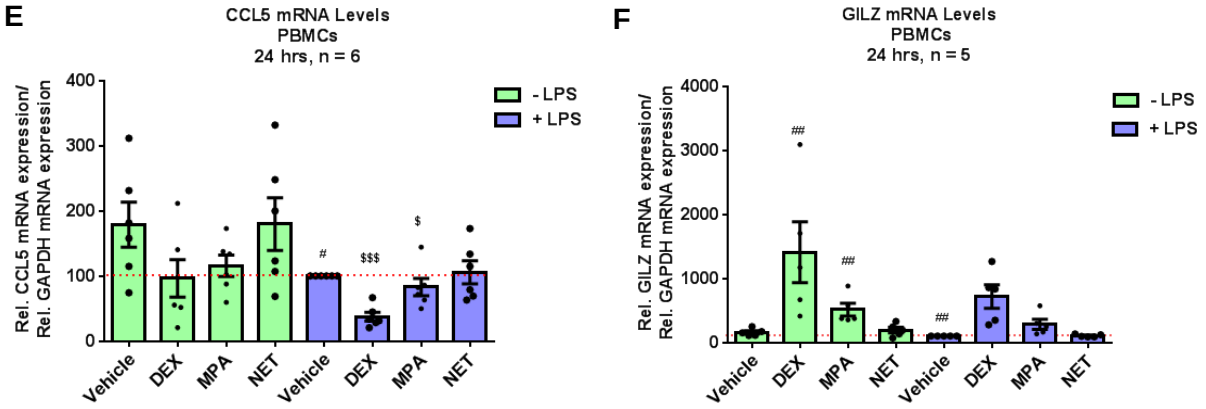
In the previous chapters, it was shown that MPA like DEX, but unlike NET, differentially regulate the expression of select immune mediators in the presence of immune activators in End1/E6E7 cells. It was next investigated whether and how DEX, MPA and NET regulate the expression of pro-inflammatory immune mediators in the presence of immune activators in PBMCs. PBMCs isolated from healthy female donors were treated with either 100 nM DEX, MPA or NET in the absence or presence of immune activators (LPS or TNF) for 24 hours. Only results from experiments involving LPS have been reported herein as TNF failed to induce the expression of immune mediators investigated (data not shown).

As shown in **Figure 5.2.4.1 A - D**, treatment with DEX significantly reduced IL1β, but did not alter CCL20, IL6 and IL8 mRNA levels. The presence of MPA did not change basal CCL20, IL6, IL1β, IL8 and CCL5 mRNA expression (**Figure 5.2.4.1 A – D** and **Figure 5.2.4.1 A - D**). NET did not significantly alter basal CCL20, IL6, IL1β and IL8 and CCL5 mRNA levels (**Figure 5.2.4.1 A – D** and **Figure 5.2.4.1 A – D**). It was also observed that MPA like DEX, but unlike NET, significantly induced GILZ mRNA expression (**Figure 5.2.4.1 F**).

Treating PBMCs with LPS resulted in an increase in CCL20, IL6, IL8 and IL1β mRNA levels (**Figure 5.2.4.1 A - D**). On the contrary, LPS significantly reduced basal CCL5 and GILZ mRNA levels (**Figure 5.2.4.1 E**, **F**). As was the case with End1/E6E7 cells, MPA like DEX significantly enhanced LPS-induced CCL20 mRNA expression; in contrast, they suppressed LPS-induced IL6 and IL8 mRNA expression (**Figure 5.2.4.1 A – D**). MPA like DEX significantly induced a reduction in CCL5 mRNA levels in the presence of LPS in the same degree as they would in the absence of LPS (**Figure 5.2.4.1 E**). Although not statistically significant, LPS appeared to inhibit DEX- and MPA-induced increase in GILZ mRNA levels (**Figure 5.2.4.1 F**). On the other hand, NET in the presence of LPS slightly reduced CCL20 and IL8 mRNA levels, slightly increased IL1β mRNA levels, but did not alter IL6 and CCL5 mRNA levels (**Figure 5.2.4.1 A – E**).



5.2.4.1: DEX, MPA and NET selectively regulate CCL20, IL6, IL1β, IL8, CCL5 and GILZ mRNA levels in a gene-specific manner in the presence of LPS in PBMCs. (continued on the next page)



5.2.4.1: DEX, MPA and NET selectively regulate CCL20, IL6, IL1β, IL8, CCL5 and GILZ mRNA levels in a gene-specific manner in the presence of LPS in PBMCs. PBMCs were treated with 100 nM DEX, MPA, NET or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 5 µg/mL LPS for 24 hrs**.** Thereafter, the cells were harvested in Tri-Reagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A**), IL6 (**B**), IL1β (**C**), IL8 (**D**), CCL5 (**E**), GILZ (**F**) were determined by qPCR using gene specific primers and normalised to GAPDH mRNA levels. Relative fold changes were calculated with reference to LPS only, which has been set to 100%. The data from at least 5 subjects was plotted in Graph Pad Prism 7 software as mean ± SEM. Statistical analysis was performed using the non-parametric Kolmogorov-Smirnov test. For comparisons between vehicle only and other treatments, statistical significance is denoted as $^{\#}$, $^{\#\#}$, $^{\#\#\#}$ or $^{\#\#\#\#}$ to indicate p<0.05, p<0.01, p<0.005 and p<0.0001, respectively. When the comparison was between LPS and other treatments, statistical significance is denoted as $^{\$}$, $^{\$\$}$, $^{\$\$\$}$ or $^{\$\$\$\$}$ to indicate P<0.05, p<0.01, p<0.005 and p<0.0001, respectively.

It has previously been established that PBMCs express the GR and not the PR (Tomasicchio *et al*., 2013). Therefore, to verify whether the GR was involved in the MPA-induced enhancement of LPS-induced CCL20 mRNA expression, PBMCs were pre-treated with 1 µM RU486 for 2 hours before co-treatment with 100 nM MPA and 5 µg/mL LPS for 24hrs. As shown in **Figure 5.2.4.2**, LPS and MPA cotreatment appeared to increase (although not statistically significant) CCL20 mRNA expression and this was prevented in the presence of RU486. This result suggests that the GR might be involved in the MPA enhancement of CCL20 transcriptional regulation by LPS in PBMCs. However, due to Inter-donor variations in response to MPA, more biological repeats would be required to improve the power of the statistical analysis.

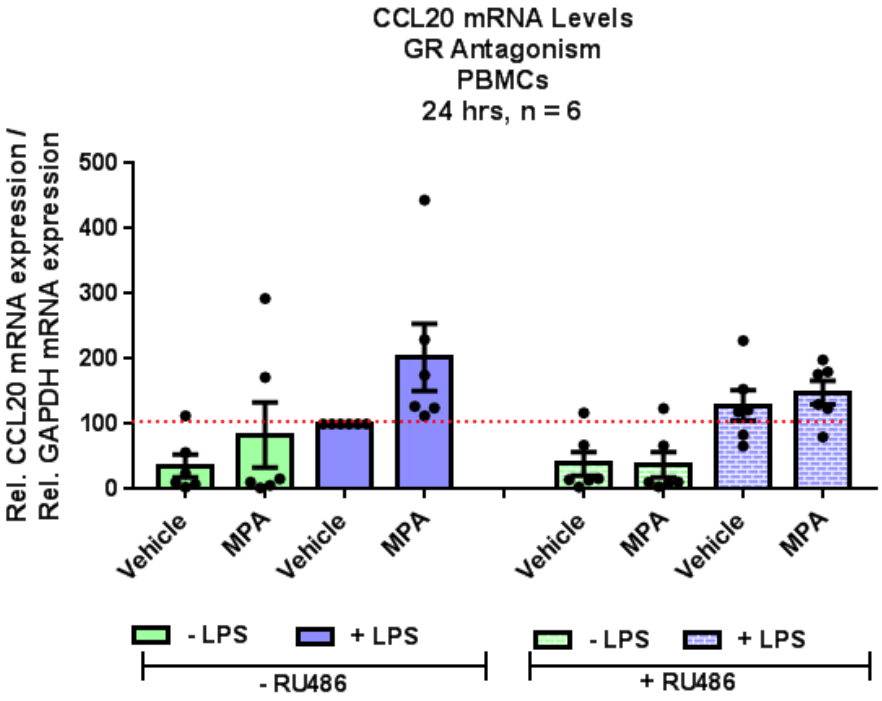


Figure 5.2.4.2: The GR appears to be required for the MPA enhancement of LPS-induced CCL20 mRNA expression in PBMCs. PBMCs isolated from 6 healthy female donors were pre-treated for 2 hours with 1 µM RU486 or 0.1% (v/v) EtOH (vehicle) and thereafter treated with 100 nM MPA or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 5 µg/mL LPS for another 24 hours. The cells were then harvested in Tri-Reagent®, total RNA isolated and converted to cDNA and relative CCL20 mRNA level was determined by qRT-PCR performed using gene specific primers and normalised to GAPDH mRNA level. The relative effects of steroid hormones and LPS on mRNA levels of the CCL20 were determined by normalising data to LPS, which has been set to 100%. The data from 6 donors was plotted in Graph Pad Prism 7 software as mean ± SEM. Statistical analysis was performed using the Kruskal-Wallis test with Dunn's multiple comparisons test.

Next, it was determined whether DEX, MPA and NET can regulate LPS-induced secretion of CCL20 by PBMCs. Supernatants collected from the experiment described in **Figure 5.2.4.2** were used to perform a CCL20 ELISA. As shown in **Figure 5.2.4.3**, LPS induced CCL20 protein secretion by PBMCs and this was strongly enhanced by DEX and MPA but not NET. Additional ELISAs also found that MPA unlike NET suppresses LPS-induced secretion of IL6, CCL5 and possibly IL1β by PBMCs.

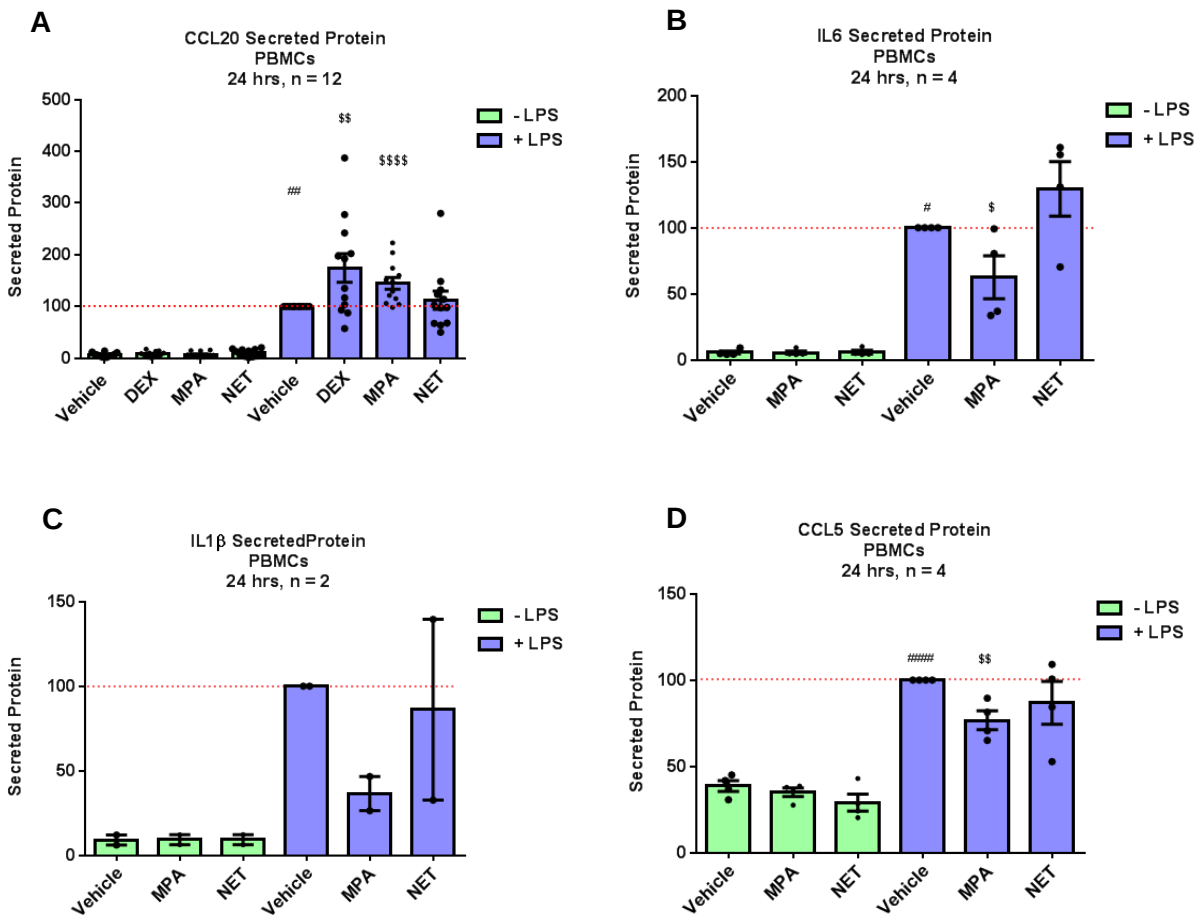


Figure 5.2.4.3: MPA, unlike NET, enhances CCL20, but suppressed IL6, CCL5 and maybe IL1β LPS-induced protein secretion by PBMCs. PBMCs isolated from 12 healthy female donors were treated with 100 nM DEX, MPA, NET or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 5 µg/mL of LPS for 24 hrs. Thereafter, supernatants were collected, and ELISA assays performed to measure the levels of CCL20 (**A**), IL6 (**B**), IL1β (**C**) and CCL5 (**D**). To determine fold changes, the data was normalised relative to LPS, which is set to 100% and plotted in Graph Pad Prism 7 software as mean ± SEM. Statistical analysis was performed using the Kolmogorov-Smirnov test. For comparison between vehicle only and other treatments, statistical significance denoted as $^{\#}$, $^{\#\#}$, or $^{\#\#\#\#}$ to indicate $p<0.05$, $p<0.01$ and $p<0.0001$, respectively. For comparisons between LPS and other treatments, statistical significance denoted as $^{\$}$, $^{\$\$}$ or $^{\$\$\$}$ to indicate $p<0.05$, $p<0.01$ and $p<0.005$, respectively.

Taken together, these results suggest DEX, MPA and NET differentially regulated LPS-induced CCL20, IL6, IL1β, IL8 and CCL5 mRNA expression in PBMCs. Like with End1/E6E7 cells, MPA like DEX enhanced LPS-induced CCL20, but inhibited LPS-induced IL6, IL1β and IL8 mRNA expression. Additionally, MPA like DEX suppressed CCL5 mRNA expression in the presence of LPS in PBMCs. This finding corresponded with the inhibition of TNF-induced CCL5 mRNA expression in End1/E6E7 cells by DEX and MPA: the effect of LPS on CCL5 mRNA expression was not investigated in End1/E6E7 cells. Whereas NET did not affect LPS-induced mRNA expression in End1/E6E7 cells, it however slightly inhibited CCL20 and IL8, but enhanced IL1β mRNA expression in response to LPS in PBMCs. Unlike

in End1/E6E7 cells, basal mRNA levels of CCL20, IL6, IL8 and CCL5 was not altered by DEX and MPA in PMBCs. Only DEX significantly inhibited IL1β mRNA expression in PBMCs.

Besides regulating mRNA expression, DEX and MPA, unlike NET, also modulated LPS-induced protein secretion. Results from this study suggest MPA like DEX enhanced LPS-induced CCL20 secretion by PBMCs, confirming previous observations in End1/E6E7 cells. In addition, the results also suggest MPA, but not NET inhibit LPS-induced IL6, CCL6 and IL1β secretion by PBMCs. By selectively enhancing CCL20, while suppressing Il6, IL8, IL1β and CCL5 expression MPA might be controlling a pathway that could enhance HIV-1 infection.

5.2.5: Supernatants from PBMCs co-treated with MPA and LPS, but not NET and LPS, enhance HIV-1 replication in TZM-bl cells

Cytokines and chemokines play an important role in HIV-1 infection particularly in reactivating HIV-1 replication in latently infected target cells (Chun *et al*., 1998; Cameroon *et al*., 2010). Changes in their secretion profile and concentrations might affect the ability of HIV-1 to infect and replicate in target cells. Considering that MPA and NET differentially regulate LPS-mediated expression of pro-inflammatory cytokines in PBMCs, it was next investigated whether supernatants from PBMCs treated with MPA or NET in combination with LPS would differentially modulate HIV-1 replication. To this end, supernatants collected from experiments described in **Figure 5.2.5** and control ligand-conditioned media were diluted 4-fold and the diluted solutions used in add-back TZM-bl HIV-1 infection assays. In brief, pre-seeded TZM-bl cells or HIV-$1_{BaL\text{-}Renilla}$ IMCs at 10 IU/mL were incubated separately in the diluted supernatants or ligand-conditioned control media for 1 hour. Thereafter, the virus was added to the cells and incubated for another 72 hours, at the end of which the cells were harvested and assayed for infection and replication. As shown in **Figure 5.2.5 A.**, when compared with supernatant from cells treated with vehicle-only, supernatants from PBMCs treated with LPS, MPA or NET had no effect on HIV-1 infection and replication. However, supernatants from PBMCs co-treated with MPA and LPS, but not NET and LPS significantly increased HIV-1 replication in TZM-bl cells when compared with LPS-only supernatants. No change in replication was observed in TZM-bl cells exposed to ligand-conditioned control media (**Figure 5.2.5 B**).

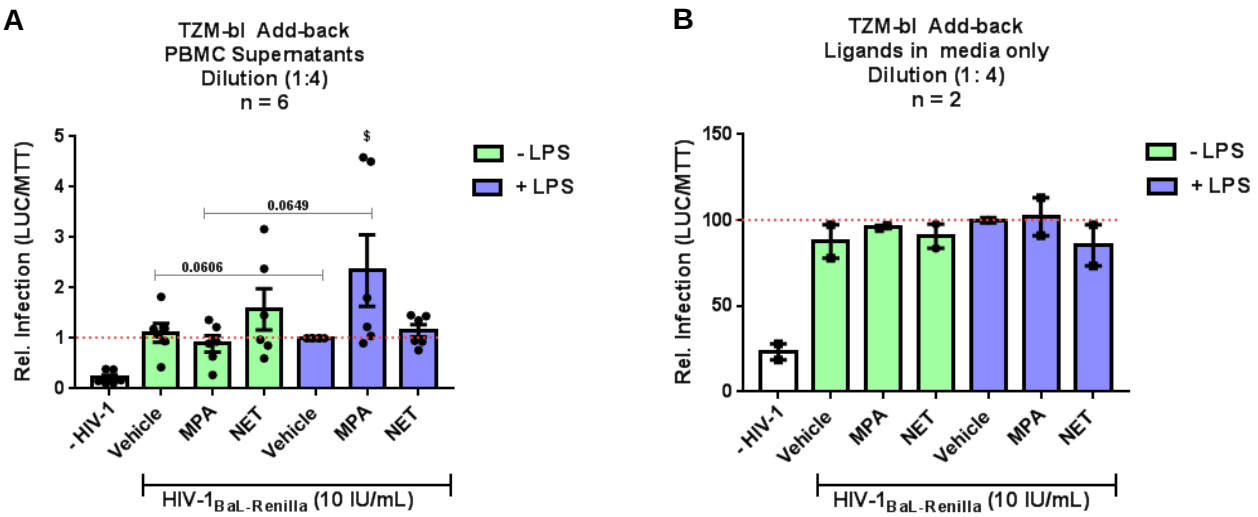


Figure 5.2.5: Conditioned media from PBMCs co-treated with MPA and LPS, but not NET and LPS increase HIV-1 infection and replication in TZM-bl cells. (**A**) TZM-bl cells were pre-exposed for 1 hour to supernatants from PBMCs incubated with 100 nM MPA, NET or 0.1% (v/v) EtOH alone or in combination LPS. The cells were subsequently treated for 72 hours with 20 IU HIV-1_{BaL} that was made up and equilibrated in PBMC supernatants. (**B)** TZM-bl cells were treated similarly as in (**A**) except that PBMC supernatants was replaced with culture media containing ligands only, which was set up in parallel with the PBMC experiment described in (**A**). At the end of the 72 hours incubation period with HIV-1, luciferase activity was quantified using the Bright-Glo™ Luciferase Assay System reagent (Promega, USA) on the Turner Biosystems Modulus Microplate reader (Promega, USA). Relative light units (RLU) were normalised relative to MTT optical density (OD) scores from a parallel experiment. The data is normalised relative to LPS, which is set to 100% and plotted in Graph Pad Prism 7 software as mean ± SEM. Statistical analysis was performed using Kolmogorov-Smirnov test to compare treatment pairs and statistical significance denoted as $ to indicate $p<0.05$.

Taken together, these results suggest that secreted soluble mediators from PBMCs treated with LPS do not affect HIV-1 infection and replication. However, the presence of MPA, but not NET modifies the secretome of LPS-treated PBMCs in a manner that favours HIV-1 infection and replication.

5.7: Summary of Findings

This chapter showed that MPA selectively supressed basal GCSF, IL8 and possibly IL6 protein secretion in ectocervical tissue explants, in a gene-specific manner. It was also found that MPA might increase TNF-induced CCL20 mRNA expression in some but not all samples due to larger sample variance. This observation, although not statistically significant, was unique to MPA as neither P4 nor NET enhanced CCL20 mRNA expression. It was also observed that MPA in the absence of TNF might enhance CCL20 protein secretion in some but not all tissue samples.

It was established that MPA like NET does not significantly regulate TJ mRNA levels; but unlike NET MPA was found to reduce desmoglein-1 mRNA expression in ectocervical tissue explants. This suggests that MPA might regulate desmosomal but not TJ gene expression in ectocervical mucosal tissues. MPA alone or in the presence of HIV-1 IMCs did not alter mucosal barrier integrity of primary genital epithelial cells. Thus, it could not be established whether synergistic interactions between MPA and HIV-1 were involved in the regulation of mucosal barrier function.

It was observed that MPA unlike NET acted singly or additively with immune activators to enhance HIV-1 infection and replication in the presence in TZM-bl cells. This suggest that MPA but not NET might cooperate with immune activators in the FGT or blood to increase HIV-I infection and replication.

This chapter also established that MPA unlike NET enhanced CCL20 mRNA levels, but inhibited IL6, IL1β, IL8 and CCL5 mRNA levels in the presence but not absence of LPS in PBMCs. In addition, MPA enhanced CCL20, but inhibited IL6, CCL5 and potentially IL1β protein secretion by PBMCs in the presence of LPS. Finally, conditioned media from PBMCs exposed to MPA and LPS enhanced HIV-1 infection and replication in TZM-bl cells when compared with supernatants from PBMCs treated with LPS or possibly MPA alone. Finally, it was observed that NET enhanced IL1β, but inhibited CCL20 and IL8 mRNA expression in the presence of LPS in PBMCs. However, these changes in mRNA expression induced by NET were not reflected in protein levels. In addition, supernatants from PBMCs co-treated with NET and LPS did not affect HIV-1 infection and replication. This suggest that NET-induced changes in protein secretion, unlike those induced by MPA, do not affect HIV-1 replication.

Chapter Six

Discussion, Conclusions and Future Perspective

6.1: Discussion

6.1.1: Limitations of the current study

One of the limitations of the current study is that all of the experiments were performed in vitro, raising the question of whether these effects occur in vivo in women. One of the initial objectives was to investigate hypotheses and establish mechanisms in End1/E6E7 cells and then investigate whether these occurred in primary endocervical epithelial cells isolated from endocervical tissues obtained from pre-menopausal women. Due to constraints in obtaining endocervical tissues, only a few experiments could be done using primary endocervical epithelial cells. Hence experiments were performed using other model systems such as primary endometrial epithelial cells and ectocervical tissue explants. Previous studies showed that End1/E6E7 cells, PBMCs and TZM-bl cells express mRNA and protein for the GR, but not the PR (Govender *et al*., 2014; Tomasicchio *et al*., 2013; Maritz *et al*., 2018). Ectocervical tissues have been reported to express both receptors (Ray *et al*., manuscript under review). Therefore, the effects elicited by MPA like those elicited by GCs in End1/E6E7 cells, TZM-bl cells and PBMCs are more likely to be mediated by the GR but not the PR. On the other hand, the GR as well as PR may mediate these effects in ectocervical tissues. Thus, the results obtained in End1/E6E7 cells may be different to those in primary endocervical cells. While in vitro studies can investigate direct effects of ligands on target cells or tissue and control for the experimental environment, whether such effects occur in women is difficult to investigate due to complex interconnected endocrine and other organismal indirect effects, as well as confounding intrinsic factors of the study population. Nevertheless, several of the findings from this study are consistent with published clinical data, as discussed in detail in this chapter. Furthermore, the in vitro findings provide novel proof of concept and mechanistic insights into possible side effects, choice of progestins and mechanisms for further investigation in primary cell and tissue models and in vivo.

6.1.2: MPA but not NET downregulates genes associated with maintaining endocervical mucosal barrier integrity and selectively upregulates expression of CCL20 and TLR2 in the endocervical epithelial cell line in the absence of immune activators

The current study has shown for the first time that MPA unlike NET downregulates the expression of select TJ genes in the End1/E6E7 endocervical epithelial cell line. While it is difficult to translate in vitro results to in vivo effects, this indicates that DMPA might increase the permeability of the endocervix via

this mechanism in women. This is consistent with findings from a clinical study showing that DMPA use disrupts the mucosal barrier of the ectocervix in women (Quispe-Calla *et al*., 2016). However, others have shown that DMPA-IM increases claudin-8 expression in the cervical transition zone in vivo (Goldfien *et al*., 2015). This suggests that MPA may enhance the integrity of the mucosal barrier of the transition zone of the cervix in women (Goldfien *et al*., 2015). Thus, it is possible that effects of DMPA on the mucosal barrier in women are tissue-specific and differ between the endocervix and other compartments of the FGT.

The results also show that MPA like GCs, but unlike NET, increases CCL20 mRNA levels against the backdrop of reducing IL6 and IL1β mRNA levels in End1/E6E7 endocervical epithelial cell lines. This is consistent with findings from a recent in vitro study showing that MPA increases CCL20 mRNA levels in primary endometrial epithelial cells (Woods *et al*., 2018). However, the increase in CCL20 mRNA levels observed in End1/E6E7 cells in the presence of MPA may not result in a corresponding increase in the levels of secreted CCL20 protein. However, it is possible that a change in protein levels occurs at a different time to that measured or under different experimental conditions. Evidence from previous clinical studies showed that DMPA-IM use did not alter the levels of CCL20 protein secreted in the FGT in heathy women in vivo (Morrison *et al*., 2014; Fichorova *et al*., 2015). Thus, it is possible that MPA, in the absence of immune activators, regulates CCL20 mRNA levels, but not CCL20 protein levels secreted by genital epithelial cells. Whether this happens in women is unclear since none of the published clinical or in vitro studies (Woods *et al*., 2018; Morrison *et al*., 2014; Fichorova *et al*., 2015) simultaneously assessed mRNA and protein expression. This study is the first to do so. However, there is evidence from an in vitro study showing that GCs, in the absence of immune stimulation, regulate both CCL20 mRNA and protein expression by bronchial epithelial cells (Zijlstra *et al*., 2014). The lack of correlation between mRNA and protein expression in End1/E6E7 endocervical epithelial cells suggests that MPA might increase the stability of CCL20 mRNA transcripts in the cytoplasm but decrease the rate at which they are translated into protein and secreted (Greenbaum *et al*., 2003). This could increase the half-life and therefore the relative abundance of CCL20 mRNA transcripts. This is consistent with findings from an in vitro study showing that DEX increased calgranulin A mRNA levels but not protein levels in thioglycolate-elicited murine macrophages; however, stimulation with LPS increased protein expression in DEX-treated compared to controls (Hsu *et a*l., 2005). Others have also described a mechanism whereby superantigens induced the formation of cytoplasmic TNF, IL8, CCL3 and CCL4 mRNA "reserves" in HA1.7 $CD4^+$ T cell clones and how these reserves are rapidly translated into protein and secreted after anti-CD3 and IL2 activation (Schall *et al*., 1992). It is possible that MPA induces the formation of CCL20 mRNA reserves in End1/E6E7 endocervical epithelial cell lines, which sensitives them towards a rapid CCL20-specific response in the presence of immune activators. Alternatively, MPA might increase CCL20 mRNA levels, but might not affect its translation to protein or the post-translational modifications that are required in order for the newly synthesised protein to be secreted.

The thesis results also suggest that by upregulating TLR2 expression in End1/E6E7 endocervical epithelial cells in vitro, MPA like DEX might enhance the sensitivity of these cells for TLR2 agonists. This agrees with results from previous studies showing that HeLa and airways epithelial cells primed with DEX responded vigorously to stimulation to TLR2 agonists. (Imasato *et al*., 2002; Shuto *et al*., 2002; Hermoso *et al*., 2004; Sakai *et al*., 2004; Homma *et al*., 2004; Busillo *et al*., 2011; Frank *et al*., 2014). The present study did not investigate whether MPA upregulates TLR2 protein levels in End1/E6E7 cells. However, Findings from a previous study showed that DEX increased TLR2 protein levels in HeLa cells (Sakai *et al*., 2004). Because MPA and DEX elicited similar effects on gene expression in End1/E6E7 and HeLa cells (Govender *et al*., 2014), it is very likely that MPA upregulates TLR2 protein levels in End1/E6E7 cells as well. Nevertheless, future experiments will investigate whether MPA indeed upregulates TLR2 protein levels in End1/E6E7 cells. It remains unknown whether DMPA upregulates TLR2 expression in vivo.

It has been reported that $CD4^+$ T cells treated in vitro with TLR2 agonists are more permissive to HIV-1 infection than control cells (Thibault *et al*., 2007; Ding *et al*., 2010). In addition, it has been demonstrated that $CD4^+$ T subsets with high levels of expression of TLR2 such as Th17 cell are more susceptible to HIV-1 infection in vitro than cells with low expression of the receptor (Bolduc *et al*., 2017). Therefore, the result that MPA induces TLR2 expression in End1/E6E7 cells suggests that MPA might enhance HIV-1 uptake by endocervical cervical epithelial cells via TLR2. Although they are not HIV-1 target cells, endocervical epithelial cells have been shown to take up HIV-1 viral particles via clathrin-dependent endocytosis – a process enhanced in the presence of MPA (Ferreira *et al*., 2015a). Interestingly, TLR2 ligation in some cell types have been shown to enhance clathrin-dependent endocytosis in vitro (Shen *et al*., 2014). Thus, it possible that the MPA-induced increase of TLR2 expression might enhance clathrin-dependent TLR2-mediated endocytosis of HIV-1 viral particles into endocervical epithelial cells, and in doing so, prime the cells for increased HIV-1 entry.

The current study did not find evidence suggesting that MPA alters the sensitivity of the End1/E6E7 cells to TRL4 ligands. However, endometrial biopsies from women using DMPA-IM have been reported to express lower levels of TLR3 and TLR4 in vivo compared to controls. This suggests the MPA might reduce the ability of the endometrium to respond to TLR3 and TLR4 ligands (Goldfien *et al*., 2015). It has been reported by a recent in vitro study that MPA upregulates TNF receptor 2 (TNFRSF1B) mRNA expression in primary endometrial cells (Woods *et al*., 2018). The current study found that MPA does not regulate basal TNFRSF1B mRNA levels in End1/E6E7 cells. Collectively these studies suggest that MPA alters the expression of specific innate immune receptors in genital epithelial cells in vivo to modulate their sensitivity to specific pathogen associated molecular patterns, including HIV-1 viral particles. Whether these effects are compartment-specific remains to be investigated.

The result that MPA does not alter CCL5 expression levels in End1/E6E7 endocervical epithelial cells in the absence of immune activators suggests that this may not be a mechanism whereby MPA might induce $CCR5^{+}CD4^{+}$T cells to infiltrate the endocervix. Current evidence from a clinical study that showed that healthy women using DMPA-IM had higher levels of CCL5 in cervical secretions compared to controls (Fichorova *et al*., 2015). Furthermore, others have shown that the proportion of $CCR5^{+}CD4^{+}$ T cells in the endocervix is higher in healthy women using DMPA-IM compared in controls (Smith-McCune *et al*., 2017; Byrne *et al*., 2016). However, the study by Smith-McCune and colleagues did not find elevated levels of CCL5 in CVL from women using DMPA-IM (Smith-McCune *et al*., 2017), suggesting that mechanisms other than CCL5 expression by endocervical epithelial cells are responsible for the increase in $CCR5^{+}CD4^{+}$ T cells in the endocervix. It is possible that DMPA-IM induces the expression of CCR5 on resident $CD4^{+}$ T cells in the endocervix. This is very likely as recent findings from two in vitro studies showed that MPA increased CCR5 expression on $CD4^{+}$T cells and in ectocervical tissues (Maritz *et al*., 2018; Ray *et al*., manuscript under review).

Collectively, the thesis results suggest that in the absence of genital tract infection or inflammation, MPA might enhance susceptibility of the FGT in vivo to HIV-1 infection by (i) enhancing endocervical mucosal permeability by downregulating claudin-4, (ii) enhancing the sensitivity of genital mucosal surfaces by upregulating TLR2 and (iii) increasing CCL20 mRNA reserves that may be rapidly converted into protein upon immune challenge with microbial or viral antigens.

6.1.3: MPA, unlike NET, acts synergistically with immune activators to upregulate select immune function, but not TJ genes in End1/E6E7 endocervical epithelial cells

This is the first study to report that TNF increases claudin-4, ZO-1 and potentially occludin mRNA levels in End1/E6E7 cells. However, this increase in TJ mRNA levels may not with associated with increases in protein levels and is consistent with observations made by others (Amasheh *et al*., 2010; Amoozadeh *et al*., 2017). As discussed in **section 6.1.2** for MPA, this suggests that TNF might have differential effects on mRNA and protein synthesis and stability.

TJ disruption is one of the potential mechanisms by which GTIs enhance the risk of HIV-1 acquisition in women (Burgener *et al*., 2015; Vitali *et al*., 2017). The present study found that MPA like DEX does not cooperate with immune activators to downregulate TJ gene expression in End1/E6E7 endocervical epithelial cells. Moreover, this study found that MPA in the presence of immune activators inhibits the expression of IL1β, which is known to disrupt TJs (Al-Sadi *et al*., 2007). This suggests that enhanced mucosal barrier impairment via TJ disruption might not be a mechanism by which DMPA further increases HIV-1 susceptibility in women with GTIs.

Unlike TJ genes, the current study is the first to show that MPA like GCs, but unlike NET, acts synergistically with immune activators to upregulate CCL20 expression in End1/E6E7 endocervical epithelial cells. This is consistent with findings from previous in vitro studies that GCs and immune activators synergised to upregulate CCL20 expression in bronchial epithelial cells and monocyte-derived macrophages (Zijlstra *et al*., 2014; van de Garde *et al*., 2014), although these studies did not investigate whether true synergism occurred. The current study also shows that MPA enhances TNFRSF1B expression by MPA in the presence of TNF in End1/E6E7 cells. Moreover, it was observed that the presence of LPS did not alter MPA-induced expression of TLR2 expression in End1/E6E7 cells. It is not clear why MPA and immune activators synergize to selectively upregulate CCL20 expression. The upregulation of innate immune receptors by MPA suggests that a feedforward regulatory loop might be involved. This is consistent with findings that GCs enhance pro-inflammatory responses by upregulating the expression of innate immune receptors specific for some immune regulators (Homma *et al*., 2004; Busillo *et al*., 2011). However, the current study did not investigate the involvement of TLR2 and TNFRSF1B in the upregulation of CCL20 expression by LPS or TNF in the presence of MPA.

The underlying molecular mechanisms for synergistic upregulation of CCL20 by MPA and TNF in End1/E6E7 cells were not investigated in the current study and so remain unclear. In addition, it is unclear why MPA, like GCs, selectively upregulates the expression of some pro-inflammatory genes but inhibits the expression of others in the same cell, although it was established that GR is involved in these responses. Basal and induced expression of pro-inflammatory genes in End1/E6E7 cells is very likely to occur via the MAPK and NFκB signalling pathways (Yang *et al*., 2015; Ayyar & Reddy, 2018). The MAPK signalling pathways is subject to feedback inhibition by MKP-1 (Shah *et al*., 2016; Newton *et a*l., 2017). Interestingly, MPA like DEX has been shown to induce MKP-1 expression in End1/E6E7 cells (Grantham, 2012). It is therefore possible that in the presence of MPA, MKP-1 mediates the deactivation of the MAPK pathway which results in the inhibition of basal IL6 and IL1β expression. This proposed mechanism is also likely involved in the repression of claudin-4 mRNA levels by MPA. However, this scheme does not explain why the opposite effect is observed with CCL20. Gene-specific responses are likely due to differences in intracellular signalling pathways, promoter architecture and chromatin structure.

It has recently been demonstrated that the phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) pathway is an alternative mechanism by which CCL20 and TNF expression are upregulated in A549 cells (Wang *et al*., 2016) and SiHa cervical epithelial cells, respectively (Yang *et al*., 2015). PI3K/AKT is deactivated by GCs in some cell types via a mechanism involving MKP-1-mediated deactivation of p38 MAPK (Lawan *et al*., 2018; Perdiguero *et al*., 2011; Zhao *et al*., 2009). This suggests that GCs should

also repress CCL20. However, in some cell types it has been demonstrated that GC-induced inhibition of PI3K/AKT prevents deactivation of forkhead box protein O1 (FOXO1) by phosphorylation. The active FOXO1 is unphosphorylated and resides in the nucleus where it has been shown to interact with the C/EBPβ binding site in the promoter of genes such as CCL20 and muscle ring finger-1 (Miao *et al*., 2012; Zhao *et al*., 2009). Therefore, it is possible that in End1E6E6 cells CCL20 is regulated by the PI3K/AKT pathway, whereas IL1β, IL6, IL8 and CCL5 are regulated by MAPK pathway. In this way, MPA like GCs can enhance CCL20 via MKP-1/p38 MAPK/PI3k/AKT-mediated activation of FOXO-1. Taken together, basal expression of proinflammation genes in End1/E6E7 cells is possibility mediated via MAPK pathway. In the presence of MPA, MKP-1 deactivates the MAPK pathway resulting in the repression of most pro-inflammatory genes. However, some genes depending on their promoters are selectively upregulated via a mechanism involving MKP-1-mediated deactivation of p38 MAKP/PI3K/AKT signalling loop.

The signalling loops described above may also explain why MPA like GCs selectively upregulate some genes, while repressing others in the presence of immune activators. However, the MKP-1/p38 MAPK/PI3K/AKT loop that activates FOXO-1 might be amplified in the presence of immune activators in order to synergistically upregulation CCL20 expression. This is possible as some immune activators induce MKP-1 activity and moreover deactivate PI3K/AKT (Shah *et al*., 2016; Newton *et al*., 2017). In addition, previous studies found that MPK-1-mediated deactivation of p38 and JNK MAPKs was required in the synergistic upregulation of TLR2 by GCs and immune activators in HeLa cells (Shuto *et al*., 2002; Imasato *et al*., 2002; Sakai *et al*., 2004; Hermosa *et al*., 2004). Interestingly, it has previously been reported that inhibiting JNK and ERK1/2, but not p38 activity enhanced TNF-induced expression of IL6 in End1/E6E7 cells in the presence of MPA (Verhoog, 2010). This suggests that MKP-1 might be an essential component in regulating the expression of pro-inflammatory genes including TNFRSF1B. How MKP-1 interacts with other signalling pathways may determine whether it's effects are pro- or anti-inflammatory.

Synergism between GCs and immune activators may amplify immune responses that impact on HIV-1 infection. For example, GCs have been shown to synergise with immune activators to upregulate HIV-1 replication in chronically infected cells in vitro (Bressler *et al*., 1993; Kinter *et al*., 2001) or to prolong the survival of activated $CD4^+$ T cells in vitro (Cima *et al*., 2006). The results shown in the current study suggest that synergism between MPA and immune activators may increase the density of $CCR6^+CD4^+$ T cells in the endocervix, rendering it more vulnerable to infection

The selective synergistic upregulation of CCL20 expression by MPA in the presence of immune activators suggests that MPA might induce $CCR6^+CD4^+$ T cells, macrophages and Langerhans cells to infiltrate the endocervix in vivo. Evidence from clinical and animal studies suggests that DMPA induces $CD4^+$ T cells and macrophages to home to mucosal tissues in the FGT (Quispe-Calla *et al*., 2018; Goode *et al*., 2014; Smith-McCune *et al*., 2017). However, it is currently unknown whether the migrating $CD4^+$ T cells express CCR6, which would suggest that CCL20 is involved in their immigration. Should they express CCR6, this would suggest that DMPA in the presence of immune activators enriches the endocervix with a $CD4^+$ T subtype that is very permissive to HIV-1 infection (Gosselin *et al*., 2010; Gosselin *et al*., 2017; Bolduc *et al*., 2017; Rodriguez-Garcia *et al*., 2014; Stieh *et al*., 2016). Other chemokines besides CCL20 could be responsible for recruiting HIV-1 target cells into the FGT. However, CCL20, more so than CCL2, CCL3, CCL4, CCL5, CCL7 and CCL8, induces the migration of Langerhans precursor cells in vitro (Dieu-Nosjean *et al*., 2000). This suggests that CCL20 might play an important role in HIV-1 target cell migration. Thus, the selective synergistic upregulation of CCL20 by MPA in the presence of immune activators, such as during a genital tract infection, might create an immune microenvironment that is favourable for HIV-1 infection.

An MPA-induced CCL20-rich microenvironment in the endocervix is very likely to promote latent HIV-1 infection in resting $CCR6^+CD4^+$ T cells (Cameron *et al*., 2010; Gosselin *et al*., 2010; Gosselin *et al*., 2017). Furthermore, the predominantly anti-inflammatory immune milieu created by MPA in vitro might restrict the ability of the virus to replicate (Guha & Ayyavoo, 2013). This might enable infected $CCR6^+CD4^+$ T cells to escape early immune detection by cytolytic $CD8^+$ T cells, which have been shown to accumulate in the endocervix of women using DMPA (Smith-McCune *et al*., 2017). However, this hypothesis is inconsistent with evidence in the literature suggesting that HIV-1 replication in enhanced in the presence of MPA (Huijbregts *et al*., 2013; Sampah *et al*., 2015; Irvin & Herold, 2015; Tasker *et al*., 2017; Maritz *et al*., 2018; Ray *et al*., manuscript under review). Latent HIV-1 infection is established when cells take up and integrate viral DNA, but do not make new RNA. Because the above studies only assessed viral replication as determined by p24 levels, it remains unclear whether MPA enhances viral DNA integration especially at time points whereby replication is not yet apparent. Findings from a recent in vitro study suggest that this may correspond to periods whereby treatment with MPA initially inhibits the expression of pro-inflammatory cytokines such as IL6 in ectocervical tissue explants (Ray *et al*., manuscript under review). Interestingly, this study also showed that at later time points MPA induces rather than inhibits IL6 expression and further showed that this correlated with HIV-1 replication in tissue samples infected in vitro with HIV-1 IMCs (Ray *et al.*, manuscript under review). This suggests that there is correlation between time-dependent expression of immune mediators and HIV-1 replication in ectocervical tissues.

The thesis results showed that MPA inhibits CCL5 expression in End1/E6E7 cells in the presence of immune activators. This is inconsistent with findings from previous clinical studies that DMPA-IM increases CCL5 protein levels in cervical secretions from women with specific GTIs using DMPA-IM compared to their counterparts not using DMPA-IM or healthy controls on DMPA-IM (Morrison *et al*., 2014; Fichorova *et al*., 2015a). In addition, findings from in vitro studies showed that MPA enhanced CCL5 expression by primary endometrial epithelial cells in the presence of HIV-1 IMCs (Ferreira *et al*., 2015a). Similarly, CCL5 has been reported to be upregulated in the vagina of DMPA-treated mice challenged with HSV-2. These results suggest that DMPA use might enhance the infiltration of $CCR5^+CD4^+$ T cells into endocervix of women with GTIs. However, the results presented herein suggest that MPA might prevent the recruitment of $CCR5^+$ cells in the FGT in the presence of immune activators. In addition, by inhibiting CCL5 expression, the results also suggest that MPA in presence of bacterial infection might reduce the ability of HIV-1 target cells to self-protect themselves or neighbouring cells against HIV-1 infection (Alvarez *et al*., 2011).

Collectively, the results presented herein suggest that MPA but not NET cooperates with immune activators to create an immune microenvironment that renders the endocervix vulnerable to HIV-1 infection.

6.1.4: MPA like GCs, but unlike NET, acts like a potent GR agonist in terms of regulating TJ and inflammatory genes at physiologically relevant concentrations in endocervical epithelial cells

The current study investigated whether the effects of MPA and NET on gene expression occurs at physiologically relevant concentrations. Dose response analyses were performed to determine the potencies (EC_{50}) and efficacies (maximal responses). Serum concentrations of MPA after a 150 mg DMPA-IM injection have been reported to range between 3 nM and 100 nM with peak concentrations averaging 21 nM before plateauing at 2.6 nM (Hapgood *et al*., 2018). However, lower peak serum concentrations of 2.3 (1.6 – 4.4) nM are reported with the low dose DMPA-SC, although comparisons were not performed in parallel (Hapgood *et al*., 2018). The current study shows that MPA significantly increases CCL20 expression in the absence of immune activators, with an EC_{50} of about 66 nM. CORT appears to be slightly more potent in activating CCL20 expression in the absence of immune activators with an EC_{50} of 31 nM. In the presence of immune activators, however, MPA at concentrations as low as 1 nM upregulates CCL20 expression with an EC_{50} of about 15 nM. Similarly, CORT at 1 nM appears to upregulate CCL20 expression in the presence of an immune activator, with an EC50 of 60 nM. This suggests that MPA like CORT at physiological concentrations may upregulate basal CCL20 expression in the endocervix in vivo. This may occur in DMPA-IM, but not DMPA-SC users shortly after injection. However, the presence of GTIs or inflammation may enhance the sensitivity of endocervical epithelial cells to MPA. Therefore, MPA concentrations higher than or equal to 1 nM will cooperate with immune

activators in the FGT to upregulate CCL20 expression in the endocervix. This could happen in women using DMPA-IM as well as those using DMPA-SC.

This study shows that MPA at 100 nM significantly reduces claudin-4 expression by 53.93% at 24 hours, with an EC_{50} of 14.27 nM. Furthermore, MPA at 1 μM represses occludin expression by 29.01% at 24 hours, with an EC_{50} of 131.2 nM. This suggests that DMPA-IM might regulate claudin-4, but not occludin at physiological concentrations Moreover, the effects of MPA on claudin-4 and occludin expression might be time-specific. Therefore, longer exposure to lower concentrates of MPA might downregulate claudin-4 and occludin expression in endocervical epithelial cells. Unlike CCL20, cotreating End1/E6E7 cells with 100 nM MPA and 1.15 nM TNF did not coregulate TJ gene expression in End1/E6E7 cells. Thus, MPA dose response curves in the presence of immune activators could not be performed in the current study.

It is very unlikely that NET-EN regulates CCL20 or TJ gene expression in vivo. This is because NET at 100 nM regulates neither CCL20 nor TJ genes in End1/E6E7 cells. Collectively, this study has revealed via dose response analyses that the downregulation of claudin-4 expression and upregulation of CCL20 expression by MPA occurs at physiological concentrations and thus may occur in vivo. However, whether tissue concentrations of MPA in DMPA users are greater or less than serum concentrations remain to be determined.

6.1.5: The GR mediates the downregulation of select TJ genes by MPA as well as the synergistic upregulation of select pro-inflammatory immune mediators by MPA and immune activators

The current study shows for the first time that the GR mediates the downregulation of claudin-4 expression by MPA and DEX in End1/E6E7 cells. However, further details of the molecular mechanisms are unknown. The GR downregulates gene expression by tethering to transcription factors such as NFκB and AP-1 or binding compositely to STATs (De Bosscher & Haegeman, 2009; Langlais *et al*., 2012; Ratman *et al*., 2013; De Bosscher *et al*., 2014). It is unclear whether there are functional NFκB sites in the promoter region of claudin-4, but it has been reported to have functional SP-1 and AP-1 sites (Honda *et al*., 2006; Ikari *et al*., 2013; Noda *et al*., 2014). It is therefore possible that the GR interacts with the AP-1 binding sites to repress basal claudin-4 expression in endocervical epithelial cells.

Previous in vitro studies have shown that the GR transactivates the occludin promoter in murine brain endothelial cEND cells and in human retinal epithelial cells in vitro via a distal imperfect GRE (Harke *et al*., 2008; Felinski *et al*., 2008). Because the GR does not transrepress via GREs, it is very unlikely that the GR mediates the repression of occludin expression by MPA in End1/E6E7 cells via this site. It is

also very unlikely the GR mediates the downregulation of occludin expression by MPA in End1/E6E7 cells. This study found that MPA represses occludin mRNA expression at doses higher than 100 nM, which is more than the Kd of MPA for the GR [between 4nM to 11 nM] (Hapgood, 2013) in the promoter region of occludin gene.

The results presented herein show that the GR mediates the downregulation of claudin-4 mRNA levels by MPA and DEX in End1/E6E7 cells, suggesting that MPA like DEX might act via the GR to disrupt the integrity of endocervical mucosal tissues in vivo. However, findings from a single animal study showing that MPA might act via the PR to increase the permeability of ectocervical and vaginal mucosal tissues in DMPA-treated mice via the downregulation of desmosomal and not TJ gene expression (Quispe-Calla *et al*., 2016). Morphological differences between the human endocervical End1/E6E7 cell line [that has been shown to express the GR and not the PR (Govender *et al*., 2014)] and the mucosal tissues of the murine lower FGT. In addition, it is possible that MPA regulates TJ and desmosomal gene expression in the different compartment of the FGT via different steroid receptors. That is MPA acts via the PR mediates the downregulation of desmosomes in ectocervical and vaginal mucosal tissues but acts via the GR mediates the downregulation of TJ genes in the endocervix. Because it was not investigated in the current study, it is currently unknown whether MPA can act via the GR to regulate the expression of desmosomes in the End1/E6E7 cell line, which do not express the PR. Future experiments are needed to provide clarity on this question. In addition, there is a need to investigate CORT can regulate TJ gene expression in the FGT. Results from a previous study show that peak serum CORT levels in healthy females is about 444.18 nM (Cho *et al*., 2017), which is higher than the concentration of GCs used in this study. This suggests that CORT like DEX might downregulate TJ gene expression.

The results from the present study show that the predominantly anti-inflammatory effects of MPA in the End1/E6E7 cell line in vitro are mediated by the GR. This is consistent with findings from a previous in vitro study that showed the GR mediates the MPA-induced suppression of pro-inflammatory gene expression in End1/E6E7 cells (Govender *et al*., 2014). Against this backdrop, however, the GR mediates the selective upregulation of CCL20 by either MPA alone or synergistically in combination with immune activators. Additional underlying molecular mechanisms involved were not investigated but could include several possibilities. Immune activators such as TNF have been shown to enhance the transcriptional activity of the GR by stabilising intracellular GR protein level (Bergann *et al*., 2009). The current study investigated and found that TNF does not stabilise GR protein levels in End1/E6E7 cells. Alternatively, some immune activators may enhance the site-specific phosphorylation of the GR at Ser-211 and Ser-226 and/or induce nuclear translocation and binding of the receptor to the promoters of target genes, in this case CCL20 (Hapgood *et al*., 2016). The CCL20 proximal promoter region has no

identifiable GRE but contains binding sites for C/EBPβ and NFκB transcription factors. It is thus possible that in the presence of MPA the GR interacts with these transcription factors to induce CCL20 mRNA expression in the presence or absence of immune activators. This is consistent with evidence that the GR interacts with C/EBPβ and NFκB to enhance IL12p40 mRNA expression in Ect1/E6E7 cells co-treated with MPA and TNF (Louw-du Toit *et al*., 2014). Therefore, the synergistic upregulation of CCL20 by MPA and immune activators in End1/E6E7 cells may result from the enhanced recruitment of GR to the promoter.

Previous research has shown that the GR mediates the upregulation of genes relevant for HIV-1 infection in the presence of MPA or GCs. These include CCR5 and CD4 in TZM-bl cells and PBMCs (Maritz *et al*., 2018) and TLR2 in HeLa cells (Shuto *et al*., 2002; Imasato *et al*., 2002; Sakai *et al*., 2004). The current study shows that the GR mediates the downregulation of claudin-4 and the upregulation of CCL20 in End1/E6E7 cells. Collectively, this suggests that GR is pivotal in modulating biological processes that are essential to HIV-1 infection in the FGT. These processes include epithelial mucosal permeability (claudin-4), migration of target cells (CCL20) and HIV-1 entry into target cell (CCR5, CD4 and TLR2).

6.1.6: MPA might cooperate with immune activators to selectively upregulate CCL20 mRNA expression in ectocervical tissues from some, but not all donors

Due to constraints in obtaining endocervical tissue, the current study could only investigate the effects of MPA on the expression of pro-inflammatory genes in ectocervical tissue explants. From results obtained it appears MPA might cooperate with immune activators to upregulate CCL20 mRNA levels in ectocervical tissues in vitro, although the effects were not statistically significant. It was observed that MPA and TNF appear to enhance CCL20 mRNA expression in some (3 out of 6), but not all samples from donors in vitro. This shows that there are high inter-donor variability samples used in the current study and suggests that about 50% of donors do respond to MPA. The different responses may be due to underlying intrinsic donor factors such as immune status, hormonal therapy, phase of menstrual cycle and age. Increasing the sample size might reduce the sample variance. Other factors such as time could have also influenced the outcome of the responses observed in ectocervical tissues in vitro. Evidence from previous studies suggest that primary genital epithelial cells or tissue explants are more responsive to MPA after 48 hours of treatment (Arici *et al.*, 1995; Ray, 2015; Cordeaux *et al.*, 2010). In the current study, however, ectocervical tissue explants were only exposed to MPA for 24 hours. Therefore, longer exposure to the ligands could change the outcome of the results reported in the current study. Further experiments are required to determine whether MPA acts additively or synergistically with immune activators to enhance HIV-1 infection and the expression of genes relevant to infection in FGT tissue from women using DMPA-IM ex vivo.

6.1.7: The downregulation of desmoglein-1 but not occludin and claudin-4 by MPA in ectocervical tissue explant might enhance mucosal permeability and HIV-1 infection.

The results presented herein show that in the absence of inflammation MPA represses desmoglein-1, but not occludin and claudin-4 expression in ectocervical tissue explants. This agrees with previous studies showing that MPA selectively downregulates desmosomal but not TJ genes in ectocervical mucosal tissues in women on DMPA (Quispe-Calla *et al*., 2016; Chandra *et al*., 2013). This suggests that MPA targets desmosomes but not TJs to disrupt the ectocervical mucosal barrier and hence increase the susceptibility of the ectocervix to HIV-1 infection.

It has been observed that MPA increases HIV-1 infection in ectocervical tissue explants from premenopausal women, via a mechanism involving CCR5 (Ray *et al*., manuscript in preparation). The current finding implies that the downregulation of desmoglein-1 is another mechanism by which MPA renders the ectocervix vulnerable to HIV-1 infection. Due to constraints on tissue availability, the current study was unable to investigate whether MPA regulates desmosomes and TJ gene expression and the molecular mechanisms thereof in primary endocervical tissue. Nonetheless, considering results from End1/E6E7 cells, it is possible that MPA selectively downregulates TJs in the endocervix, but not in the ectocervix to enhance mucosal permeability. Therefore, TJs may be relevant in maintaining the integrity of mucosal surfaces of the upper but not the lower FGT.

The current study investigated whether MPA enhances mucosal permeability of endocervical and endometrial epithelial monolayers in the absence or presence of HIV-1. The results show that MPA does not affect endocervical and endometrial mucosal permeability in vitro. However, they should be interpreted with caution. It was observed that exposure to HIV-1 IMCs failed to disrupt the integrity of PGEC monolayers, which is inconsistent with findings from previous studies (Nazli *et al*., 2010; Ferreira *et al*., 2015b; Nazli *et al*., 2013). The primary genital epithelial cells used in the current study took between two to three weeks to establish confluent monolayers. This is in contrast with what has been reported previously that PGECs establish confluent monolayers within five to seven days after seeding (Nazli *et al*., 2010; Kaushic *et al*., 2011; Nazli *et al*., 2013; Ferreira *et al*., 2015b). The disruptive effects of HIV-1 on mucosal barrier integrity have been shown to be mediated via TLR2 and TRL4 in vitro (Nazli *et al*., 2013). Because the expression of TLR4 in primary endocervical epithelial cells has been shown to be time dependent (Ma & Yang, 2010), it is possible that these receptors are lost when primary genital epithelial cells are cultured for a very long time. This could restrict their ability to respond to HIV-1.

This study was also unable to verify whether MPA regulates mucosal permeability in End1/E6E7 cells. This cell line was generated by immortalising primary endocervical epithelial cells isolated from a pre-

menopausal woman with the HPV E6E7 oncogene (Fichorova *et al*., 1997). Compared to other endocervical epithelial cell lines, End1/E6E7 cells are non-cancerous in origin and closely resemble primary endocervical epithelial cells (Fichorova *et al*., 1997; Fichorova *et al*., 1999). One of the initial goals of the current study was to verify whether End1/E6E7 cells can be used as an in vitro model to study the barrier functions of endocervical mucosal epithelial tissue. The results shown in this study suggest that End1/E6E7 cells do not form confluent monolayers but grow into multilayers. This is consistent with findings from some previous studies (Gali *et al*., 2010; Nold *et al*., 2012; Nold *et al*., personal communication), but in contrast to other findings (Sathe & Reddy, 2014; Das *et al*., 2014). The reason for this discrepancy remains unclear, but variations in growth condition may account for these differences. However, it could be that End1/E6E7 cells do no longer form polarised cultures after several passages. This growth tendency is typical of squamous and not columnar epithelial cells. The cells used in this study were between passage 9 and 40. It was beyond the scope of this study to investigate the effect of passage number on the ability of End1/E6E7 cells to form confluent monolayers.

6.1.8: MPA acts additively with select immune acivators to increase HIV-1 replication in TZM-bl cells

The current study shows that MPA like DEX, but unlike NET, acts alone or additively with immune activators to increase HIV-1 replication in TZM-bl cells. This is consistent with fndings from previous studies that showed GCs cooperate in a synergistic manner with immune activators to upregulate HIV-1 replication in chronically infected U1 cells in vitro (Bressler *et al*., 1993; Kinter *et al*., 2001). This suggests that target cells from women with GTIs using DMPA-IM are more likely to be infected with HIV-1 compared to those of their healthy counterparts. This study used TNF and LPS to induce inflammatory responses in TZM-bl cells and found that they both cooperated with MPA but not NET to increase HIV-1 replication in HIV-1 exposed cells. Some meta-analyses of observational studies have reported that DMPA-IM use enhanced the risk of HIV-1 acquisition associated with BV, but not HSV-2 (Ralph *et al*., 2015; Morrison *et al*., 2015; Haddad *et al*., 2018). This suggests that not all GTIs are likely to cooperate with DMPA to further enhance the risk of HIV-1 infection in women. TNF and LPS elicit similar responses on HIV-1 replication in the presence of MPA because they target the same signalling pathways although via different receptors. However, during an infection the signalling pathways activated are more complex and may result in different inflammatory responses. Therefore, DMPA use may modify the risk of HIV-1 acquisition associated with some GTIs but not others due to differences in soluble immune mediator expression profiles.

The mechanism(s) involved in the additive upregulation of HIV-1 replication by MPA and immune activators in TZM-bl cells exposed to HIV-1 IMCS were not investigated in this study. Because TZM-bl cells are endocervical in origin, it is possible that MPA increases TLR2 expression in this indicator cell

line and makes them more permissive to HIV-1 infection and replication, as shown in $CD4^+$ T cells (Ding *et al*., 2012; Bolduc *et al*., 2017; Thibault *et al*., 2007). Furthermore, since MPA has been found to increase CCR5 and CD4 expression in TZM-bl cells (Maritz *et al*., 2018), this may explain why MPA increases TNF- and LPS-induced HIV-1 replication in TZM-bl cells. Future studies could investigate whether these receptors play a role in the additive upregulation of HIV-1 replication by MPA and select immune activators.

Collectively, the result suggests that MPA alone enhances the risk of HIV-1 acquisition. This is consistent with findings from previous laboratory and clinical studies that suggest MPA increases the risk of HIV-1 acquisition (Hijbregst *et al*., 2013; Sampah *et al*., 2015; Irvin & Herold, 2015; Tasker *et al*., 2017; Maritz *et al*., 2018; Quispe-Calla *et al*., 2018; Martin *et al*., 1998; Baeten *et al*., 2007; Leclerc *et al*., 2008; Wand & Ramjee, 2012; Heffron *et al*., 2012; Crook *et al*., 2014; Noguchi *et al*., 2015; Ralph *et al*., 2015; Morrison *et al*., 2015; Brind *et al*., 2015; Polis *et al*., 2016; Hapgood *et al*., 2018; Hickey *et al*., 2016). In addition, they also suggest that DMPA-IM may cooperate with GTIs to further enhance the risk of HIV-1 acquisition in women. This is consistent with findings from observational studies suggesting DMPA-IM enhances the risk of HIV-1 acquisition associated with bacterial vaginosis (Haddad *et al*., 2018; Morrison *et al*., 2015). Therefore, DMPA-IM use may not be a better choice of hormonal contraception in women living in regions with or without a high prevalence of GTIs. However, NET alone or in the presence of immune activators did not increase HIV-1 infection in TZM-bl cells. This suggests that NET-EN might not cooperate with GTIs to enhance the risk of HIV-1 acquisition in women and could serve as a safer alternative to DMPA-IM.

6.1.9: Soluble factors secreted by PBMCs in the presence of MPA and immune activators increase HIV-1 replication in TZM-bl cells.

In the absence of immune activation, this study shows that MPA does not regulate the expression of pro-inflammatory immune mediators investigated herein in PBMCs after 24 hours of treatment. This could be time dependent as some studies have shown that MPA unlike NET regulates the expression of select pro-inflammatory immune mediators in PBMCs after 48 hours of treatment (Maritz *et al*., 2018; Hapgood *et al*., 2014; Ray, 2015). The current studies collectively showed that MPA selectively enhances the expression of innate immune receptors and markers of cellular activation but inhibits or does not alter the expression of soluble immune factors by PBMCs. Evidence from clinical and animal studies also suggest that DMPA-IM reduces serum levels of some select pro-inflammatory soluble factors, but mostly does not the alter serum levels of others (Michel *et al*., 2016; Batista *et al*., 2017; Goode *et al*., 2014). There is no consensus on which pro-inflammatory immune mediators are regulated by DMPA-IM in women. This may be attributed to interindividual variability of the samples and

differences in study design including the fact that different studies investigate the different immune mediators.

This study investigated the effects of MPA on LPS-induced gene expression in PBMCs in vitro and found that MPA is anti-inflammatory for several modulators investigated. This is consistent with findings from other in vitro studies that showed that MPA mostly suppressed the expression of pro-inflammatory immune mediators in the presence of immune activation (Kleynhans *et al*., 2011; Huijbregts *et al*., 2013; Huijbregts *et al*., 2014; Michel *et al*., 2016). While it may be predominantly anti-inflammatory in vitro, MPA nonetheless upregulates the expression of select pro-inflammatory immune mediators in the presence of immune activators, as shown in this study for CCL20. However, others have shown that CXCL10 and G-CSF are co-regulated in vitro by MPA and immune activators (Kleynhans *et al*., 2011). This suggests that MPA modifies the cytokine and chemokine secretion profiles of PBMCs in the absence or presence of LPS. How this affects HIV-I infection remains unknown.

This study thus investigated the effects of soluble immune mediators secreted by PBMCs treated with MPA alone or in combination with immune activators on HIV-1 infection in TZM-bl cells. The results show that secretions from PBMCs treated with MPA do not affect HIV-1 replication in TZM-bl cells. This can be explained by the fact that MPA at 100 nM does not alter the levels of soluble immune mediators secreted by PBMCs after 24 hours of treatment in the absence of LPS. Others have some that secretions from vaginal epithelial cells incubated in vitro with 388 µM MPA increased HIV-1 replication in the chronically infected Jurkat-Tat-CCR5 (JT-CCR5) cells as did recombinant IL6, TNF and GM-CSF (Irvin & Herold, 2015). This suggest that pro-inflammatory soluble factors secreted by vaginal epithelial cells in response to MPA mediated the increase in HIV-1 replication. Contrary to the current study that saw no changes in the levels of soluble immune mediators, Irvin and Herold (2015) found that supernatants from vaginal epithelial Vk2/E6E7 cells incubated with 388 µM MPA overwhelmingly had high levels of IL6, IL8, TNF, CCL3, CCL4, CCL5 and GM-CSF. Variations in cell type and the concentration of MPA used may explain these differences. However, in the current study, secretions from PBMCs co-treated with 100 nM MPA and LPS did enhance HIV-1 replication in TZM-bl cells. Although secretions from PBMCs co-treated with MPA and LPS had low levels of IL6 and CCL5, they also were found to have high levels of CCL20 compared to secretions from untreated cells. It remains unknown which soluble factors are involved, but they could include CCL20 in conjunction with some unidentified factors not investigated herein. Current evidence suggests that CCL20 enhances HIV-1 entry and integration into target cells but suppresses viral replication by inducing the expression of APOBEC3G (Lafferty *et al*., 2010). This suggests that MPA and LPS also induce a yet to be identified soluble factor that prevent APOBEC3G-mediated suppression of viral replication but one that equally activates viral replication.

Future experiments could investigate whether involvement of CCL20 secreted by PBMCs co-treated with MPA and LPS in enhancing HIV-1 replication.

Previous studies have shown that serum levels of CCL20 are elevated in HIV-1 positive patients (Fontaine *et al*., 2011; Aziz *et al*., 2016; Valverde-Villegas *et al*., 2018). The results presented herein suggest that DMPA-IM use might further increase CCL20 serum levels in HIV-1 positive patients. High CCL20 serum levels in HIV-1 infected patients have been associated with the homing and depletion of Th17 cells in mucosal tissues of the gut (Loiseau *et al*., 2016; Lee & Körner, 2017; Valverde-Villegas *et al*., 2015; Peng *et al*., 2013; Falivene *et al*., 2015; Singh *et al*., 2014). This suggests that MPA-induced upregulation of systemic CCL20 protein levels in HIV-1 patients might enhance the homing and subsequent depletion of Th17 cells at mucosal surfaces. A drop in Th17 cell numbers in HIV-1 patients has been associated with poor disease outcome (Falivene *et al*., 2015). While no direct association has been established between DMPA-IM use and HIV-1 disease progression (Richardson *et al*., 2007; Stringer *et al*., 2009; Morrison *et al*., 2011; Heffron *et al*., 2013; Whiteman *et al*., 2016), DMPA-IM use might indirectly influence an unfavourable disease prognosis via upregulating CCL20 expression in HIV-1 positive patients, followed by a lowering of Th17 cell numbers.

Plasma LPS levels may be elevated in HIV-1 infected women with concentrations ranging between 87.2 pg/mL and 170.8 pg/mL (Brenchley *et al*., 2006; Funderburg *et al*., 2012; Valiathan *et al*., 2016). However, the current study used LPS at a concentration of 5 µg/mL. This raises the question whether lower concentrations of LPS can cooperate with MPA to upregulation CCL20 expression in End1/E6E7 cells and PBMCs. Moreover, it also questions whether the in vitro effects observed in the current study can occur in vivo in HIV-1 negative women. In HIV-1 infected women, the source of plasma LPS is predominantly the gut (Vassallo *et al*., 2012; Zhang *et al*., 2015). However, the vaginal microbiome may also contribute to plasma LPS levels, especially in women with bacterial vaginosis (Si *et al*., 2017). One study reported that women in the Texas, USA with bacterial vaginosis had vaginal LPS levels of 323.5 ng/mL (Aroutcheva *et al*., 2008). Given that bacterial vaginosis in prevalent in Sun-Saharan Africa and that women of Sub-Saharan African descent have a more diverse vaginal microbiome compared to women elsewhere, it is possible that higher serum and vaginal LPS can be detected.

Taken together, although others have shown that in vitro MPA is predominantly anti-inflammatory, this study shows that it upregulates the expression of select pro-inflammatory immune mediators in the presence of immune activators in vitro. This may create a microenvironment that allows (i) more HIV-1 target cells to become infected and/or (ii) migrate to mucosal tissues where they are depleted in women.

6.1.10: MPA like LNG but unlike NET may not be a better choice of hormonal contraceptive for women at risk of HIV-1 infection.

The results in the this and previous studies show that MPA differs from other progestogens in the manner in which it regulates gene expression in vitro (Koubovec *et al*., 2014; Ronacher *et al*., 2009; Africander *et al*., 2011; Hapgood *et al*., 2014; Govender *et al*., 2014; Maritz *et al*., 2018; Tomasicchio *et al*., 2013; Huijbregts *et al*., 2013; Huijbregts *et al*., 2014; Shen *et al*., 2017). This study shows that MPA unlike NET, LNG and P4 downregulates claudin-4 expression in vitro in endocervical epithelial End1/E6E7 cells. This suggests that unlike MPA, neither NET, LNG nor P4 might enhance the permeability of the endocervix in vivo and thus render it vulnerable to HIV-1 infection. However, evidence from clinical studies suggest that DMPA-IM and LNG-IUD use increase the permeability of ectocervix and vagina in vivo (Quispe-Calla *et al*., 2016; Quispe-Calla *et al*., 2017). Because it has not been investigated, the in vivo effects of NET-EN on genital mucosal permeability remains unknown. Nonetheless, based on available evidence in the literature and findings from the current study, DMPA-IM might not be safe for use by women at high risk of HIV acquisition.

Data from animal studies suggest that female macaques treated with exogenous P4 or in luteal phase are more susceptible to SHIV infection (Sodora *et al*., 1998; Vishwanathan *et al*., 2011 Kersh *et al*., 2014; Marx *et al*., 1996; Smith *et al*., 2000). This suggest that a progestogen-dominant state enhances the risk of infection. However, not all progestogens used in hormonal contraception increase the likelihood for HIV-1 infection in women. Evidence from observational studies suggests that DMPA-IM but not NET-EN significantly increases the risk of HIV-1 acquisition in women (Ralph *et al*., 2015; Morrison *et al*., 2015). While there is paucity in the literature on the effect of LNG-IUD use on the risk of HIV-1 acquisition, evidence from the few studies that investigated this question found no association between HIV-1 acquisition and LNG-IUD. However, evidence from clinical studies show that LNG-IUD use increased the proportion of $CCR5^{+}CD4^{+}$ T cells in the blood (Sciaranghella *et al*., 2015) and FGT (Shanmugasundaram *et al*., 2016), besides increasing the permeability of the ectocervix (Quispe-Calla *et al*., 2017). This suggest that LNG-IUD may also enhance the risk of HIV-1 infection

The recent in vitro findings showing that MPA but not NET acts via the GR to increase HIV-1 infection of $CD4^{+}$ T cells and TZM-bl cells (Maritz *et al*., 2018). The current study extends these findings by showing that MPA but not NET acts alone or additively with immune activators to increase the infectibility of TZM-bl cells. Taken together with the published literature, the thesis findings suggest that NET-EN may be a safer choice of hormonal contraception use by women at risk of HIV-1 infection than DMPA-IM, while the LNG-IUD may also not be a suitable choice

6.2: Conclusions

The in vitro results presented herein show for the first that physiologically relevant concentrations of MPA, unlike NET and LNG, acting as a partial GR agonist relative to DEX, may increase the permeability of endocervical epithelial End1/E6E7 cells in the absence of immune activators by downregulating the expression of the tight junction gene claudin-4. In contrast, MPA unlike NET may disrupt the ectocervical mucosal barrier by downregulating desmoglein-1 but not claudin-4 expression, suggesting that different mechanisms may operate in the endocervix and ectocervix. This study also suggests that physiologically relevant concentrations of MPA, unlike NET, acts as a full GR agonist like CORT to upregulate CCL20 and TLR2 expression in End1/E6E7 cells in the absence of immune activators, suggesting that MPA might increase the sensitivity of the endocervical epithelial cell line toward CCL20 and TLR2 specific inflammatory responses. Taken together, this study provides new insights into plausible mechanisms by which MPA but not NET and LNG may increase the susceptibility of the FGT.

In the presence of immune activators, this study showed for the first time that MPA, unlike NET, at physiologically relevant concentrations acts via the GR to cooperate, in some instance synergistically, with immune activators to upregulate CCL20 and TNF receptor 2, but not tight junction gene expression in End1/E6E7 cells. This occurred against the backdrop of MPA suppressing IL1β, IL6, IL8 and CCL5 expression in the presence of immune activators and suggests that MPA might enhance immunological responses mediated via TNF receptor 2 and moreover enhance the recruitment of CCL20-responsive HIV-1 target cells in the endocervix in women. The in vitro results show that MPA but not NET might cooperate with immune activators to upregulate CCL20 expression in the ectocervical tissues in some, but not all women. This indicates interindividual variations in responses induced by MPA. Nonetheless, MPA selectively potentiated LPS-induced expression of CCL20, but repressed LPS-induced expression of IL1β, IL6 and IL8 in PBMCs in vitro. The study shows that synergistic effects of GR ligands like MPA with proinflammatory modulators are highly gene-specific, while the precise intracellular mechanism for CCL20 remains to be determined.

This study found that MPA, unlike NET, can act alone or additively with immune activators to enhance HIV-1 replication in TZM-bl cells. In addition, this study found that secretions from PBMCs cotreated with MPA and LPS, but not MPA alone or NET and LPS, had high CCL20 but low CCL5 and enhanced HIV-1 replication in TZM-bl cells. This suggests that women with GTIs using DMPA-IM but not NET-EN are more likely to acquire HIV-1.

The current study was unable to establish via statistical analysis whether MPA cooperates with immune activators to upregulate CCL20 expression in ectocervical tissue explants, although this was shown in

PBMCs. The small sample size coupled with a large degree of inter-donor variability could have confounded the results. Increasing the sample size will improve the significance of the data.

The in vitro findings herein show that when responses to MPA are obtained, these are different to those of NET, or LNG or P4 where investigated, are GR-mediated and are plausibly likely to increase HIV-1 acquisition and/or pathogenesis. This adds to the accumulating body of evidence that MPA acts as a potent partial to full GR agonist, in multiple cell and tissue models, and is biochemically and functionally different to other progestins used in endocrine therapy, as well as luteal phase P4 levels.

6.3: Future perspectives

This study has raised many intriguing questions and identified several potentially important mechanisms whereby MPA, unlike NET, could affect immune function and HIV-1 acquisition or pathogenesis. There are many possible avenues to explore in future, and this section will focus on those that appear to be the most relevant and interesting. Because all the experiments described herein are in vitro, using cell lines, PBMCs and ectocervical tissue explants, there is a need to investigate whether the results shown in the current study can occur in vivo in women using injectable hormonal contraception. Thereafter, molecular mechanisms will be established in vitro using primary cells.

6.3.1: Investigate the effects of DMPA-IM and NET-EN on claudin-4, desmoglein-1, TLR2 and CCL20 expression where possible in the endocervix, ectocervix and PBMCs

The results shown in the current study suggest that DMAP-IM use might increase the permeability of the endocervix by downregulating claudin-4 expression. Due to paucity of data for NET-EN, it's effects on mucosal permeability in the FGT remain unknown. However, results shown in the current study suggest that NET-EN might not disrupt the mucosal barrier of the endocervix. Therefore, future experiments could assess and compare the mucosal permeability of endocervical tissue biopsies from healthy women using DMPA-IM, NET-EN or no HC, using methods previously described by others (Quispe-Calla *et al*., 2016; Quispe-Calla *et al*., 2017).

Evidence shown in the current study suggests that MPA downregulates the expression of desmoglein-1 but not TJ genes in ectocervical tissues in vitro. This is consistent with findings from a previous study in mice showing that DMPA increased the permeability of the ectocervix by downregulating desmosomes but not TJ genes (Quispe-Calla *et al*., 2016). However, it remains unclear whether MPA differentially regulate desmosomes and TJ genes in the endocervix. Therefore, the expression of

desmoglein-1 and claudin-4 mRNA and protein could be investigated in endocervical tissue biopsies from healthy women using DMP-IM, NET-EN or no HC.

Another intriguing question to investigate is whether MPA and NET regulate CCL20 and TLR2 expression in the endocervix, ectocervix and blood in vivo. Tissue biopsies and PBMCs from healthy women using DMPA-IM, NET-EN or no HC could be evaluated for CCL20 and TRL2 mRNA and protein levels. In addition, cervical tissue biopsies and PBMCs from women using DMPA-IM and NET-EN could be treated with TRL2 ligands ex vivo and assessed for the expression of soluble immune mediators particularly CCL20. This will confirm whether progestogens can cooperate with immune activators to upregulate CCL20 expression. In addition, it could be investigated whether TLR2 ligands enhance HIV-1 infection in cervical tissue biopsies and PBMCs from women using DMPA-IM and NET-EN ex vivo.

An intriguing question is whether DMPA or NET-EN can alter the proportion of $CCR6^{+}CD4^{+}$ T cells in the FGT in vivo in the absence or presence of immune activation. The results shown in the current study suggest that MPA is more likely than NET to cause $CCR6^{+}CD4^{+}$ T cells to infiltrate the FGT in the presence of immune activators via upregulating the expression of CCL20. Therefore, endocervical and ectocervical tissue biopsies could be obtained from using women DMPA-IM, NET-EN or no HC with or without GTIs. Immunohistochemistry and confocal microscopy will then to use to establish the effects of HC use alone or in combination with GTIs on the density of $CCR6^{+}CD4^{+}$ T cells in the tissue samples. Furthermore, in vitro migration assay could be used to investigate whether secretions from tissue biopsies from women using DMPA-IM, NET-EN or no HC treated ex vivo with TLR2 ligands can induce the migration of $CCR6^{+}CD4^{+}$ T cells.

6.3.2: Investigate molecular mechanisms in primary endocervical epithelial cells

Future experiments could investigate whether the GR is involved in the regulation of claudin-4, desmoglein-1, CCL20, TLR2 and TNF receptor 2 expression by MPA in primary genital epithelial cells in the absence or presence of immune activators. Preliminary results from studies in the Hapgood Laboratory suggest that primary genital epithelial cells express both the GR and the PR (Hapgood *et al*., unpublished). Thus, it remains unclear which steroid receptor would mediate the regulation of the target genes by MPA in primary genital epithelial cells. Because is RU486 antagonises both receptors, the role of GR could be investigated using GR knockdown with siRNA.

The results shown herein suggest that MPA might enhance CCL20 mRNA levels in the cytoplasm in the absence of immune activators. Whether this contributes to the synergistic upregulation of CCL20 mRNA

levels in the presence of immune activators remains unclear. Therefore, future experiments using actinomycin D (transcription inhibitor) could be performed to establish the effects of MPA on CCL20 mRNA stability in the absence and presence of immune activators.

This thesis showed that MPA upregulates the expression of TLR2 and TNF receptor 2 but suppresses claudin-4 and desmoglein-1. Although a requirement for the GR was established in the current study, the molecular mechanisms remains unknown. The GR has been shown to be recruited to a GRE in the proximal promoter region of TLR2 in HeLa cells in responses to GCs (Hermoso *et al*., 2002). It possible that in the presence of MPA the GR transactivates TLR2 expression via this site in End1/E6E7 cells. However, it is unclear which sites does the GR interacts with to regulate CCL20, claudin-4, desmoglein-1 and TNF receptor 2 expression in response to MPA. The promoter region of CCL20 has been shown to contain a C/EBPβ binding site. It is possible that the GR is recruited to this site to transactivate basal expression in response to MPA. In addition, potential GREs or binding sites of other transcription factors could be identified in the proximal promoter regions of using in silico transcription factor binding site prediction tools. Thereafter, chromatin immunoprecipitation (CHIP) assays could be used to established whether treatment with MPA causes the GR to interact with these sites in the promoter of the target genes.

It could also be investigated whether co-treatment with immune activators and MPA results in an enhances recruited of the GR to the CCL20 promoter to result to synergistic upregulation of CCL20 expression. Because the results shown herein suggest that the effects of MPA and immune activators on CCL20 expression are mutual, it is possible that co-treatment with MPA and immune activators also results to the recruitment of transcription factors such as p65 to the CCL20 promoter. Interestingly, GCs have been shown to enhance the recruitment of p65 to the CCL20 promoter. Conversely, immune activators may also enhance the interaction of the GR with the CCL20 promoter. Therefore, future experiments will use assays investigate whether the GR is recruited to C/EBPβ site. Furthermore, CHIP assays will also be used to investigate whether MPA enhances p65 recruitment to the CCL20 promoter in the presence of immune activators or whether immune activators enhance GR recruitment to the promoter.

Future research will also investigate the role of MKP-1/p38 MAPK and MKP-1/p38 MAPK/PI3K/AKT signalling pathways in the selective synergistic upregulation of CCL20 by MPA and TNF in End1/E6E7 cells. Previous studies have shown that MKP-1/p38 MAPK signalling pathway is involved in the synergistic upregulation of TRL2 expression by GCs and immune activators in HeLa cells (Shuto *et al*., 2002; Imasato *et al*., 2004; Sakai *et al*., 2004; Hermosa *et al*., 2004). However, this study is proposing

that the MKP-1/p38 MAPK pathway mediates MPA-induced inhibition of basal and TNF-induced IL6, IL1β and IL8 expression by MPA in End1/E6E7 cells. On the other hand, the MKP-1/p38 MAPK/PI3K/AKT pathway selectively mediates MPA-induced upregulation of basal and TNF-induced expression of CCL20 in End1/E6E7 cells. To verify this hypothesis, initial experiments could be performed to investigate the role of MKP-1 in MPA-induced regulation of IL6 and CCL20 in End1/E6E7 cells using siRNA knockdown technology. In addition, specific pharmacological inhibitors assign roles to specific MAPKs and PI3K/AKT.

Besides CCL20, chemokines such as CCL21 and CCL19 have been shown to induce latent HIV-1 infection in resting $CD4^+$ T cells (Cameroon *et al*., 2010; Saleh *et al*., 2007). Unlike $CCR6^+CD4^+$ T cells that respond uniquely to CCL20, $CCR7^+CD4^+$ T cells respond to CCL21 as well as CCL19. However, there are populations of $CD4^+$ T cells that express both receptors and so are capable of responding to all three chemokines. These are mainly Th17 cells of central memory phenotype. A previous study reported that MPA increased basal CCL21 mRNA levels in the endocervix of macaque monkeys (Goode *et al*.., 2014). It remains unknown whether CCL19 is regulated by MPA, but there is evidence that GCs upregulate its expression in airways epithelial cells in the presence of TNF (Oakley *et al*., 2017). The intriguing question to pursue is whether MPA can act alone or via these chemokines to induce latent infection in HIV-1 target cells. To answer this question, Th17 cells could be differentiated from PBMCs. The cells could then be pre-treated with MPA and then stimulated with TNF. The cotreated cells could then be exposed to HIV-1 IMCs and cultured at specific time points. At the end of each time point, cells could be harvested for DNA and RNA extraction to assess HIV integration and mRNA levels of select immune mediators, respectively. In addition, cell culture supernatants could be collected and used to quantify by ELISA the levels of HIV-1 reverse transcriptase and select immune mediators This time-course approach could facilitate the identification of the window in which latent infection is established and how changes in immune mediator secretion profiles affect this over time. Specific neutralising antibodies could be used to established whether MPA acts via CCL19, CCL20 and CCL21 to induce latent HIV-1 infection.

This study also showed that secretions from PBMCs cotreated with MPA and LPS enhanced HIV-1 replication in TZM-bl cells. These secretions were found to have high levels of CCL20, but low levels of CCL5, IL6 and possibly IL1β. Future experiments could investigate the role of CCL20 or other soluble immune mediators in the upregulation of HIV-1 replication by secretions from PBMCs cotreated with MPA and LPS using neutralising antibodies.

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Appendix A

Supplementary data for chapter three

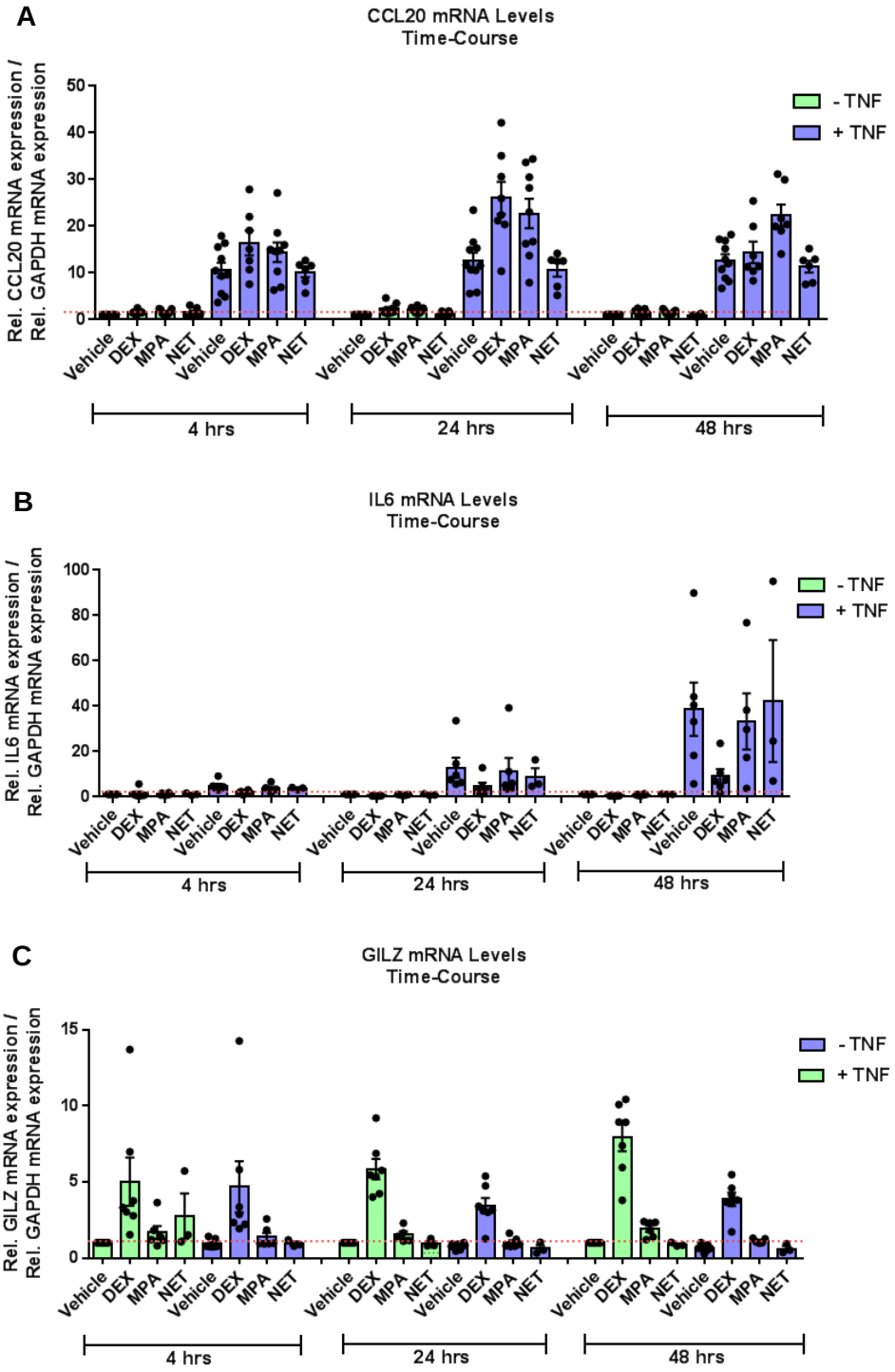

Figure 1: The regulation by DEX, MPA and NET of basal and induced CCL20, IL6 and GILZ mRNA levels in End1/E6E7 cells is time-dependent. End1/E6E7 cells were stimulated with 100 nM DEX, MPA, NET or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15nM human TNF for 4, 24 and 48 hours. At

the end of each incubation time, cells were harvested in TriReagent® and Stored at -80˚C until total RNA isolated and converted to cDNA. The relative mRNA levels of CCL20 (**A**), IL6 (**B**), and GILZ (**C**) were determined by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones and TNF on mRNA levels of the selected genes were determined by normalising to the vehicle control of each time point, which have been set to 1. The data include at least 3 independent biological repeats plotted as mean ± SEM in Graph Pad Prism 7 software.

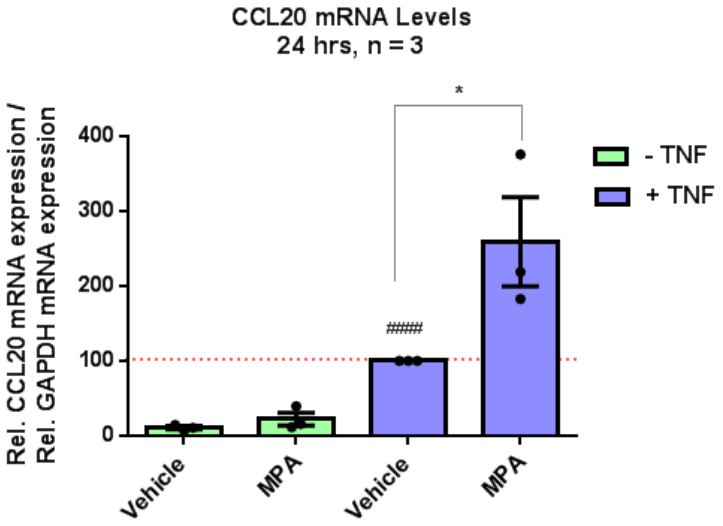

Figure 2: MPA and TNF jointly upregulate CCL20 mRNA expression in End1/E6E7 samples used in RT^2 PCR Array analysis. End1/E6E7 cells were treated with 100 nM MPA or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF for 24 hours. Thereafter, total RNA was isolated using RNeasy Mini kit (Qiagen, USA), assessed for integrity by Bioanalysis and then converted to cDNA using the RT^2 First Strand kit (Qiagen). The relative mRNA levels of CCL20 was determined by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones on mRNA levels were determined by normalising to TNF only, which has been set to 100%. Statistically analysis was performed using one-way ANOVA with Tukey's multiple comparison and statistical significance is denoted as * to indicated $p<0.05$ (TNF vs TNF + MPA). In some cases, the unpaired Student's t-test was used, and statistical significance is denoted as #### to indicate $p<0.0001$ (Vehicle vs TNF).

Table 1: The effects of MPA and TNF on mRNA levels of genes relevant to HIV-1 pathogenesis in End 1 E6E7 cells

Position	Gene Symbol	Fold Change (comparing to vehicle control set to 1)								
		TNF			MPA			TNF + MPA		
		Fold Change	p-value	Comments	Fold Change	p-value	Comments	Fold Change	p-value	Comments
A01	APEX1	0.69	0.566953		0.78	0.709290		0.57	0.528825	
A02	APOBEC3G	0.86	0.773880	C	0.93	0.947102	C	1.07	0.961797	C
A03	BAD	0.91	0.804554		0.76	0.630536		0.97	0.776030	
A04	BANF1	0.90	0.694157		0.98	0.819093		0.93	0.688958	
A05	BAX	0.91	0.692354		0.88	0.855164		1.02	0.838513	
A06	BCL11B	0.57	0.462289		0.84	0.923458		0.53	0.369369	
A07	BCL2	0.90	0.681536		0.99	0.882180		0.69	0.590751	
A08	BTRC	0.73	0.547760		0.77	0.653562		0.85	0.609549	
A09	CASP3	0.78	0.688821		0.94	0.767208		0.97	0.800565	
A10	CASP8	0.79	0.637932		0.96	0.907481		0.79	0.642802	
A11	CBX5	0.80	0.704360		0.77	0.708837		0.97	0.795792	
A12	CCL2	1.29	0.642777	B	0.89	0.865563	B	1.24	0.707658	B
B01	CCL3	0.93	0.842015	B	0.93	0.947102	C	1.07	0.961797	C
B02	CCL4	0.86	0.773880	C	0.93	0.947102	C	1.12	0.926316	B
B03	CCL5	3.69	0.229996	A	0.41	0.468883	B	2.60	0.293128	B
B04	CCL8	0.86	0.773880	C	0.93	0.947102	C	1.07	0.961797	C
B05	CCNT1	0.75	0.607005		0.82	0.742427		0.85	0.646911	
B06	CCR2	0.86	0.773880	C	0.93	0.947102	C	1.07	0.961797	C
B07	CCR3	0.64	0.481925	B	0.69	0.543583	B	0.80	0.578428	B
B08	CCR4	0.75	0.561261	B	1.05	0.818144	B	2.10	0.609705	B
B09	CCR5	0.86	0.773880	C	0.93	0.947102	C	1.07	0.961797	C
B10	CD209	0.86	0.773880	C	0.99	0.925627	B	1.07	0.961797	C
B11	CD247	0.60	0.465384	B	0.89	0.903950	B	0.53	0.338800	B
B12	CD4	0.72	0.781230	B	0.58	0.647430	B	0.97	0.755550	B
C01	CD44	1.03	0.881389		0.98	0.823304		1.19	0.996040	
C02	CD69	2.39	0.301556	B	0.93	0.948652	B	3.38	0.174812	B
C03	CD74	3.41	0.352820		1.03	0.898560		3.58	0.387811	
C04	CDK7	1.07	0.741983		1.30	0.955650		1.22	0.870574	
C05	CDK9	0.85	0.744979		0.90	0.758517		0.89	0.676216	
C06	CDKN1A	1.07	0.955046		0.85	0.731819		1.26	0.974219	
C07	CEBPB	1.32	0.956748		0.84	0.703234		1.32	0.874793	
C08	COPS6	0.76	0.607523		0.85	0.785303		0.89	0.724971	
C09	CR2	1.06	0.740023	B	2.38	0.559231	B	1.18	0.665613	B
C10	CREBBP	0.74	0.379961		0.94	0.568696		0.90	0.565962	
C11	CX3CL1	1.42	0.605603	B	0.93	0.947102	C	1.79	0.397519	B
C12	CXCL12	0.86	0.773880	C	0.93	0.947102	C	1.07	0.961797	C
D01	CXCR4	0.56	0.692755	B	0.40	0.433418	B	0.83	0.994917	B
D02	ELANE	0.82	0.701371	B	0.89	0.858970	B	1.56	0.573067	B
D03	EP300	1.08	0.941727		1.12	0.943733		1.28	0.954505	
D04	FCAR	0.86	0.773880	C	0.93	0.947102	C	1.07	0.961797	C
D05	FOS	0.68	0.570625		1.29	0.825717		0.89	0.933635	
D06	GADD45A	0.92	0.871267		0.76	0.600842		0.97	0.847735	
D07	HCK	0.99	0.923509	B	0.93	0.947102	C	1.07	0.961797	C
D08	HTATSF1	0.69	0.635756		0.57	0.806585		1.22	0.800354	
D09	IFNA1	0.79	0.582851		0.68	0.859505		0.95	0.776255	
D10	IFNB1	1.24	0.925179	B	0.80	0.907435	B	0.89	0.969059	B
D11	IFNG	0.77	0.540798	B	0.51	0.507023	B	1.59	0.432693	B

D12	IL10	0.86	0.773880	C	0.93	0.947102	C	1.07	0.961797	C
E01	IL12B	0.75	0.540638	B	0.71	0.610448	B	1.13	0.830816	B
E02	IL16	0.86	0.766090	B	0.93	0.938008	B	1.87	0.383458	B
E03	IL1B	0.89	0.752639	B	0.45	0.339375	B	1.22	0.988359	B
E04	IL2	3.47	0.246307		1.23	0.991180		3.29	0.290000	
E05	CXCL8	0.86	0.773880	C	0.93	0.947102	C	1.07	0.961797	C
E06	IRF1	5.98	0.229158		0.93	0.828674		7.22	0.226727	
E07	IRF2	1.77	0.645576		0.94	0.811082		1.99	0.571660	
E08	KLRD1	1.39	0.722563		1.02	0.932455		1.59	0.640749	
E09	LTBR	0.86	0.739689	B	0.89	0.884121	B	0.95	0.906611	B
E10	MAP3K5	0.84	0.662812		0.99	0.931167		1.00	0.820074	
E11	MBL2	0.75	0.498105		1.12	0.915475		0.93	0.818110	
E12	NFATC1	1.00	0.922962	B	0.93	0.947102	C	1.07	0.961797	C
F01	NFKB1	1.08	0.886728		1.08	0.920545		1.13	0.965401	
F02	NFKBIA	2.55	0.285442		1.05	0.961026		3.41	0.185347	
F03	PPIA	0.88	0.751514		0.87	0.902017		1.16	0.857912	
F04	PRDX1	1.02	0.895047		0.38	0.924716		1.15	0.911979	
F05	PTK2B	0.93	0.769421		0.28	0.908156		1.15	0.795774	
F06	RBL2	0.94	0.676546		1.13	0.869129		1.18	0.968140	
F07	SELL	2.42	0.373382	B	0.93	0.947102	C	3.44	0.348633	B
F08	SERPINA1	5.66	0.231363	A	0.79	0.582216	B	6.83	0.285199	A
F09	SERPINC1	0.73	0.525841	B	1.04	0.825516	B	0.71	0.477332	B
F10	SLPI	1.20	0.936995		0.99	0.918523		1.62	0.631448	
F11	SMARCB1	0.83	0.657977		1.01	0.911114		0.97	0.783471	
F12	STAT1	1.09	0.987071		1.16	0.942652		1.27	0.776042	
G01	STAT3	1.22	0.926631		1.07	0.981203		1.67	0.582451	
G02	TFCP2	1.62	0.600129		1.94	0.342448		1.94	0.316682	
G03	TGFB1	1.16	0.929176		1.04	0.976662		1.46	0.743320	
G04	TNF	10.66	0.187637	B	1.09	0.718594	B	16.40	0.120217	B
G05	TNFRSF1B	2.19	0.380946	B	0.96	0.669255	B	3.66	0.237804	A
G06	TNFSF10	0.98	0.924582		1.13	0.890301		1.31	0.780171	
G07	TRIM5	0.81	0.637268		0.95	0.765587		0.95	0.716534	
G08	TSG101	0.95	0.717690		1.07	0.931220		1.20	0.837136	
G09	VPS4A	0.85	0.592572		0.97	0.879363		0.89	0.693001	
G10	XCL1	0.86	0.773880	C	0.93	0.947102	C	1.07	0.961797	C
G11	XPO1	0.77	0.559885		1.03	0.861393		0.91	0.748066	
G12	YY1	0.97	0.828124		1.23	0.899896		1.09	0.998045	
H01	ACTB	1.02	0.816577		0.97	0.909324		1.19	0.192713	
H02	B2M	2.60	0.591403		2.31	0.738273		3.36	0.361168	
H03	GAPDH	1.00	0.000000		1.00	0.000000		1.00	0.000000	
H04	HPRT1	0.85	0.589640		1.02	0.904048		1.10	0.809794	
H05	RPLP0	0.86	0.579716		0.98	0.924038		1.05	0.962510	

A: This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample and is reasonably low in the other sample (< 30).

These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result.

This fold-change result may also have greater variations if p value > 0.05; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

B: This gene's average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the p-value for the fold-change is either unavailable or relatively high (p > 0.05).

This fold-change result may also have greater variations; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

C: This gene's average threshold cycle is either not determined or greater than the defined cut-off (default 35), in both samples meaning that its expression was undetected, making this fold-change result erroneous and un-interpretable. (Explanatory notes from Qiagen Data Analysis Centre website)

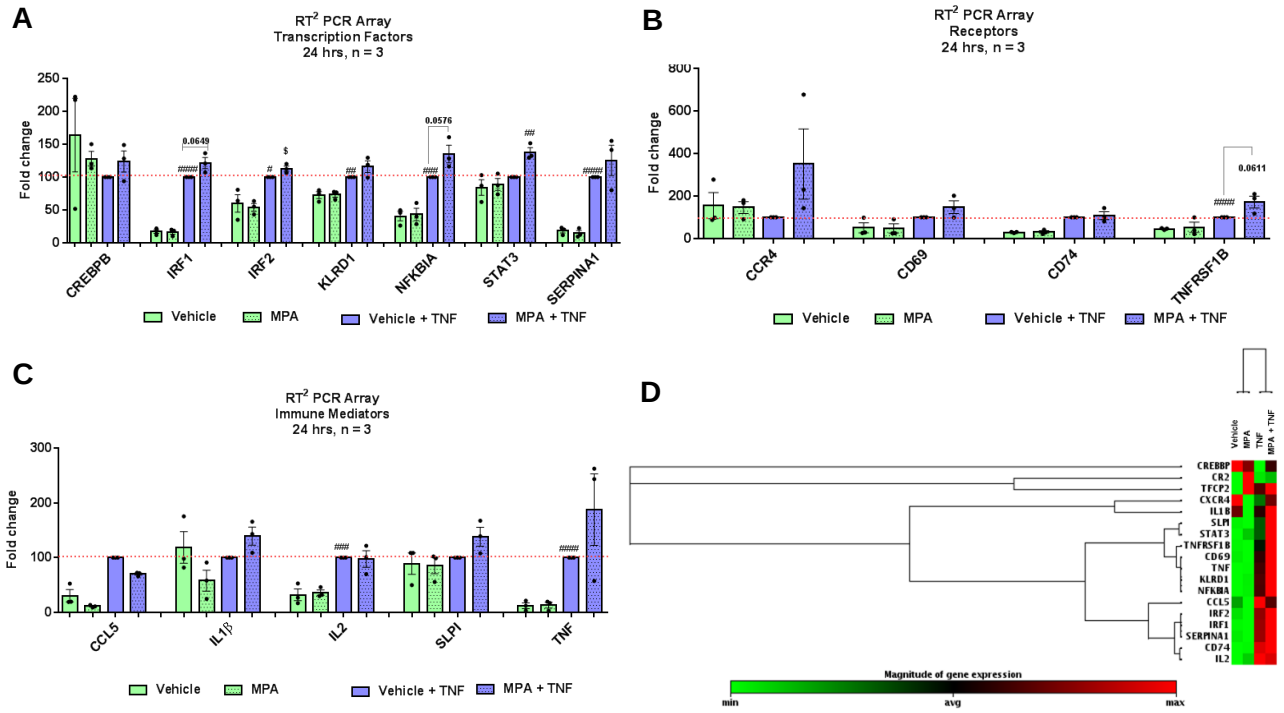

Figure 3: MPA differentially regulates TNF-induced transcription of genes relevant to HIV-1 pathogenesis in End1/E6E7 cells. The Human Host HIV-1 Response RT² Profiler PCR array was used to study the effect of MPA on TNF transcriptional regulation of genes relevant to HIV-1 pathogenesis. End1/E6E7 cells were treated with 100 nM MPA or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF for 24 hours. Thereafter, total RNA was isolated using RNeasy Mini kit (Qiagen, USA), assessed for integrity by Bioanalysis and then converted to cDNA using the RT² First Strand kit (Qiagen). Samples were loaded onto the plates and sent to a commercial facility for analysis. (**A - C)** show the Fold changes of genes plotted relative to TNF, which was set to 100%. The data include three independent biological repeats plotted as mean ± SEM in Graph Pad Prism 7 software. Statistical analysis was performed using the unpaired Student's t-test for comparisons between vehicle only and other ligands with statistical significance denoted by #, ##, ###, or #### to indicate p<0.05, p<0.01, p<0.005 and p<0.0001, respectively. When the comparison was between TNF and other

ligands statistical significance is denoted by $ to indicate p<0.05. (**B**) is the hierarchical clustering heatmap with genes (rows) and ligands (columns) showing similar effects clustered together.

Appendix B

Supplementary data for chapter four

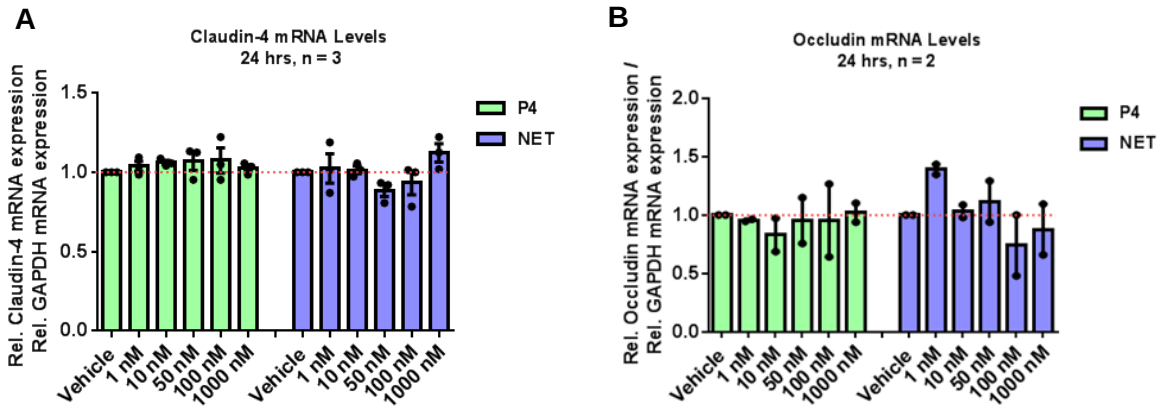

Figure 1: Neither P4 nor NET regulates claudin-4 nor occludin mRNA levels in End1/E6E7 cells. Confluent End1/E6E7 cells were stimulated with increasing concentrations of P4, NET or 01% (v/v) EtOH (vehicle) for 24 hours. Cells were then harvested in TriReagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of claudin-4 (**A**) and occludin (**B**) were determined by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroids on mRNA levels were determined by normalising to vehicle control, which has been set to 1. The data include at least two independent biological repeats plotted as mean ± SEM in GraphPad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison.

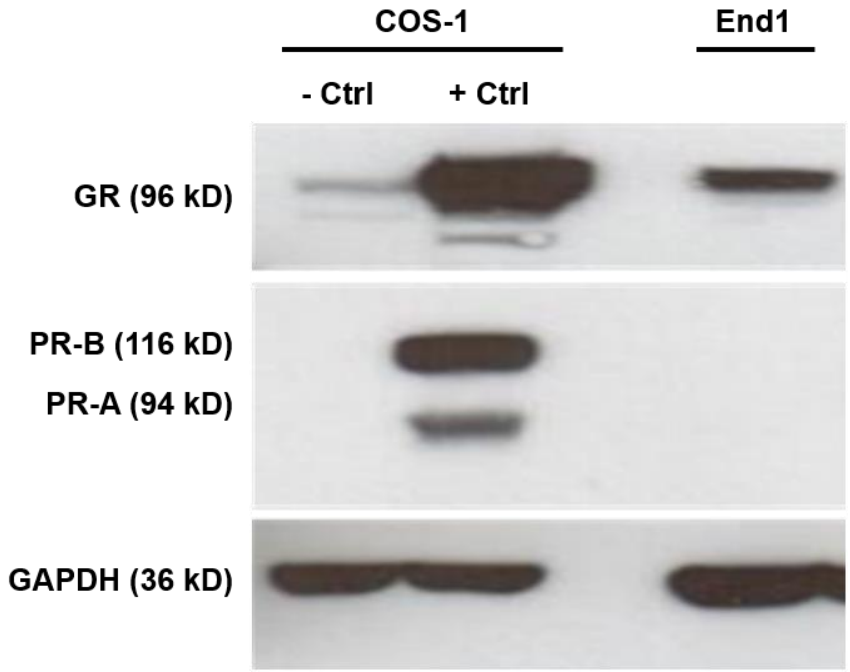

Figure 2: End1/E6E7 express detectable levels of GR protein, but not PR-A or PR-B. End1/E6E7 cells were harvested in 2X SDS Loading Buffer, boiled for 10mins and resolved on an 10% SDS-PAGE. Resolved

proteins were then transferred onto nitrocellulose membrane, blocked and probed using antibodies specific for the GR, PR-A, PR-B and GAPDH. Control samples were generated by transiently transfecting COS-1 cells with plasmids expressing the GR, PR-A, PR-B or empty vectors. The blot above is representative of 3 independent experiments.

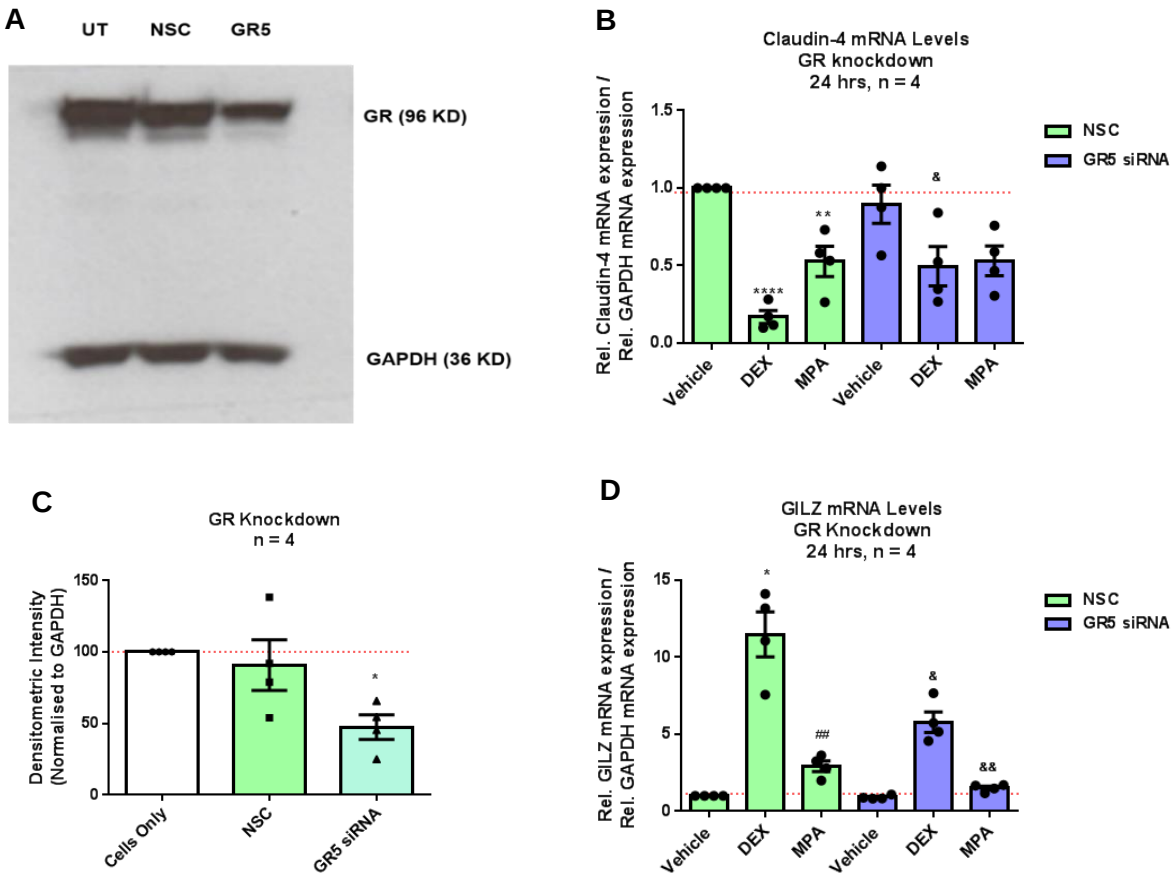

Figure 3: GR knockdown partially lifts the DEX- but not MPA-induced repression of claudin-4 mRNA levels End1/E6E7 cells. Confluent End1/E6E7 cells were transiently transfected with 10 nM of GR HS_NR3C1_5 (GR5) siRNA or non-silencing scrambled sequence control (NSC) siRNA for 48 hrs. Thereafter, cells were treated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (Vehicle) for another 24 hrs. Cells were then harvested in TriReagent®, total RNA isolated and converted to cDNA. The relative mRNA levels of claudin-4 (**B**) and GILZ (**D**) were determined by qRT-PCR using gene specific primers and normalised to GAPDH mRNA levels. The relative effects of steroid hormones on mRNA levels were determined by normalising to vehicle NSC control, which has been set to 1. In (**A**), extra wells of cells transiently transfected with10 nM of GR HS_NR3C1_5 (GR5) siRNA or non-silencing scrambled sequence control (NSC) siRNA for 72 hrs were harvested in 2X SDS Loading Buffer, resolved on an 10% SDS-PAGE and western blot analysis was done using antibodies specific for the GR and GAPDH. The blots from four biological repeats were scanned, quantified and for comparisons expressed relative to the untransfected (UT) cells (**C**). The data was plotted as mean ± SEM in Graph Pad Prism 7 software. Statistical analysis was performed using one-way ANOVA with Turkey's multiple comparisons and statistical significance denoted as *, ** and **** to indicate $p<0.05$, p,0.01 and $p<0.0001$, respectively. In some cases, the unpaired Student's t-test was used to compare corresponding treatments in the NSC and GR5 siRNA groups with statistical significance denoted as $ or $$ to indicate $p<0.05$ and $p<0.01$, respectively.

Appendix C

Supplementary data for chapter five

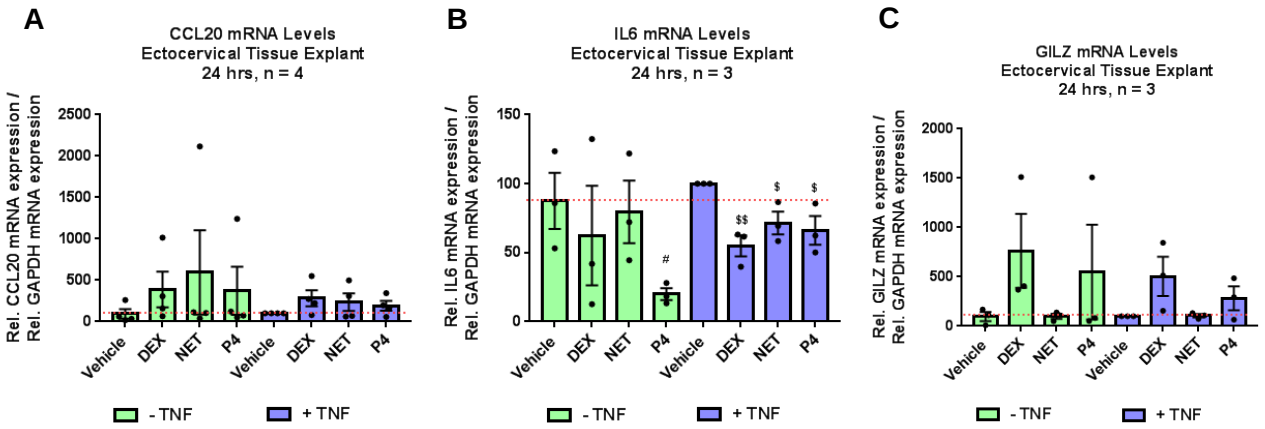

Figure 1: DEX, NET and P4 differentially regulate CCL20, IL6 and GILZ mRNA expression in the presence of TNF in human ectocervical tissue explants. Ectocervical tissue explants from pre-menopausal women were treated with 100 nM DEX, NET, P4 or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF for 24 hours. Thereafter, supernatants were collected and stored at -80°C until used and the rest of the tissue pieces were homogenised in TriReagent®, total RNA isolated and converted to cDNA. In (**A** – **C)**, CCL20 (**A**), IL6 (**B**) and GILZ (**C**) mRNA levels were quantified by qPCR and normalised to GAPDH mRNA levels. The relative effects of the ligands on mRNA levels of the selected genes were determined by normalising to TNF, which has been set to 100%. The data was plotted in Graph Pad Prism 7 software as mean ± SEM. Statistical analysis was performed using the Kolmogorov-Smirnov test and statistical significance is denoted as $^{\#}$ to indicate $p<0.05$ for comparisons between vehicle only and TNF; or as $^{\$}$ or $^{\$\$}$ to indicate $p<0.05$ and $p<0.01$ for comparison between TNF and other treatments.

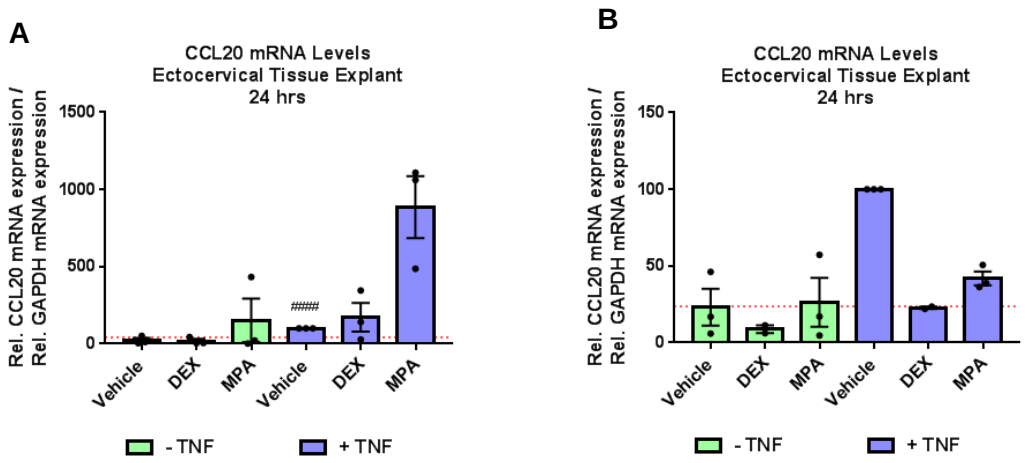

Figure 2: Inter-individual variations of human ectocervical tissue explant responses to DEX and MPA in the absence and presence of TNF. Ectocervical tissue explants from pre-menopausal women were treated with 100 nM DEX, MPA or 0.1% (v/v) EtOH (vehicle) in the presence or absence of 1.15 nM TNF for 24 hours. Thereafter, supernatants were collected and stored at -80°C until used and the rest of the tissue pieces were homogenised in TriReagent®, total RNA isolated and converted to cDNA. CCL20 mRNA levels were quantified by qPCR and normalised to GAPDH mRNA levels. The relative effects of the ligands on mRNA levels of the selected genes were determined by normalising to TNF, which has been set to 100%. (**A)** shows results obtained from tissue samples in which MPA and TNF appear to coregulate CCL20 mRNA expression, while (**B**) shows results from tissue samples in which MPA and TNF appears not to coregulate CCL20 mRNA levels. The data was plotted in Graph Pad Prism 7 software as mean ± SEM.

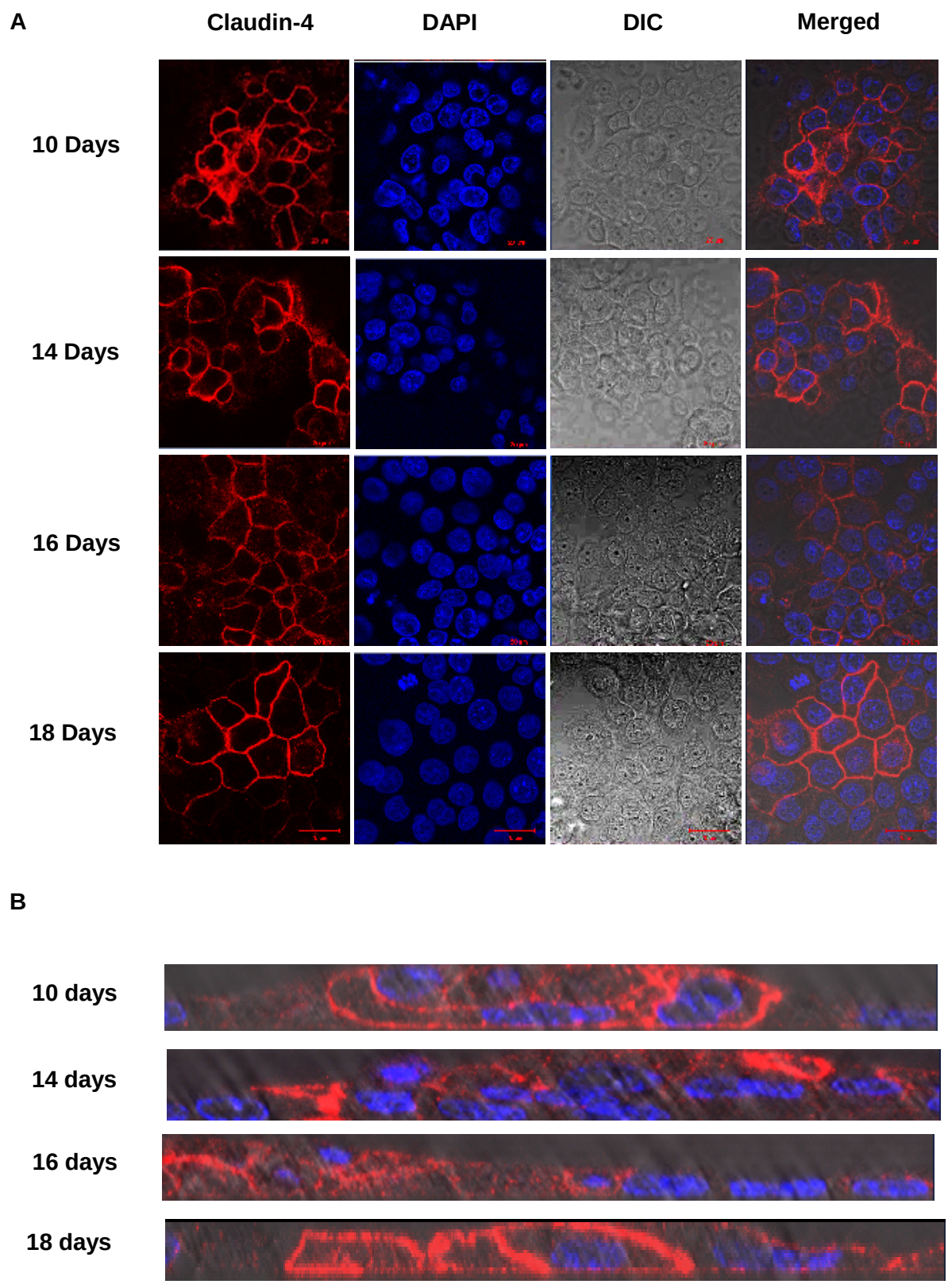

Figure 3: Subcellular localization of claudin-4 between cell-cell junctions of confluent End1/E6E7 cells. End1/E6E7 cells were seeded at 100,000 cells/mL onto glass cover slips and grown from 5 to 18 days. Thereafter, cells were harvested by washing 1X with cold PBS, fixed with cold methanol and stained with anti-claudin4 (red) and DAPI (blue). Scale bars: 20 µM. (**A**) Z stack images were acquired for each day and *en face* 2D images (representing the middle slice) were generated using the Carl Zeiss Zen 2.3

software. (**B**) XZ projections of Z stacks were obtained using the Image J software. The figure above is representative of two independent experiments (n = 2). Differential Interference Contrast (DIC) is used to enhance he contrast in unstained, transparent samples.

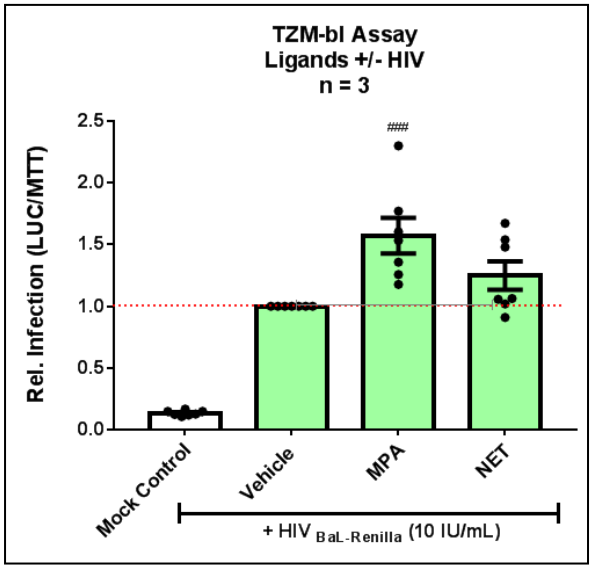

Figure 4: MPA but not NET enhances HIV-1 infection and replication in TZM-bl cells. TZM-bl cells were treated with 10 IVU of HIV-$_{1BaL\_Renilla}$ IMCs or Mock control for 24 hours. Thereafter, the cells were co-stimulated with 100 nM of DEX, 100 nM MPA or 0.1% v/v EtOH (vehicle) for another 48 hours. At the end of the 72 hours incubation period with HIV, luciferase activity was quantified using the Bright-Glo™ Luciferase Assay System reagent (Promega, USA)) on the Turner Biosystems Modulus Microplate reader (Promega, USA). The data represent at least three independent biological repeats plotted as mean ± SEM in GraphPad Prism 7 software. Statistical analysis was performed using the unpaired Student's t-test was used to compare pair of treatments; when comparison was between vehicle and other treatments, statistical significance is denoted as ### to indicate $p<0.005$. This figure includes pooled data from the minus LPS or TNF groups from experiments described in **Figure 4.3.8** (Chapter 4).