

Investigation of the underlying molecular mechanisms of immune modulation by the contraceptive Medroxyprogesterone acetate (MPA) on immune responses to mycobacteria

By

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature:

Lizaan Ehlers

Date: 29 November 2013

Abstract

Background

Individuals who are latently infected with *Mycobacterium tuberculosis* (M.tb) are able to quell the infection by balancing the innate and adaptive immune responses. Glucocorticoids (GCs) can affect this balance and can increase the risk of reactivation of TB. The three month injectable contraceptive medroxyprogesterone acetate (MPA) is widely used by women in developing countries, where TB is rife. MPA, unlike the two monthly contraceptive norethisterone enanthate (NET), possesses selective glucocorticoid activity, and could therefore alter immune responses to TB.

Aims

The aim of my investigation was to elucidate the immune modulatory effects of the synthetic progestins, MPA and NET, compared to the endogenous hormones, cortisol and progesterone, in *Mycobacterium bovis Bacillus Calmette–Guérin* (BCG) or anti-CD3 stimulated peripheral blood mononuclear cells (PBMC). I aim to determine the effects of MPA, NET, cortisol and progesterone on the receptor expression of glucocorticoid and various progesterone receptors. I investigate the effect of the above mentioned hormones on the downstream signalling cascades in the presence or absence of either BCG or anti-CD3. The overall immune modulation will be determined with regard to the cytokine production in PBMCs.

Methods

The presence of receptors for these steroid hormones in PBMCs was verified and BCG, anti-CD3 and hormone induced changes in receptor expression determined through RT-PCR. The impact of cortisol, MPA, NET and progesterone on BCG or anti-CD3 mediated activation of downstream signalling molecules were determined by Western blot as well as Luminex analysis.

Results and Conclusion

My results show that BCG and anti-CD3 mediated activation of the T cell receptor associated signalling molecules, Lck, ZAP-70, LAT was inhibited by the steroid hormones. Similarly several kinases including JNK, ERK and p38 and transcription factors including STAT3,

STAT5 and CREB were differentially affected by the hormones. The inhibition of phosphorylation seen in the different signalling molecules indicated an inhibition of activation of downstream signalling cascades. To investigate the impact of the hormone induced changes in the signalling cascades on the expression of inflammatory and anti-inflammatory cytokines Luminex analysis was performed on the supernatant of the BCG and anti-CD3 stimulated PBMC cultures. Cortisol and MPA, but not NET and progesterone, significantly inhibited the secretion of IL-1 α , IL-1 β , IL-6, IL-10, TNF- α , IL-12 and IL-13. These results suggest that the immune suppressive effects of MPA are likely mediated through a combination of direct genomic GR action as well as through direct or indirect inhibition of several signalling molecules.

The inhibition of the IFN- γ , IL-12, IL-1 and IL-6 secretion by MPA could potentially increase the risk of susceptibility to TB in women using this contraceptive. Therefore the absence of glucocorticoid activity seen with NET could make this contraceptive a better choice for women in TB endemic areas.

Opsomming

Agtergrond

Individue wat latent met Mikobakterium tuberkulose (M.tb) geïnfekteer is, is in staat om die infeksie te onderdruk deur die ingebore en aanpasbare immuunrespons te balanseer. Glukokortikoïede (GCs) kan hierdie balans beïnvloed en kan die risiko van heraktivering van tuberkulose (TB) verhoog. Die drie maande inspuitbare voorbehoedmiddel medroksiprogestroon-asetaat (MPA) word algemeen gebruik deur vroue in ontwikkelende lande, waar TB volop is. MPA, in teenstelling met die twee maandelikse voorbehoedmiddel noretisteroon enantaat (NET), beskik selektiewe glukokortikoïed aktiwiteit, en kan dus die immuunrespons teenoor TB verander.

Doelwitte

Die doel van my studie was om die immuunregulerende effekte van die sintetiese progesterone, MPA en NET, toe te lig, in vergelyking met die endogene hormone, kortisol en progesteron, in *Mycobacterium bovis Bacillus Calmette - Guerin* (BCG) of anti-CD3 gestimuleerde perifere bloed mononukleêre selle (PBMS_e). Ek het beoog om die gevolge van MPA, NET, kortisol en progesteron op die reseptor uitdrukking van glukokortikoïede en verskeie progesteron reseptore te bepaal. Ek het ondersoek ingestel op die effek van die bogenoemde hormone op die sein transduksie in die teenwoordigheid of afwesigheid van óf BCG of anti-CD3. Die algehele immuun -modulasie sal bepaal word met betrekking tot die produksie van sitokiene in PBMS_e.

Metodes

Die teenwoordigheid van reseptore vir die steroïedhormone in PBMS_e is geverifieer en BCG en anti-CD3 en die veranderinge deur die hormone in verband met die reseptor uitdrukking bepaal deur RT-PCR. Die impak van kortisol, MPA, NET en progesteron op BCG of anti-CD3 aktivering van sein transduksie molekules is bepaal deur 'Western blot' asook Luminex analise.

Resultate en gevolgtrekking

My resultate toon dat BCG en anti-CD3 die aktivering van die T-sel reseptor wat verband hou met sein molekules, LCK, ZAP-70, en LAT word geïnhibeer deur die steroïedhormone. Van die kinases insluitend JNK, ERK en p38 en transkripsie faktore, insluitend STAT3, STAT5 en CREB is beïnvloed deur die hormone. Die inhibisie van fosforilering gesien in die verskillende sein molekules dui daarop aan dat 'n inhibisie van aktivering van sein transduksie. Die impak van die hormoon veroorsaak veranderinge in die sein transduksie met betrekking tot die uitdrukking van inflammatoriese en anti-inflammatoriese sitokiene. Luminex analise is uitgevoer op die supernatant van die BCG en anti-CD3 gestimuleerde PBMS kulture. Kortisol en MPA, maar nie NET en progesteron, het aansienlik die produksie van IL-1 α , IL-1 β , IL-6, IL-10, TNF- α , IL-12 en IL-13 geïnhibeer. Hierdie resultate dui daarop dat die immuunstelsel se onderdrukkende effekte van MPA is waarskynlik bemiddel deur 'n kombinasie van direkte genomiese GR interaksie sowel as deur direkte of indirekte inhibisie van verskeie sein molekules.

Die inhibisie van die IFN- γ , IL-12, IL-1 en IL-6 sekresie deur MPA kan potensieel die risiko verhoog van vatbaarheid vir TB in vroue wat hierdie voorbehoedmiddel gebruik. Daarom oor die afwesigheid van glukokortikoïede aktiwiteit wat gesien is met NET, kan maak laat hierdie voorbehoedmiddel 'n beter keuse vir vroue in TB endemiese gebiede.

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List of Abbreviations and Symbols

ACTH:	Adrenal corticotrophin hormone
ANOVA:	Analysis of variance
AP-1:	Activator protein-1
APC:	Antigen-presenting cells
AR:	Androgen receptor
BCG:	Bacille Calmette-Guérin
Anti-CD3:	Anti-CD3 antibody
CFU:	Colony forming units
CI:	Confidence intervals
CNS:	Central Nervous System
CRE:	cAMP response elements
CREB:	cAMP response element-binding protein
CRH:	Corticotrophin releasing factors
DC:	Dendritic cells
DEX:	Dexamethasone
dH ₂ O:	Distilled water
DNA:	Deoxyribonucleic acid
E2b:	17 beta-estradiol benzoate
ELISA:	Enzyme-linked immunosorbent assay
ER:	Estrogen Receptor
ERID:	Estrogen receptor interacting domain

ERK:	Extracellular-signal-regulated kinases
FBS:	Fetal Bovine Serum
Fyn:	Tyrosine-specific phospho-transferase
GAP:	GTPase activating protein
GC:	Glucocorticoid
G-CSF:	Granulocyte colony-stimulating factor
GEF:	Guanine nucleotide exchange factor
GR:	Glucocorticoid Receptor
GRE:	GR response element
HIV:	Human immunodeficiency virus
HPA:	Hypothalamus pituitary axis
IFN- γ :	Interferon-gamma
IL:	Interleukin
IL-6RE	IL-6 responsive element
ITAM:	Immunoreceptor tyrosine-based activation motifs
I κ B:	Inhibitor of kappa B
JNK:	c-Jun N-terminal kinases
LAT:	Linker for activation of T cells
Lck:	Lymphocyte-specific protein tyrosine kinase
LPS:	Lipopolysaccharide
M.tb:	Mycobacterium Tuberculosis
ManLAM:	Mannose capped lipoarabinomannan
MAPK:	Mitogen-activated protein kinase

MEK:	Mitogen activated protein kinase
mGR:	Membrane GR
MHC:	Major histocompatibility complex
MOI:	Multiplicity of infection
MPA:	Medroxyprogesterone acetate
mPR- α :	Membrane progesterone receptor alpha
mPR- β :	Membrane progesterone receptor beta
mPR- γ :	Membrane progesterone receptor gamma
MR:	Mineralocorticoid receptor
NET:	Norethisterone
NET-A:	Norethisterone Acetate
NET-EN:	Norethindrone Enanthate
NFAT:	Nuclear factor of activated T cells
NF κ B:	Nuclear factor- κ Beta
nGRE:	Negative GRE
NK:	Natural Killer cells
nPR:	Nuclear Progesterone receptor
OACD:	Oleic acid albumin dextrose catalase
OD:	Optical density
PBMC:	Peripheral blood mononucleocyte
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PHA:	Phytohaemagglutinin

PI3K:	Phosphatidylinositol-3-kinases
PKB:	Protein kinase B
PKC:	Protein kinase C
PLA-2:	Phospholipase A2
PMA:	Phorbol 12-myristate 13-acetate
PMGRC1:	Progesterone membrane receptor component 1
PR:	Progesterone Receptor
PRE:	Progesterone response element
RBA:	Relative binding affinities
RNA:	Ribonucleic acid
RTK:	Receptor tyrosine kinase
SEM:	Standard error of the mean
SIV:	Simian immunodeficiency virus
SHIV:	Simian human immunodeficiency virus
SRC-2:	Steroid receptor coactivator-2
STAT:	Signal transducers and activators of transcription
STI:	Sexually transmitted infection
SUN-IRG:	SUN Immunology Research Group
Syk:	Spleen tyrosine kinase
TB:	Tuberculosis
TBS:	Tris buffered saline
TCR:	T cell receptor
TEMED:	Tetramethylethylenediamine

Th-1:	T helper-1 cells
Th-2:	T helper-2 cells
TLR:	Toll-like Receptor
TMB:	Tetramethylbenzidine
TNF- α :	Tumor necrosis factor-alpha
TST:	Tuberculin skin test
US:	Unstimulated
WHO:	World Health Organisation
ZAP-70:	ζ -associated protein 70 tyrosine kinase
ZN:	Ziel-Neelsen
α :	Alpha
β :	Beta
γ :	Gamma
δ :	Delta
ϵ :	Epsilon
ζ :	Zeta

Chapter 1: Introduction

1.1 Tuberculosis

More than 2 billion people worldwide are latently infected with *Mycobacterium tuberculosis* (M.tb), these amounts to roughly one third of the world's population (1). Despite the high number of recorded M.tb infected individuals, merely 10% of these will convert to active disease.

Tuberculosis is an infectious disease spread through airborne droplets containing M.tb, which is introduced to the surrounding air when an individual infected with M.tb coughs or sneezes. During 2009 according to the World Health Organisation (WHO) 1.7 million people died from TB, 35% of these recorded deaths were among woman infected with M.tb (1). The Global Tuberculosis Report 2012 places South Africa in the top five countries with the largest number of TB incidence cases in 2011, 1) India (2.0 – 2.5 mill), 2) China (0.9 – 1.1 mill), 3) South Africa (0.4 – 0.6 mill), 4) Indonesia (0.4 – 0.6 mill) and 5) Pakistan (0.3 – 0.5 mill). South Africa had 389 974 new TB case notifications in 2011 (1). Figure 1.1 shows that Africa is amongst the high incidence regions in the world with regard to TB disease.

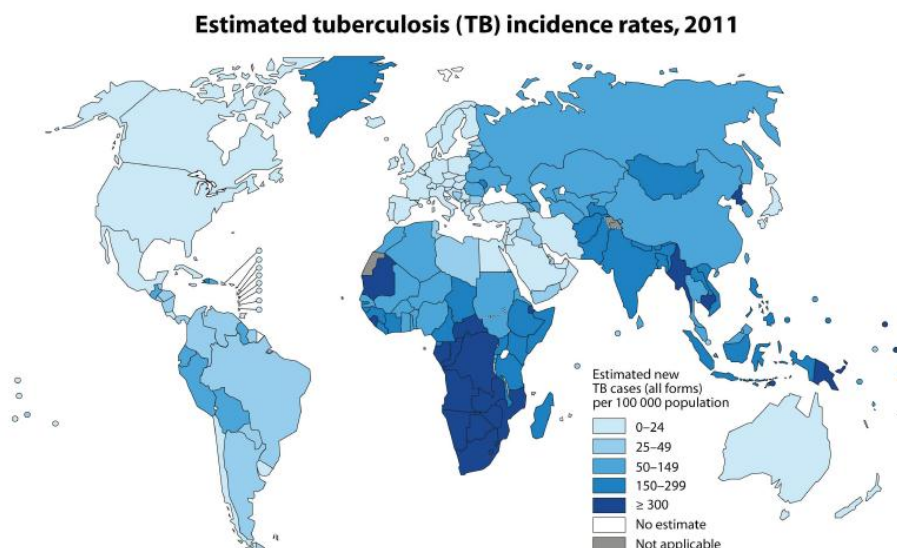


Figure 1.1: Estimated TB incident rates as recorded in 2011 by the WHO (1).

1.2 Immunology of TB

The *M.tb* bacilli gain entry into the hosts system through the respiratory tract by the inhalation of droplets allowing it to enter the lower respiratory tract. When *M.tb* bacilli enter the lungs they endure four possible fates (2). The initial host response can completely eradicate the bacteria so that the patient does not develop TB. Secondly, the bacilli could begin to multiply and grow within the host after infection causing clinical disease which is known as primary TB. Thirdly, once in the host the bacilli could remain dormant and never cause clinical disease. This phenomenon is called latent TB infection and the individuals only presents with a positive tuberculin skin test (TST). Lastly, the latent bacilli may begin to grow at a later stage resulting in the manifestation of clinical disease referred to as reactivation TB (2).

Upon introduction of an antigen to the host, the body will launch a nonspecific defence mechanism, either immediately or within hours after the introduction to the host. This initial immune response is known as innate immunity (3). Cells of the innate immune system are neutrophils, macrophages and dendritic cells (DC) which phagocytose and kill pathogens. The innate immune response contributes to the activation of the adaptive immune response through antigen presentation. Antigen presentation and interleukin (IL)-12 production by the macrophage leads to activation of T helper cells, which in turn produce interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α) (figure 1.2). These classical Th-1 cytokines stimulate the macrophages to produce reactive oxygen species (ROS) that kill the intracellular bacteria.

The host responds to *M.tb* infection with a cell mediated immune response, aiming at growth inhibition of the bacteria through phagocytosis as well as cellular immunity through antigen presentation and recruitment of T-cells (4). The DC and macrophages are responsible for the binding and uptake of the pathogen. Macrophages play a fundamental role, by secreting cytokines and chemokines and the presentation of antigens to T-cells (5). The diagram in figure 1.2 shows the cytokine production by *M.tb* infected macrophages to activate and inhibit T-cell functions.

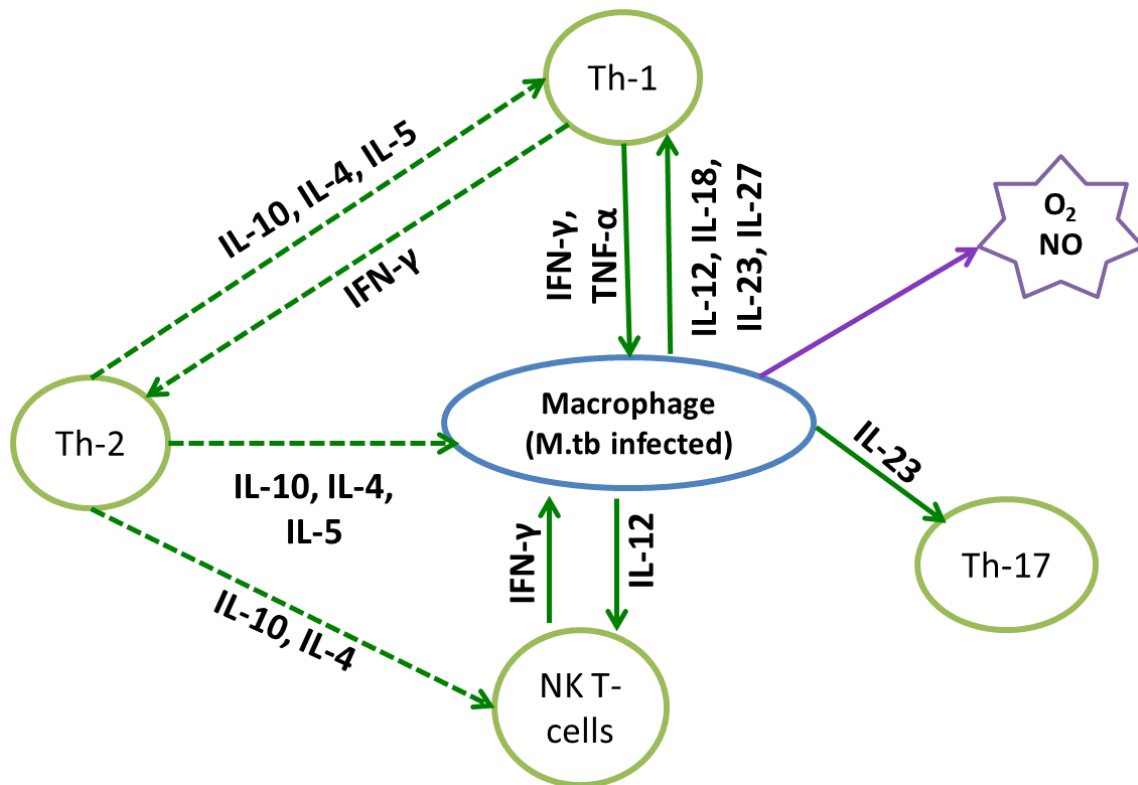


Figure 1.2: Schematic representation of the interaction between macrophage and T-cells in tuberculosis adapted from (3, 6).

Dotted lines represent an inhibitory effect, the solid lines represents activation.

Cell-mediated immunity is known to be vital for protection against TB disease. Following M.tb infection, M.tb resides mainly in the alveolar macrophages phagosome that provide the first line of defense, a vacuolar compartment associated with major histocompatibility complex (MHC) II antigen processing and presentation. MHC class II presentation of mycobacterial antigens by macrophages to CD4⁺ T cells is an essential part of the protective response against the infection.

Phagocytosis is the internalisation of the bacteria into endosomes/phagosomes. Acidification of the phagosome prevents bacterial growth and increases the activities of antimicrobial hydrolases and directs the fusion of phagosomes with lysosomes. As a result phagosome acidification is a crucial event that prompts the destruction of invading pathogens (7). The phago-lysosome fuses with vesicles from the golgi apparatus after which antigen is incorporated and displayed on the cell surface by MHC-II. The antigen presenting cells (APC) detect the pathogen through pattern recognition receptors (8).

In the case of M.tb the bacterium is able to inhibit the acidification of the phagosome and largely evades being killed by the macrophage (9). The lack of acidification in the

mycobacterial phagosome that is seen with *M.tb* infection is in part due to the absence of the V-ATPase on the phagosomal membrane. *M.tb* is speculated to be able to sense its engulfment by macrophages and subsequently interferes with the host signalling to promote its intracellular survival (7).

1.3 T-cell functions

Eradication of the *M.tb* infection mainly depends on the successful interaction between the infected macrophages and the T cells. CD4⁺ T cells produce IFN- γ and TNF- α after activation with mycobacterial antigens by APCs. CD8⁺ T cells, contribute as well through secretion of cytokines and lysing infected cells (10). CD4⁺ T cells are essential for the control of the *M.tb* infection. Alternatively CD8⁺ T-cells recognize antigens, mainly viruses, processed in the cytosol and presented by MHC-I molecules on their cell surface, which are found on the surface of most nucleated cells. CD4⁺ T-cells assist the host immune response by activating effector cells and recruiting additional immune cells to the site of disease. CD8⁺ T-cells are more likely to be cytotoxic to target cells and participate directly in the lysis of infected cells and induction of apoptosis of the target cells (11). CD4⁺ T cells are essential for the control of the *M.tb* infection. T-cells become activated upon recognition of mycobacterial antigens presented on the cell surface APCs. T helper type 1 CD4⁺ T-cells (Th-1) and natural killer (NK) cells secrete IFN- γ that activate macrophages to produce reactive oxygen and nitrogen species to assist in the killing of the *M.tb* bacilli. Alternatively CD8⁺ T-cells recognize antigens processed in the cytosol and presented by MHC-I molecules on their cell surface, which are found on the surface of most nucleated cells. CD4⁺ T-cells assist the host immune response by activating effector cells and recruiting additional immune cells to the site of disease. While CD8⁺ T-cells are more likely to be cytotoxic to target cells and participate directly in the lysis of infected cells and induction of apoptosis of the target cells (11).

CD4⁺ Th-cells can be separated into different subsets, Th-1, Th-17, Treg, TFh and Th-2, however for the purpose of this study we focus on Th-1 and Th-2 cells. These cells derive from Th-0 cells, the differentiation from these precursor cells are thought to be stimulated through cytokines such as interleukin-12 (IL-12) (12). Phenotypically, Th-1 cells are characterized mainly by their ability to produce the cytokines IFN- γ and IL-2, whereas Th-2 cells produce cytokines such as IL-4, IL-5, and IL-10. Th1-type cytokines are those that

activate other inflammatory and phagocytic cells capable of inhibiting the growth of intracellular bacteria. Th-2 cells are involved in the production of IgE and recruitment of eosinophils. Certain cytokines can be secreted by both Th-1 and Th-2 such as, IL-3, lymphotoxin, and granulocyte-macrophage-colony stimulating factor (GM-CSF) (12).

i. IL-12p40

IL-12 is a key player in host defence against M.tb, IL-12 is produced mainly by phagocytic cells, such as macrophages. The phagocytosis of M.tb appears to be required for the secretion of IL-12 (10). IL-12 induces the Th-1 response and down-regulates the Th-2 response by increasing IFN- γ production from activated T cells. The IFN- γ secretion along with Th-1 cytokines antagonizes the IL-4 and IL-10 production (13). It has been suggested that IL-12 is a key cytokine in immune regulation by changing the Th-0 to the Th-1 phenotype and can be seen as a marker of active TB disease (13).

IL-12 acts on the T-cells, hence it serves as a link between the innate and adaptive immune systems (4). IL-12 naturally occurs as a heterodimer consisting of two subunits namely p40 and p35, IL-12p40 plays a central role in the formation of granulomas, Th-1 development and M.tb infection control (14). IL-12 binds to the IL-12R β 1 and IL-12 β 2 complexes respectively which are located on the surface of the NK cells and the Th1 cells (4). Three cytokines produced by APCs namely IL-23, IL-18 and IL-27 have been identified to have IL-12 like activities. The p40 subunit from IL-12 is shared with IL-23 which is coupled to a second chain p19, the IL-23 receptor also shares the IL-12R β 1 subunit of the IL-12 receptor to form a unique receptor complex IL-23R β 3. All three cytokines are responsible for the production of IFN- γ , IL-18 mainly works together with IL-12 for optimal IFN- γ production; IL-27 causes the proliferation of naïve T-cells and produces significant amounts of IFN- γ in synergy with IL-12 and IL-18 (4). It has been shown that IL-12 is vital in TB, and that the IL-12p40 subunit plays a part in the migration of DCs from the lung to the mediastinal lymph nodes which are required for the activation of naïve T-cells (15). People with a congenital defect in IL-12p40 or in the β 1 subunit of the IL-12 receptor have increased susceptibility to mycobacterial infection (14).

A study was performed in a murine model to determine the importance of IL-12 within the context of M.tb infection (16). Administration of IL-12p70 therapy to CD4^{-/-} mice after M.tb infection resulted in a reduced bacterial burden in the lungs and overall survival of these

mice. This implicates that IL-12 is beneficial in infectious disease models that require a Th-1 immune response in order to clear the infection. This data indicates that endogenous IL-12 is important for the priming of a T-cell response against M.tb infection. The addition of exogenous IL-12 over a period of time however did not result in a stronger T-cell response in the C57BL/6 mice (16). Administration of IL-12 therapy increased the expression of IFN- γ at an early time point, 10 days post M.tb infection, however after 1 month there was a notable reduction in the frequency and number of IFN- γ producing T-cells present in the lungs. This led to the hypothesis that the initial peak in IFN- γ production from the exogenous IL-12 may be the cause of the overall enhanced outcome in the CD4^{-/-} mice (16).

Another study used an IL-12p40^{-/-} mouse model infected with M.tb to determine the capability of the mice to control the bacilli and determine the effects that the knock out would bring on the immune response (17). The study found that the absence of IL-12p40 resulted in the uninhibited growth of M.tb bacilli in all target organs after a systemic infection. The growth correlated with a reduction in the expression of mRNA of IFN- γ , this was also reflected in the decrease in nonspecific as well as antigen specific IFN- γ protein production. The lack of IFN- γ production was linked to the delay in antigen specific T-cell activation within the mice. The authors concluded that IL-12 plays a key role in the generation of antigen-specific T-cells that are able to produce IFN- γ (17). The conclusion that was drawn from the results was that the initial interaction of the bacilli with the host induces IFN- γ production. The amount of IFN- γ produced is dependent on the concentration of IL-12 and TNF- α within cells. An increased production of IFN- γ is necessary to induce the expression of IL-12R β 2 chain on naïve T-cells. The expression of the receptor on the naïve T cells is needed for the T cells to act in response to the IL-12 and thus become antigen-specific IFN- γ producing cells (17).

A human study found that people with genetic defects in their IL-12/IL-23/IFN- γ system, had increased susceptibility to mycobacteria (18). This led to the conclusion that the IL-12/IL-23/IFN- γ system plays an important role in immunity against mycobacteria (18).

ii. IFN- γ & TNF- α

With regard to M.tb infection a lot of focus has been placed on the role of IFN- γ and its ability to activate the macrophages to inhibit the growth of mycobacteria. It has been suggested that IFN- γ plays an important part in host defence and its primary role is to serve

as a macrophage activator (3). The secretion of IL-12 by macrophages acts on the cells to produce IFN- γ during the initial phases of the immune response, this is essential for the activation and differentiation of the antigen specific Th-1 cells (4). The activation of macrophages is dependent on IFN- γ and tumour necrosis factor- α (TNF- α) in this manner activating the mechanism of cell mediated immunity, to allow phagocytes to contain the pathogen and allow protection to the host. The host's immune system tries to control the M.tb infection through activation of the CD4⁺ T-cells by IFN- γ dependant activation of macrophages, while IL-12 plays a role in creating and maintaining the host's protective immune response (14), IL-12 is an important factor in the release of IFN- γ from the natural killer cells (19). Individuals who have lower levels of IFN- γ and IL-12 have a larger risk of acquiring TB (4). A study done on patients with severe clinical TB who were classified radiographically, presented with low levels of circulating IFN- γ in peripheral blood. The low levels of IFN- γ are associated with a severe state of disease (20).

IFN- γ is characteristically secreted by Th-1 cells, the higher detectable titers of IFN- γ and TNF- α normally observed for the duration of M.tb infection indicates that a Th-1 response is favoured by M.tb. This is confirmed by the nearly undetectable levels of IL-4, a Th-2 cytokine (13). The raised IFN- γ levels in TB patients is essential for killing intracellular mycobacterium through macrophage activation, as well as stimulating the release of TNF- α in addition to 1, 25 dihydroxycholecalciferol (vitamin D3) (21). Both of these assist in the inhibition of the mycobacterium, possibly through the production of reactive oxygen and nitrogen species (21). TNF- α is a proinflammatory cytokine produced by monocytes, macrophages, and dendritic cells when it encounters M.tb or mycobacterial antigens, TNF- α plays a role in granuloma formation and macrophage activation.

The interaction between NK T-cells, macrophages and T-cells is shown in figure 1.2 above. The production of IFN- γ from Th-1 and NK T-cells activates the macrophage which produces the ROS. The production of IL-12 acts as a positive feedback loop to NK T-cells. The production of IL-4 and IL-10 by Th-2 cells inhibits the activity of macrophages, Th-1 and NK T-cells but to a small extent, as IFN- γ secreted by the Th-1 cells supresses the function of Th-2 cells (3).

iii. IL-1 & IL-6

IL-1 β is a proinflammatory cytokine that is involved in the host response to M.tb. IL-1 β similar to TNF- α , is largely produced by monocytes, macrophages, and dendritic cells. IL-1 β is expressed in excess at the site of disease in TB patients (10). The cytokine IL-6 has properties of both pro- and anti-inflammatory cytokines. IL-6 is produced at the site of infection at an early stage during M.tb infection. It is postulated that IL-6 may be harmful in M.tb infections, as it inhibits the production of TNF- α and IL-1 β . However, contradicting reports suggest a protective role for IL-6. A study conducted on a mouse model deficient in IL-6, presented with an increased susceptibility to infection with M.tb, which seemed to be equivalent to a lack of IFN- γ production at an early stage of infection (10).

iv. IL-10

The cytokine IL-10 is produced by the macrophages following phagocytosis, however T cells are able to produce IL-10. IL-10 antagonizes the proinflammatory cytokine response by downregulating the production of IFN- γ , TNF- α and IL-12, these cytokines are essential for a protective immune response in a TB patient, thus it can be stated that IL-10 will obstruct the host's defence mechanism to M.tb (10). Elevated IL-10 levels have been reported in patients with TB, the raised level of IL-10 production is associated with an increase in the disease prevalence. During TB an increased IL-10 production is seen more frequently in anergic patients, proposing that M.tb induces IL-10 production, suppressing an effective immune response (22).

1.4 Glucocorticoids and TB

i. Endogenous Glucocorticoids

Glucocorticoids (GC) are a class of steroid hormones. In humans the main GC is cortisol and this hormone regulates numerous processes within the body such as, cardiovascular, metabolic, immunological responses and homeostatic functions (23). Cortisol in layman terms is often referred to as the 'stress hormone' as it primes the body to respond and cope in situations presenting with physical and emotional stress. It is also an essential regulator of inflammatory processes and immune functions; cortisol plays a key role in several processes associated with host defence mechanisms (24). In PBMCs of TB patients it was reported that

the mRNA of the human glucocorticoid receptor (hGR), consisting of the two isoforms hGR α and hGR β ratio was higher than those in the control group (25). Upon analysing the ratios against the disease severity, the severe TB cases showed the lowest mRNA hGR α / β ratio. The findings in the severe cases are compatible with a certain degree of GC resistance, which could potentially explain the increased inflammation and tissue destruction that occurs in these patients (25).

ii. Control of secretion of Glucocorticoids

Cortisol is synthesized from cholesterol in the zona fasciculata cells of the adrenal cortex (23). The release of the hormone follows a distinct circadian rhythm, with maximum levels reached just prior to waking up, levels decrease throughout the day with lowest levels reached in the early phase of sleep (23). In a situation posing a threat of emotional and/or physical trauma cortisol is released. The extent of the stress response is dependent on different factors regarding the stress stimulus such as the nature, duration, intensity and the person's previous experience of the stimulus (23).

The hypothalamus pituitary axis (HPA) is responsible for the secretion of GCs for both the stress response and the normal circadian rhythm (figure 1.3). The hypothalamus acts as a sensor of changes in the external and internal environment. It receives and combines neural and humoral information from different sources. The hypothalamus responds to the received stimuli, circadian factors and either physical or emotional, through activation of a signalling cascade leading to glucocorticoid synthesis (24).

The first step is the release of a hypothalamic neurohormone, corticotrophin-releasing hormone (CRH) from parvocellular neurones. CRH moves from the hypothalamus through the hypophyseal-portal blood vessels to the anterior pituitary gland where it stimulates the release of adrenocorticotrophic hormone (ACTH) from the corticotrophs. ACTH acts on the adrenal cortex via type 2 melanocortin receptors to start the synthesis of cortisol, once synthesised it is released into the systemic circulation. Negative feedback ensures the sensitivity of the HPA axis to incoming stimuli. The glucocorticoids use the negative feedback loop to ensure that the secretion of CRH and ACTH from the hypothalamus and anterior pituitary gland respectively is suppressed (24).

It has previously been shown that a decrease in dehydroepiandrosterone (DHEA) levels is accompanied by an increase in the cortisol/DHEA ratio in individuals with active TB (26). DHEA is a circulating steroid hormone, produced by the adrenal glands, the gonads, and the brain, where it functions predominantly as a metabolic intermediate in the biosynthesis of the sex steroid hormones, androgen and estrogen. The increase in GCs exerts anti-inflammatory properties that oppose protective immune responses against intracellular pathogens. The decrease in DHEA in TB patients can account for the inhibitory effect of GCs on the immune response, although it does not account for the control of inflammatory processes. Thus the increases in cortisol levels are insufficient to correct for the loss of anti-inflammatory responses due to the reduction of DHEA in TB patients (26).

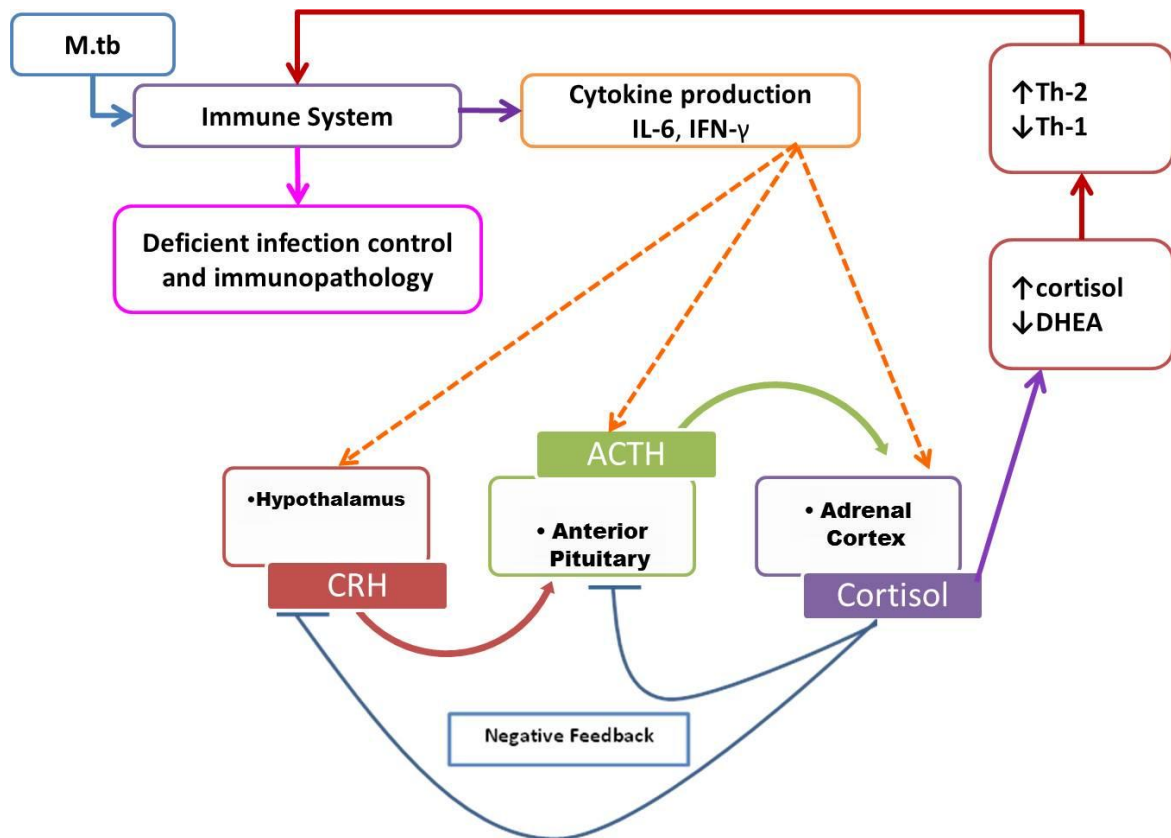


Figure 1.3: Diagram showing the HPA axis and the feedback loop responsible for the release of cortisol adapted from (18).

1.5 Mechanism of action

1.5.1 Hormone signalling

Glucocorticoids act largely through a nuclear receptor called the glucocorticoid receptor (GR). The mechanisms of action can either occur through genomic mechanisms that involve the nuclear receptors, or the non-genomic mechanism which acts through the classical receptors or membrane associated receptors and signalling cascades.

i. Genomic mechanisms of action

If there are no ligands present the GR resides within the cytoplasm bound to various chaperone molecules composed of heat shock proteins and immunophilins, forming a large multi-protein complex (27). Upon interaction between the ligand and GR, the receptor dissociates from the heat shock proteins leading to nuclear translocation of the active receptor-ligand complex. Once in the nucleus the GR controls the transcriptional activity of the GC responsive genes by binding to the GR response element (GRE) or the negative GRE (nGRE) in the promoter regions of target genes (28). The transcriptional regulation by the GR is exerted through the classical transcriptional activity on GC responsive promoters through the binding of a homodimer ligand-receptor complex to the GREs (29). In the trans-repression hypothesis, ligand-activated GR interacts with, or tethers to, key transcription factors, such as NF- κ B, AP-1 and STAT, to switch off inflammatory gene transcription such as IL-1 and IL-6 (30)(29). The transcription factors are involved in regulating the expression of pro-inflammatory genes therefore binding to these transcription factors results in the trans-repression of cytokine secretion such as, IFN- γ , TNF- α , IL-1 and IL-2 involved in the host inflammatory responses (31). The mechanisms describes above indicates various mechanisms of GC repression could either be independent of the requirement for a gene expression (classical trans-repression) or dependent on gene expression (repression through the trans-activation of anti-inflammatory genes). The GRE facilitated trans-activation of transcription is important for the up-regulation of several genes which are involved in the regulation of immune functions. The ligand-receptor complex can bring about transcription, upon binding to the GREs, for instance I κ B, annexin-1, mitogen-activated protein kinase (MAPK) and IL-10 (28). Changes induced by genomic mechanisms usually take hours or days. However, some anti-inflammatory and immunosuppressive effects of GCs occur rapidly thus non-genomic effects have been suggested as an explanation. A schematic representation of the genomic signalling can be seen in figure 1.3.

ii. Non-genomic mechanism of action

Non-genomic GC signalling mechanisms (figure 1.3) work independently of their effects on transcriptional regulation promoted inside the nucleus, but utilize other pathways of action (32). Such actions of GCs are rapid and take place within seconds to minutes after initiation in contrast to genomic actions that usually require many hours. Three alternative non-genomic GC mechanisms have been suggested: Firstly, signalling through a membrane GR (mGR). Secondly, direct membrane effect of the GCs and lastly, molecules released from GR-hsp-complex are said to mediate rapid effects (32).

Direct membrane effects of glucocorticoids have been observed at high doses of GCs in red blood cells, leukocytes, a number of cancer cell lines and certain neuronal cells. The non-genomic effects of GCs have been described in different cellular processes for example, actin structures, neuronal membranes, transmembrane currents, intracellular Ca^{2+} mobilization and signal transduction pathways. Very high concentrations of GCs seem to change cell membrane fluidity and other physico-chemical properties through insertion in the lipid bilayer of cell membranes (32). The inhibition of Lck and Fyn in CD4⁺ lymphocytes serves as an example of GR-mediated modulation of the activity of signalling molecules that are connected to the plasma membrane. By blocking the recruitment of Lck and Fyn to the T-cell receptor (TCR), affecting the stimulation of downstream kinases MAPK, JNK, and protein kinase B and C and so inactivating the TCR signalling (28,33). The GR also translocates into the mitochondria and regulates their activities possibly in a non-genomic fashion. The way in which the GR-mediated apoptosis in the mitochondria works is not well understood. However, the reduction of the membrane potential of the mitochondria, the interaction with pro-apoptotic B-cell lymphoma 2 (Bcl-2) family proteins, and the stimulation of the Bcl-2-associated X protein (Bax) / Bcl-2 homologous antagonist (Bak) assembly may play a role. Additionally, the GR could influence the induction of apoptosis by directly changing the transcription of the mitochondrial genes through interaction with their GRE-like sequences (32).

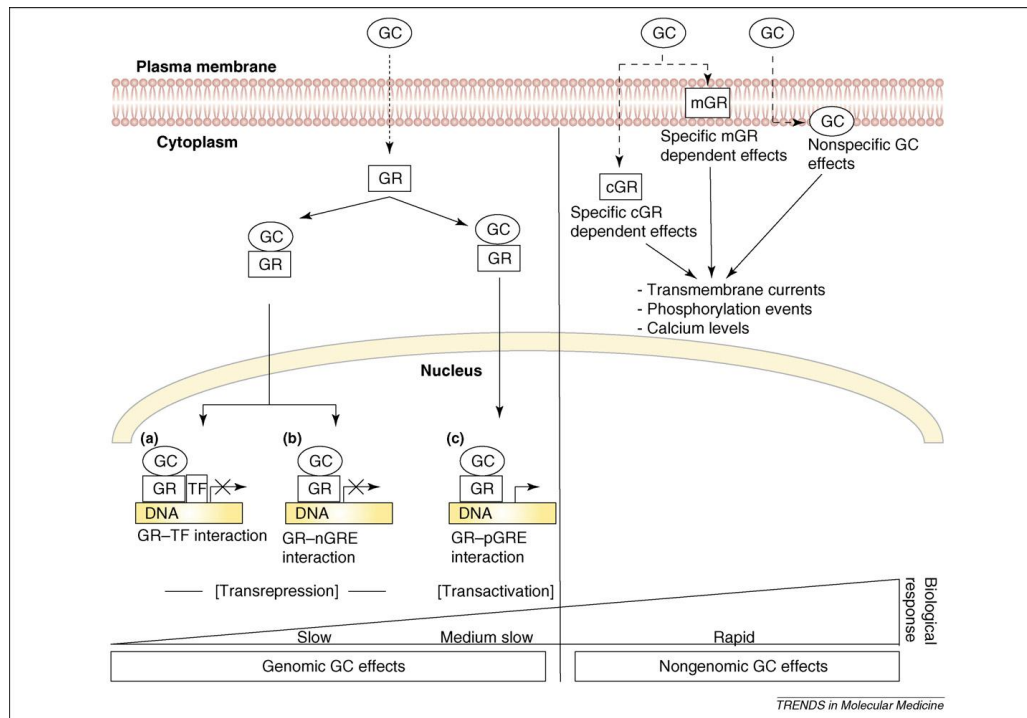


Figure 1.4: Diagram shows the genomic and non-genomic mechanism of action of GCs (28).

1.5.2 GR and TCR interaction

It has been reported that in human T-cells (figure 1.4) the GR is linked to the early signalling complex of lymphocyte-specific protein tyrosine kinase (Lck) and the proto-oncogene tyrosine protein kinase (Fyn) molecules of the TCR after TCR stimulation (32).

The activation of T-cells occurs in response to the presentation of antigen. Stimulation of the TCR is triggered by MHC II molecules on APCs that present antigen peptides to TCR complexes, leading to the activation of the TCR complex. This happens through the activation of Lck and Fyn which results in TCR phosphorylation of the tyrosine residues (33). The kinases Lck and Fyn are essential for TCR signalling, Lck binds to CD4 or CD8 co-receptor and Fyn has been known to bind to CD3 cells. Upon the initiation of the T-cell signalling cascade through the activation of Lck, ζ -associated protein 70 tyrosine kinase (ZAP-70) is recruited to the TCR complex where it is phosphorylated and activated by Lck. Once activated ZAP-70 in turn phosphorylates linker for activation of T-cells (LAT) which activates the downstream signalling cascades that includes protein kinase C (PKC), protein kinase B (PKB) and MAPKs such as p38 MAPK, extracellular-signal-regulated kinase (ERK) and JNK (33). ERK (Extracellular signal-Related Kinase) is a family of two, homologous

proteins known as Erk1 (p44, MAPK3) and Erk2 (p42, MAPK1) both of them function in the same signalling pathway. The two proteins are often collectively referred to as ERK1/2 or p44/p42 MAP kinase. The activation of ERK-1 and ERK-2 is mediated by MEK. Upon activation ERK MAP kinase 1/2 can induce a wide range of cellular processes, proliferation, differentiation and transcription. Once ERK 1/2 is activated it translocates to the nucleus where it phosphorylates its nuclear targets. The ERK pathway is considered the classical MAPK (Mitogen-Activated Protein Kinase) signalling pathway (34). This signalling pathway controls and regulates the growth and survival through the promotion of cell proliferation and the prevention of apoptosis. ERK is activated by growth factor stimulation of receptor tyrosine kinases (RTKs), this leads to the activation of the Ras-Raf-MEK,-ERK pathway that results in MEK, and the phosphorylation and activation of ERK1/2 (p44/044) on the threonine-x-tyrosine (TxY) motif (Thr185/Tyr187 for Erk1 and Erk2, respectively). The transcription factor cAMP response element-binding protein (CREB) binds to DNA sequences called cAMP response elements (CRE) to increase or decrease the transcription of downstream genes. Once activated the CREB protein binds to a CRE region, it is then bound by a CBP which then co-activates CREB to turn gene expression on or off.

An example of non-genomic GR-mediated modulation linked with the plasma membrane is the inhibition of Lck and Fyn in T-cells, preventing the recruitment of Lck and Fyn to the TCR, thus preventing downstream TCR-mediated signaling events, such as the stimulation of downstream kinases MAPK and JNK (33). The properties of GC-induced rapid effects on signal transduction in T-cells were investigated (33). It was reported that there was clear differences in the phosphorylation patterns between the dexamethasone (DEX) treated and non-DEX-treated cells. This led to the conclusion that there are rapid DEX dependant effects within the signal transduction. The largest observed effect was the decrease in phosphorylation of Lck/Fyn kinases in the DEX treated group, as well as an impairment of recruitment of these substrates to the TCR complex. Thus impairing the recruitment of Lck/Fyn disrupts the formation of the GR-TCR-Lck-Fyn multi-molecular complex, suggesting that Lck and Fyn play a key role in TCR activation. Lck and Fyn act as rapid molecular targets of GC action in activated human T-cells through a GR dependant mechanism (33). GCs can also obstruct TCR signaling further downstream at the level of 1,4,5-triphosphate (IP3) assisted release of Ca^{2+} from the endoplasmic reticulum. Calcium responses were proposed to be mediated to some extent by a protein-protein interaction between Lck and IP3 receptor (35). A knock down of Lck led to a loss of Lck expression and activity and resulted in IP3 receptor down-regulation. T cells stimulated with DEX showed

rapid conversion in calcium signalling induced by GC stimulation. The authors concluded that the GC mediated inhibition of Lck controls the pattern of TCR responses by negatively regulating IP3 receptor expression (35).

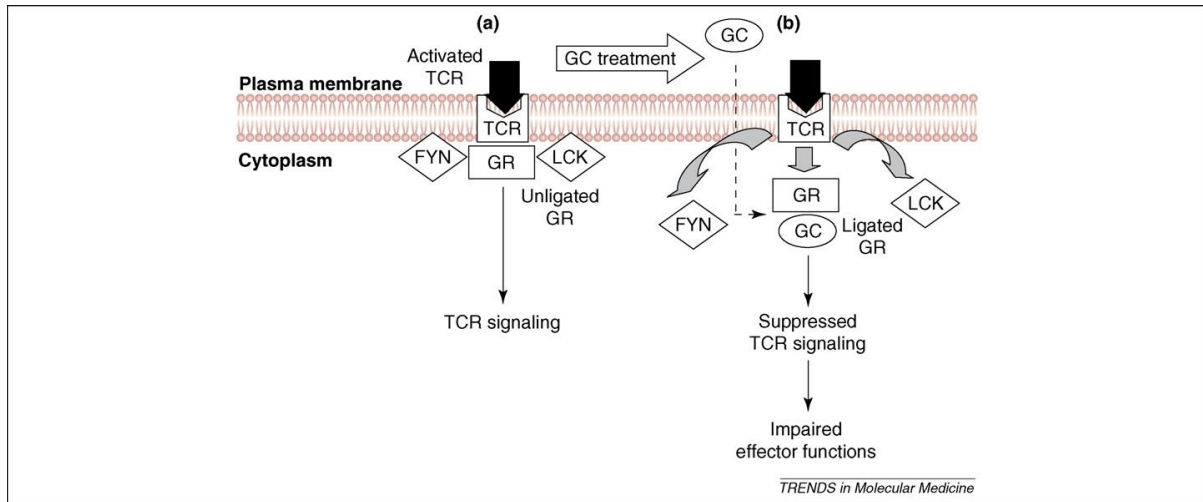


Figure 1.5: Diagram shows the suppressed immune signalling through TCR as a result of GC treatment (28).

1.5.2.1. Signalling molecules not specific to TCR signalling

Signalling through the TCR signalling pathway is not the only way in which cells can react to a change within their environment; cells can act in many different ways through a selection of intracellular signalling pathways. Some signalling molecules will signal through the Receptor Tyrosine kinases (RTK) pathways. The activation of the Signal Transducers and Activators of Transcription (STAT) proteins, STAT5A and STAT5B are activated through tyrosine phosphorylation usually via JAK proteins. The two proteins are often collectively referred to as STAT5A/B. STAT5A/B plays a key role in immune cell development and regulation, and facilitates IL-2 and IL-15 signalling in regulatory T-cells. The p70S6 kinase acts downstream of PIP3 in the PI3 kinase pathway. The kinase p70S6 targets the substrate in the S6 ribosomal protein, the phosphorylation of S6 leads to protein synthesis at the ribosome. The activation of mTOR leads to the phosphorylation of p70S6 therefore leading to its activation. The STAT3 protein is activated through the phosphorylation of the tyrosine, in response to various cytokines and growth factors. STAT3 facilitates the expression of a variety of genes in response to cell stimuli and therefore plays an important role in many cellular processes such as cell growth and apoptosis. The p38 MAP kinase (further just referred to as p38) forms part of the MAP kinases that respond to stress stimuli, such as inflammatory cytokines,

heat shock or osmotic shock. The functions of p38 include the involvement in cell differentiation, apoptosis and autophagy. MKK3 and SEK are responsible for the activation of p38 by the phosphorylation at threonine and tyrosine residues. Activated p38 can phosphorylate transcription factors such as ATF2, Mac and MEF2, p38 can also phosphorylate post-transcriptional regulating factors such as TTP. The activation of JNK occurs through dual-phosphorylation of threonine and tyrosine residues within a Thr-Pro-Tyr motif. JNK alters the activity of various proteins that located at the mitochondria or acts in the nucleus. JNK activates downstream molecules such as, c-Jun, ATF2, ELK1, p53 and HSF1. The downstream molecules that are inhibited by JNK activation include NFAT4, NFATC1 and STAT3. Through the activation and inhibition of molecules JNK regulates cellular functions including cell growth, differentiation, survival and apoptosis. I κ B α is part of a group of cellular proteins that function to inhibit the transcription factor NF- κ B. I κ B α inhibits NF- κ B by hiding the nuclear localization signals of the NF- κ B proteins and ensuring they remain in an inactive state within the cytoplasm. I κ B α blocks the capacity of the transcription factor NF- κ B to translocate to the nucleus.

1.5.3 Progesterone

Progesterone is a steroid hormone that is involved in the female menstrual cycle, pregnancy and embryogenesis. Progesterone is produced by the corpus luteum, the adrenal glands and the placenta during pregnancy. Progesterone exerts its primary action through the intracellular PR, although the presence of membrane bound progesterone receptor has been suggested. The progesterone receptor (PR) is a protein found inside cells, and is activated by the steroid hormone progesterone. The effects of progesterone are mediated by two functionally different isoforms of the progesterone receptor, PR-A and PR-B. Progesterone bound PR-A and PR-B have different transcription activating properties. The PR-B functions as a transcriptional activator in most cell and promoter environments, whereas PR-A is transcriptionally inactive and functions as a strong ligand dependent trans-dominant repressor of steroid hormone receptor transcriptional activity. An inhibitory domain, which maps to the amino terminus of the receptor, exists within both PR isoforms. The PR has three known membrane receptors, mPR- α , mPR- β and mPR- γ , the membrane PR's are thought to assist with the signalling of progesterone (36). The signalling through membrane bound receptors are more rapid than the genomic method of the classic steroid receptors. The androgen

receptor (AR) is a type of nuclear receptor that is activated by binding of either of the androgenic hormones testosterone or dihydrotestosterone in the cytoplasm, where upon they then translocate to the nucleus. The AR is closely related to the PR. The progesterone receptor membrane component 1 is involved in the heme binding as well as binding and activating p450 proteins, which play a key role in drug, hormone and lipid metabolism (36).

A recent study hypothesized that intracellular PR plays a role in regulating the CD4⁺ T cell activity and adaptive immune responses *in vivo* (37). A mouse model was used where the use of the intracellular PR had been knocked out. They found that the knock of the intracellular PR suppressed the T-cell dependant antibody responses. It was speculated that the suppression occurred largely by reducing CD4⁺ effector T cells activity, potentially through the transcriptional repression of the IFN- γ (37). There is increasing evidence that progesterone modulates immune functions in mammals (38), such as the changes in the symptoms of autoimmune diseases for example rheumatoid arthritis. In women changes in the cellular immune response to infection and the expression of IFN related genes have been observed during the menstrual cycle and pregnancy. Dosiou et al. suggested that the observed increase in membrane progesterone receptor alpha (mPR- α) expression could potentially contribute to the immune modulation of progesterone during the second half of the menstruation cycle and during pregnancy (39). When T cell clones that have a known Th-1 cytokine profile were stimulated with CD3 and progesterone, detectable amounts of IL-4 mRNA was found as well as the expression of CD30 on their surface membrane (40). These results show that progesterone can support the development of Th cells producing Th-2 cytokines (40). Progesterone promotes differentiation of Th-2 cells and production of type 2 cytokines like IL-10. Nonetheless, most studies fail to detect the presence of the nuclear progesterone receptor (nPR) in immune cells, this led to the suggestion that there must be alternative mechanisms of action for progesterone (figure 1.5) (38).

mPRs are suggested as alternative progesterone receptors, another group of proteins proposed to mediate non-genomic signalling. The different forms alpha, beta, and gamma were first identified in fish. They play a role in oocyte maturation and contribute to the pathogenesis of epithelial ovarian tumors as it is differentially expressed in ovarian carcinoma (41). The expression of mPR- α in human breast cancer cells was reported to decrease cAMP levels after progesterone stimulation by activating the MAPK pathway (42). In agreement,

expression of the seatrout mPR- α in human breast cancer cells was shown to rapidly activate the MAPK (ERK1/2) signalling cascade and lower intracellular cAMP levels in response to progesterone (43). These findings are contradicting to the findings that describe that cAMP concentrations in isolated plasma membrane preparations from breast cancer cells who stably express human mPR- α were down-regulated upon treatment with progesterone (44). Contradicting results are seen in the function of mPRs in relaying MAPK activation in response to progesterone. The mPR- α from seatrout and zebrafish were reported to activate ERK1/2 when expressed in breast cancer cells (43). However, the human mPRs appear to not signal through ERK1/2 but activate p38 MAPK. Though, this speculation was only based on the observation that treatment of cultured human myometrial cells with cell-impermeable progesterone-BSA led to rapid phosphorylation of p38, but not ERK1/2 (45).

In peripheral blood leukocytes and T lymphocytes from women of reproductive age and in the Jurkat cell line, the transcripts of membrane progesterone receptor (mPR)- α and mPR- β have been identified (38). A study done using flow cytometry reported that the mPR- α protein is localized on the cell membrane and the N-terminus is situated extracellularly (46). It further reported that the expression of the mPR- α appeared to be hormonally regulated in the CD8⁺ T cells, but this was not observed in the CD4⁺ T cells. During the state of high progesterone levels, in the mid-luteal phase, there was an increase in mPR- α expression on the CD8⁺ T cells. The expression of mPR- β does not change in either the CD8⁺ or the CD4⁺ T cells between the follicular and mid-luteal phase. This led to the hypothesis that the increase in the expression of mPR- α in the CD8⁺ T cells during the luteal phase could be a contributing factor for the immune modulatory effect of progesterone in the second half of the menstrual cycle and during pregnancy (46).

Progesterone receptor membrane component (PGRMC1) has been proposed as alternative to progesterone receptor, this protein is expressed in various human tissues including heart, liver and placenta (45). PGRMC1 is strongly expressed in different kinds of cancer including lung, breast and ovarian cancer and is discussed to promote cell survival and insensitivity to chemotherapy (47). It has been shown that progestins are capable of activating a variety of signalling pathways through mPR- α and progesterone membrane component 1 (PGRMC1) (38). In human myometrial cells upon progestin binding to the mPR- α , leads to the activation of the p38 MAPK, which leads to the phosphorylation of myosin light chain protein in these cells. Activation of a pertussis toxin sensitive pathway via mPR- α and mPR- β in human

myometrial cells alters the nPR transactivation activity, by activating this pathway it also alters the expression of nuclear steroid receptor coactivator (SRC-2). There have been indications that progestins initiate Ca^{2+} mobilization from intracellular stores through both mPR- α and PGMRC1 (38). However there are studies that contradict these findings, another study failed to verify that mPRs are expressed on the cell surface or that they mediate progesterone dependant signalling events, for example activation of p38 MAPKs, Ca^{2+} mobilization or the inhibition of cAMP production (36).

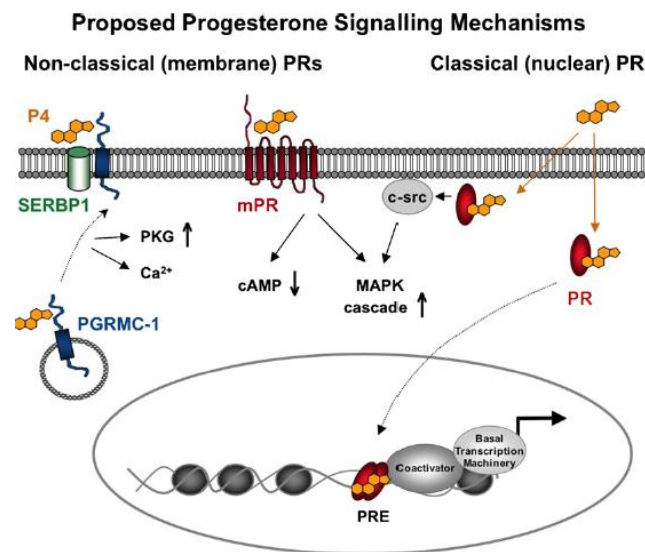


Figure 1.6: Schematic overview of the proposed signalling mechanism of the non-genomic progesterone actions mediated by the classical or nuclear PR. Alternatively signals through the non-classical putative progesterone receptors, PGRMC1 and the mPRs (48).

1.5.4 Injectable progestins

Worldwide there are roughly 150 million women at present who use hormonal contraceptives (49). Of these ≥ 100 million women use combination oral contraceptive pills and ≥ 50 million women are using synthetic progestin injectables, predominantly medroxyprogesterone acetate (MPA), MPA is used mainly in developing countries (49)(50)(46). MPA is a synthetic progestin used by woman as a hormonal contraceptive that is injected intramuscularly every 3 months. MPA is favoured amongst women as it requires fewer clinic visits throughout the course of the year, and is administered in local clinics to women free of charge. Norethisterone enanthate (NET) is the two month injectable contraceptive that is available at

clinics, but is used less frequently as contraception of choice amongst woman. However for women who suffer from TB and are receiving Rifampicin as part of their course of TB treatment, MPA and NET, which are progestin only contraceptives and the recommended choice during the course of TB treatment. An estradiol containing form of hormonal contraception is not recommended. This is due to it being rapidly metabolized as a result of the up-regulation of p450 cytochromes by Rifampicin during the course of the treatment and therefore ineffective as a form of contraception (51).

1.5.5 Molecular mechanisms of action of synthetic progestins

MPA can bind to both the glucocorticoid receptor (GR) and the progesterone receptor (PR) respectively (52). Both MPA and NET have been shown to bind to the androgen receptor (AR) and mineralocorticoid receptor (MR) (53). From a structural point of view MPA, a 17 α -hydroxyprogesterone derivative is more comparable to progesterone than to NET which is a 19-nortesterone derivative (54).

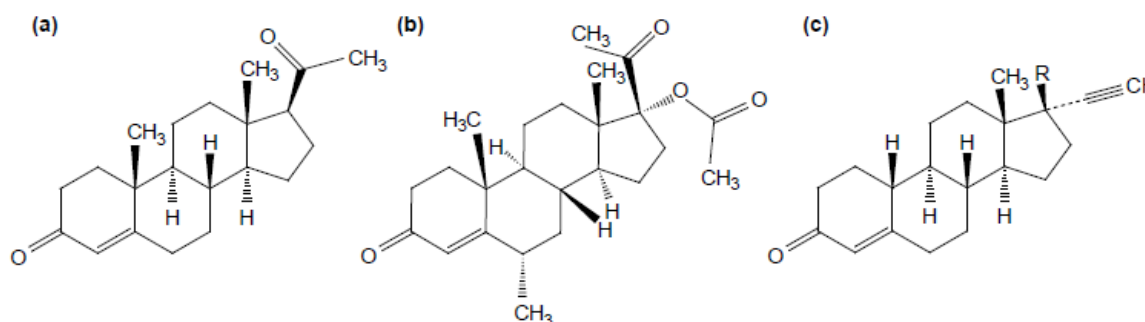


Figure 1.7: Depicts the chemical structure of the following hormones. (a) Progesterone, (b) MPA and (c) NET (54).

MPA in itself is the main progestogenic compound, rather than its metabolites, although in the case of NET, NET-A and NET-EN are hydrolysed to NET and its metabolites which together have progestogenic action. It has been reported that NET metabolites appear to have more non-progestogenic actions than MPA metabolites (54). MPA shows a higher binding affinity to the GR, and MPA mimics GC activity by repressing cytokine genes *in vitro*. MPA is very effective as a GR agonist, greater than both progesterone and NET, for both transrepression and transactivation (54). The agnostic properties of the hormones are reported, according to their trans-activation and trans-repression potency and efficacies (55). Upon the binding of the receptor homodimer to the GREs an increase in gene transcription occurs, known as trans-activation. A monomeric ligand-receptor complex changes gene

expression through the process of protein to protein interactions with transcription factors which is referred to as trans-repression (27). The following parameters were used to classify the hormones; $\geq 85\%$ efficacy as a full agonist; $< 85\%$ efficacy as a partial agonist when compared to the synthetic GC, DEX. The hormones were classified as follows; cortisol – full agonist; MPA – full agonist or partial agonist; Prog – partial agonist and NET – antagonist. The hormone was deemed an antagonist when it showed no activity on any of the three promoters (55).

Kontula *et al.* experimentally determined the binding affinity of certain steroid hormones to the GR and PR. They found that biologically active progestins that contain a pregnane structure (progesterone and MPA) can bind to both receptor groups, while steroids like DEX only have a binding affinity for the GR. However MPA has a much higher binding affinity to the GR than the PR (52). Within the experimental study conducted by Kontula *et al.* it was shown that progesterone decreases the lymphocyte functions such as antigen presentation, cytokine production and pathogen elimination. While under the same stimulatory conditions MPA showing a higher affinity for the GR, produced a different response pattern, similar to the effects seen by DEX (52). It was found that progesterone showed a general suppressive activity on the lymphocyte function when experimentally tested with mitogens for activating T cells, T and B cells or B cells only. Whereas MPA displayed an affinity for the GR, however when stimulated with T cell mitogens lymphocytes become more sensitive to inhibitory activity. While the inhibitory activity of the B and T cell mitogens was suppressed to a lesser degree than the lymphocytes response to the B cell mitogen only (52).

MPA and NET can bind to the GR, PR, AR and the MR. Table 1.1 is a summary from a recent review article published in *Steroids* by Africander *et al* (56). The table shows the relative binding affinities (RBA) of progesterone and the synthetic progestins to steroid receptors. The RBA shown here were determined by recombinant human steroid receptor binding *in vitro* in a study performed by Philibert *et al.* The biological activity listed below was determined in preclinical models by different study groups.

Table 1.1: Relative Binding Affinities (RBA) to steroid receptors, and biological activity of progesterone and synthetic progestins.

	Progesterone	MPA	NET-A
PR (RBA)	100 ^a	298 ^a ; 230 ^b	134 ^a ; 150 ^b
Progestogenic activity	+	++	++
AR (RBA)	3 ^a ; 62 ^g ; 80 ^f	35 ^a ; 5 ^b ; 151 ^f ; 75 ^g	55 ^a ; 15 ^b ; 134 ^f
Androgenic activity	±	±	+
MR (RBA)	1000 ^a	3.1 ^a ; 160 ^b ; 134 ^f	2.7 ^a ; 0 ^b ; 0.005 ^f
Anti-mineralcorticoid activity	+	-	-
GR (RBA)	11 ^a ; 6 ^c	58 ^a ; 29 ^b ; 79 ^c	1.4 ^a ; 0 ^b ; 0.88 ^c
Glucocorticoid activity	±	+	-

- RBAs are shown as a percentage of total specific binding with that of the natural ligand. Symbols of biological activity (-) not effective; (+) effective; (++) strongly effective; (±) literature inconsistent. **a)** Determined by recombinant human steroid receptor binding in vitro. **b)** Means of RBA determination not defined. **c)** RBAs were determined by overexpressing the hGR in COS-1 cell line. **d&e)** Rat uterine cytosols were used to determine RBAs for the PR and estrogen receptor (ER), rat prostate and rat thymus cytosols were used to measure RBAs for the AR and GR respectively. RBAs for the MR were determined by overexpressing the hMR in COS-1 cells. **f)** RBAs were determined by overexpressing the hAR and hMR in COS-1 cells. **g)** RBA was determined by overexpressing the hAR in COS-1 cells.

From the above table it can be seen that the binding affinity for the PR is highest for MPA followed by NET and lastly progesterone. Both the synthetic progestins exhibit strong effective progestogenic activity. Although they are made to act like their natural occurring ligand progesterone, the possibility remains that they could act differently as a result of their affinities for different steroid receptors (56).

The AR is found within many different tissues for example, vaginal tissues, testes, mammary glands, prostate and muscle (57). The AR plays a key role in normal ovary and breast development in females, and male reproductive functions. Bamberger *et al.* found that the lymphocytes expressed AR at mRNA levels, but the AR protein expression was found to be below the detection limit of Western blot experiments (58). From table 1.1 it can be seen that MPA and NET bind to the AR with relatively high binding affinities, and display androgenic effects in the rat model. This could put forward the notion that the androgenic effects are AR mediated (56) .

The MR is found within the epithelial tissues of the kidneys and the colon, can also be found in the heart and adipocytes. It has been reported that MR on myeloid cells are necessary for efficient classical macrophage activation by pro-inflammatory cytokines (59). Macrophages

from mice lacking MR in myeloid cells exhibited a transcription profile of alternative activation. *In vitro* studies showed that MR deficiency corresponded with inducers of alternatively activated macrophages (for example, IL-4 and agonists of PPAR γ and the GR) to enhance alternative activation. *In vivo* the MR deficiency in macrophages mimicked the effects of MR antagonists (59). Aldosterone is the natural ligand for MR, known to regulate blood pressure through sodium and water retention systems in the kidney (57). Progesterone acts as an effective MR antagonist, this is not reflected upon binding of MPA and NET to the MR. They have a fairly low binding affinity and do not exhibit any agonist or antagonist properties at the MR (54).

1.5.6 MPA and infectious diseases

Numerous studies have indicated that the use of hormonal contraceptives can increase susceptibility to HIV infection. A 10 year cohort study conducted in Kenya amongst women sex workers showed that women using MPA or oral contraceptives had a higher risk of acquiring HIV-1 than non-contraception users (50). Another study conducted amongst African women that were MPA users, showed that they had higher seroprevalence for HIV-1 and roughly 6% of the HIV risk was due to MPA use (50).

A study done on 342 adolescent girls aged 14-17years in the United States, found no association between MPA use and an increased risk in an acquiring an sexually transmitted infection (STI) (60). The cohort displayed equal risk of acquisition of a STI between non-users and MPA users alike. This finding was contradictory to a study done among an adult women cohort, here MPA use and STI infection a significant association was found (61).

A recent study looked at the possible mechanism through which MPA could affect the acquisition of HIV-1 (62). MPA suppressed the proliferation of T-cells after antigen presentation, and reduced the ability of the plasmacytoid dendritic cells to respond to stimulation. Additionally, MPA prevented the down-regulation of HIV co-receptors CCR5 and CXCR4 on activated T-cells, and increased the rate of HIV-1 replication in PBMCs. These mechanisms can stimulate HIV-1 proliferation in the genital tissues in the early stages of infection, and add to the increased shedding of HIV-1 in the genital tract of infected women (62). The HIV-1 virus accessory protein, viral protein R (Vpr), can modulate the transcriptional response of the GR. GCs and Vpr have been reported to induce apoptosis in various cells, including T-cells (63). They have previously shown that the injectable

contraceptive, MPA is a partial to full agonist for the GR, unlike NET. They found that the C-terminal part of the Vpr peptide, or HIV-1 pseudovirus, along with stimulation with DEX or MPA amplified the apoptotic phenotype, unlike NET and progesterone. They conclude that contraceptive doses of MPA but not NET or physiological doses of progesterone could potentially accelerate depletion of CD4⁺ T-cells in a GR-dependent manner in HIV-1 positive women, thus adding to immunodeficiency seen within the women. This suggests that careful must be made in the choice of progestin used in contraception may be critical to susceptibility and progression of diseases such as HIV-1 (63).

Animal studies have been conducted to look at the effect that progesterone has on viral STI susceptibility. Sodora *et al* reported that Rhesus macaques were more susceptible to intravaginal simian immunodeficiency virus (SIV) infection during the high progesterone luteal phase compared to the high estrogen phase of the follicular phase. Administering MPA to the Rhesus macaque monkeys had a 7-fold increase in the acquirement of SIV, accompanied by a significant increase in viral shedding during acute phase (64). Studies have also been performed in murine models, where they found that under the influence of progesterone the mice became more susceptible to sexually transmitted bacterial and viral infections. The treatment of the mice with MPA increased their susceptibility to genital HSV-2 infections by a 100-fold compared to the 10-fold increase seen with progesterone (65). However, there are some contradicting findings, recently it was found that MPA did not eliminate the control of viral replication but might have enhanced viral control after IV challenge in simian human immunodeficiency virus (SHIV)-immunized rhesus macaques (66). MPA was reported to stimulate T cell activation and T regulatory responses prior and just after SIV challenge. This led to the conclusion that the observed decrease in protection in vaginal SIV challenge in MPA treated SHIV immunized animals can be attributed to the changes in genital tract such as thinning of vaginal epithelium that enhance transmission (66).

A recent study done on woman, who received MPA as part of their injectable contraceptive regime, revealed that the production of IL-12, IL-1 α , IL-10 and IL-13 was inhibited in these women (67). It was speculated that the decrease in cytokine secretion could be ascribed to the lower levels of circulating monocytes within these women using MPA. This led to the conclusion that the lower levels of monocytes in circulation accompanied by the inhibition of IL-1 and IL-12 production, could lead to an increased risk of TB infection as a result of an increased susceptibility to TB (67).

Problem Identification

MPA is currently the leading choice of hormonal contraceptive amongst the women in low socio economic classes, as it is provided free of charge at South African health care clinics and only requires 4 clinic visits during the course of a year. Female MPA contraceptive users, that are TB household contacts have shown to respond differently to antigens of mycobacterial origin than control individuals as found through previous studies conducted (67). It has been found that the MPA contraceptive users produced significantly lower levels of IL-12p40, TGF-alpha, IL-13, IL-1alpha, sCD40L when analysed in a 3 day PBMC stimulation assay (67). The women using MPA showed a decrease in the amount of circulating monocytes potentially attributed to GC activity of this contraceptive. The suppression of cytokines and reduced monocyte count could add to the susceptibility of MPA users (67).

My study wishes to show the immune modulatory mechanisms underlying the observed cytokine inhibition that was seen in the MPA users studied previously. My study will be looking at the signalling pathways that are involved in the presence of anti-CD3 and BCG stimulation, and the influence that the presence of the hormones will have on the activation of the signalling molecules.

Rationale

In my study the effect of the hormones cortisol, progesterone, MPA and NET on the secretion of cytokines and phosphorylation of signalling molecules was determined after stimulation with BCG and CD3 respectively. PBMCs were stimulated with BCG to induce an antigen specific response, which occurs through recognition of the antigen bound to MHC on antigen presenting cells by the T-cell receptor (TCR) complex. This form of stimulation also leads to co-receptor activation and activation of more complex signal transduction cascade besides TCR signalling. Leading to cellular responses aimed at eliminating the encountered pathogens. Antigen presentation forms part of the acquired immune system. Whereas CD3 stimulation induces a non-specific response, which only leads to TCR activation and signalling without co-receptor activation. The cells of the innate immune system recognize and react to encountered pathogens in a non-specific way, by recruiting immune cells to sites of infection through the production of cytokines.

Hypothesis

I hypothesise that due to its selective GC activity MPA but not NET modulates BCG and anti-CD3 induced cytokine responses and signalling pathways in human PBMCs.

Aim

The aim of the study is to compare the underlying immune modulatory properties of MPA and NET and compare them to the endogenous hormones cortisol and progesterone. I will investigate whether MPA and/or NET affect BCG and anti-CD3 induced cytokine expression and activation of signalling molecules in human PBMCs.

Research Objectives

1. To determine the effect of cortisol, MPA, progesterone and NET on the BCG and anti-CD3 induced cytokine secretion in human PBMCs.
2. To determine the presence of GR and PR protein in human PBMCs through Western blot.
3. To determine the mRNA of GR, PR, AR, mPR- α , mPR- β , mPR- γ and PGRMC1 in human PBMCs, as well as the effect that the addition of cortisol, MPA, progesterone and NET on the receptor mRNA levels.
4. To determine whether after BCG and anti-CD3 induced activation of signalling pathway is altered by cortisol, MPA, progesterone and NET.

Research Plan

The schematic diagram below depicts the research plan set out to achieve our aims.

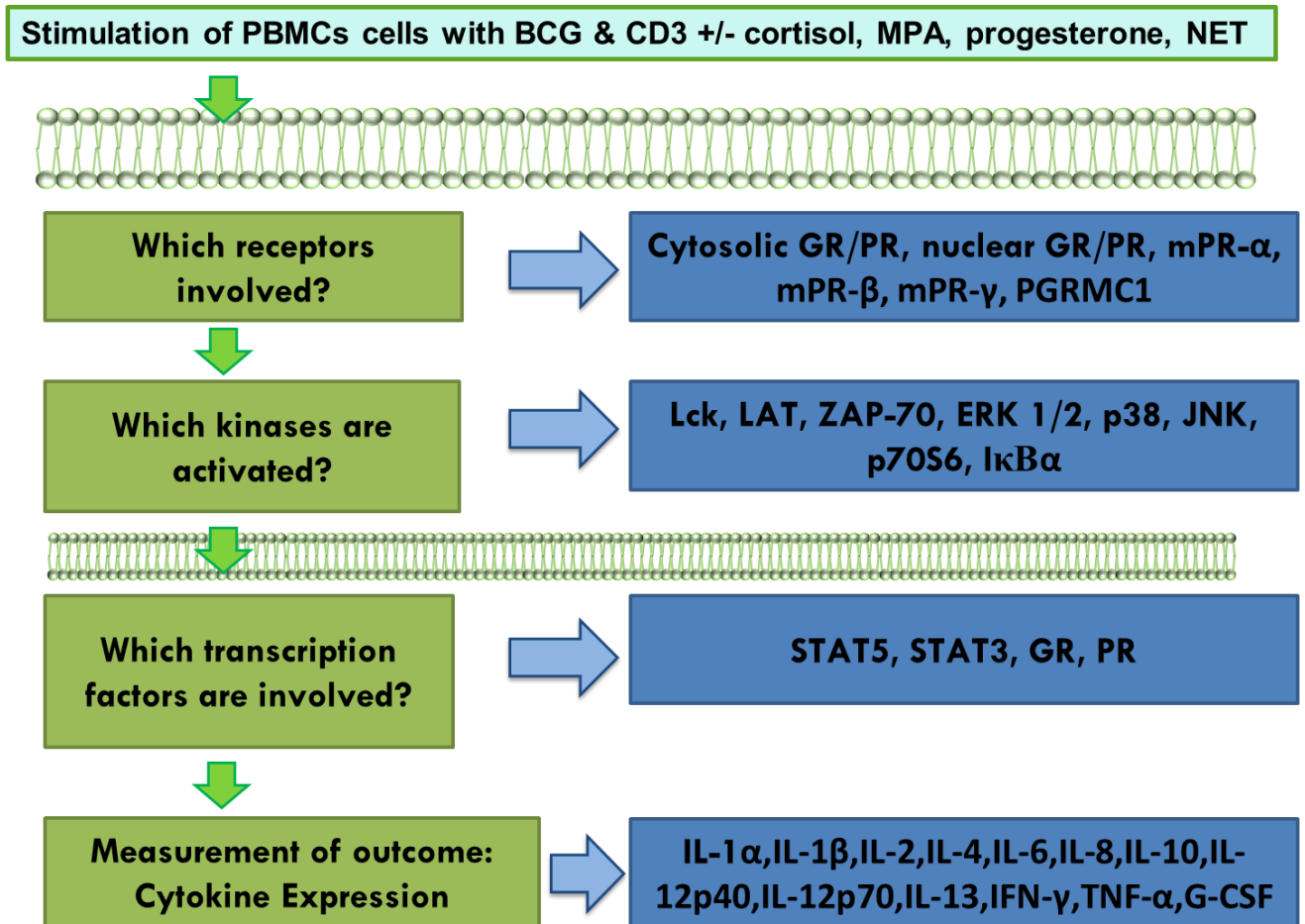


Figure 1.8: A diagram of the proposed research plan to meet the research aims for the study.

Chapter 2: Materials and Methods

2.1 Cell Culture

2.1.1 Peripheral Blood Mononuclear Cells (PBMCs)

2.1.1.1. Study Participants

Participants were chosen on the following criteria: women who are non-contraceptive users and have a positive Tuberculin Skin Test (TST). Blood was collected from these women throughout the duration of the project. To be eligible for study participation the women had to be between 15 and 45 years of age. They were not using contraceptives or receiving any form of steroid treatment, not pregnant and had not been sterilized.

2.1.1.2 PBMC Isolation

PBMCs were isolated from 40ml whole blood collected from each of the participants for every time point, following the protocol implemented in our laboratory. Ethical clearance for the sample processing and PBMC isolation was granted to the SUN-Immunology Research Group (SUN-IRG) under the ethics number: N05/11/187. The ethical clearance was awarded to the project entitled '*Biomarkers of protective immunity and surrogate markers of TB disease in Africa – Gates Grand Challenge project 6-74.*' The ethically approved protocols for the GC6-74 project for PBMC isolation was used for the duration of this project. The protocol followed is explained here in brief. Whole blood sample collected was diluted in a ratio of 1:1 in sterile phosphate buffered saline (PBS) (Lonza. Basel, Switzerland), and layered on 15ml Histopaque (Sigma-aldrich. South Africa). The blood was centrifuged at a low speed, 1500rpm, to allow for density gradient separation. The buffy coat containing the white blood cells was harvested into a 50ml conical tube (Becton Dickenson. New Jersey, USA) with 40ml PBS and centrifuged for 10minutes at 1500rpm, the cell pellet was re-suspended in 10ml PBS, that serves as the final wash step. The cell pellet obtained at the end of the last wash step was re-suspended in 1ml pre-warmed AIM-V media (Invitrogen, Life Technologies. California, USA), a 1:4 dilution is made with cells and trypan blue dye (Sigma-Aldrich) and 10µl of diluted cells was loaded onto a haemocytometer to allow for cell counting.

2.1.2 Cell Stimulations

Isolated PBMCs were plated at a concentration of 1×10^6 cells per well in 24 well plates respectively (Greiner Bio-one, England, UK) in a final volume of 2 ml pre-warmed AIM-V media (Invitrogen). The PBMCs were treated with the different stimulants listed below to induce responses.

i. Hormones

Medroxyprogesterone acetate (MPA), hydrocortisone, progesterone, norethisterone (NET) were purchased from Sigma-aldrich, and were re-suspended in 96% ethanol to a concentration of 10^{-2} M and stored in aliquots at -20°C .

Steroids were added at a final concentration of 10^{-5} M per well. The high concentration of steroids ensures the saturation of the receptors present in the cells. This results in all the receptors being bound by a hormone, and accounts for the different binding affinities of the hormones to their receptors. The steroids were added in the presence and absence of Bacille Calmette-Guérin (BCG) and anti-human anti-CD3 antibody.

ii. BCG

Mycobacterium bovis Bacille Calmette-Guérin (BCG) was grown from a stock that was given as a gift by Dr Léanie Kleynhans, BCG is categorised as a biosafety category two bacteria, and therefore all experiments conducted within a biosafety level two laminar flow cabinet.

BCG used to stimulate cells was grown in liquid cultures of Middlebrook 7H9 broth (Difco, Becton Dickensen), the media was supplemented with 0,2% glycerol, 0,05% Tween 80 and 10% oleic acid albumin dextrose catalase (OADC) enrichment media (Becton Dickensen) in 75cm^3 tissue culture flasks (Corning, Corning, New York) and placed in an Incubator at 37°C .

Regular OD readings at 600nm were taken, until the OD readings fell within the range 0.7-1.0 nm, indicating the start of the logarithmic growth phase. An acid fast Ziel-Neelsen (ZN) stain is done on an aliquot of the BCG to ensure that there was no other bacterial contamination present within the BCG aliquots. A 10% glycerol concentration is added to the liquid cultures and aliquoted into 200 μl aliquots and stored at -80°C .

After two days in the -80°C freezer, the number of viable bacteria had to be determined by plating serial dilutions of the thawed aliquots on Middlebrook 7H11 (Difco. Becton Dickensen) agar plates supplemented with 0,2% glycerol and 10% OADC (Becton Dickensen). The inverted plates were semi-sealed in plastic bags and incubated for 3 weeks at 37°C , after which the number of colony forming units (CFUs) were determined. The frozen aliquots were used in subsequent experiments ensuring the same number of bacteria at the same stage of growth was used in each successive experiment thus it is assumed that the results obtained are reproducible.

Prior to stimulating the PBMCs an aliquot of BCG is thawed and de-clumped by being drawn up and down five times with a 29G syringe. Within one well of 1×10^6 PBMCs it was assumed that 1×10^5 macrophages (10%) were present, therefore a multiplicity of infection (MOI) of 1 macrophage to 5 bacilli was used for experimental conditions. PBMCs were therefore stimulated with 5×10^5 CFU/ml.

ZN Stain Method

To ensure the BCG aliquots were not contaminated a ZN stain has to be performed, method described below briefly. Placed a drop of the suspension onto microscope slide and allowed to air dry for ten minutes at 60°C , slide was heat-fixed for ten minutes at 90°C . The prepared slide was placed on the staining rack and flooded with carbol fuchsin (Sigma-Aldrich). The slide was heated to steaming point over an open flame; the slide was left to stand for five minutes. The fixed slide was rinsed with distilled water (dH_2O) and drained. Slides were flooded with acid-alcohol and allowed to stand for two minutes. The dH_2O rinsing step was repeated, and the slide flooded with methylene blue and allowed to stand for two minutes. The slides were rinsed with dH_2O and air dried. The completed slides were examined by placing a drop of immersion oil onto the microscope slide and visualizing at an objective of 100X.

iii. Anti-CD3

1×10^6 PBMCs per well were stimulated with an anti-human CD3 antibody (eBioscience. San Diego, California), from here on forth it will only be referred to as anti-CD3. Anti-CD3 was added to the cells at a final concentration of $1 \mu\text{g/ml}$ per well in the presence and absence of steroid hormones, cortisol, progesterone, MPA and NET.

After the PBMC stimulations were done with either BCG or anti-CD3 respectively. The supernatants and cells were harvested on different time points: Day 1, Day 3 and Day 6 and stored at -80°C until subsequent analysis by enzyme-linked immunosorbent assay (ELISA), Luminex and RT-PCR analysis.

2.2 Cytokine Determination

2.2.1 ELISA

ELISA was used to determine cytokine production from harvested Day1, Day 3 and Day 6 supernatants. Two cytokines IL-12p40 (Becton Dickenson) and IFN γ (BD Pharmingen, San Diego, California) were selected for ELISAs.

i. IL-12p40 ELISA

A human IL-12p40 ELISA set was purchased from BD (BD Pharmingen). Ninety six well plates (Greiner Bio-one) were coated with 100 μ l purified anti-human IL-12p40 capture antibody overnight at 4°C. The plates were washed with a PBS wash buffer containing 0,05% Tween-20 (Sigma-Aldrich) and there after blocked with assay diluent (PBS containing 10% Fetal Bovine Serum (FBS); (Biochrom AG, Berlin, Germany) for 1 hour at room temperature. Standards and cell supernatants were thawed and added to the plate in duplicate for 2 hours at room temperature. The detection limits of the top and bottom standards are 2000 pg/ml and 31,3 pg/ml respectively. The plates are washed with PBS wash buffer and 100 μ l working detector, consisting of biotinylated anti-human IL-12p40 detection antibody and streptavidin-horseradish peroxidase conjugate added, and incubated for one hour at room temperature. The plate was washed 7 times and 100 μ l Tetramethylbenzidine (TMB) substrate solution (Becton Dickenson) added for a final 30 minute incubation in the dark and 50 μ l of 1M H₂SO₄ (Merck, Johannesburg, South Africa) stop solution was added prior to reading plate at 450nm. The absorbance was read on a Bio-Rad Benchmark Microplate Reader and data generated was managed through Microplate Manager Software (version 5.2, Build 103, Bio-Rad Laboratories).

ii. IFN- γ ELISA

A standardized optimized protocol used within our laboratory was used to determine the level of IFN- γ . Brief description of ELISA protocol followed. A 96 well flat-bottomed plate (Greiner-Bio one) coated with 50 μ l of 2 μ g/ml of purified mouse anti-human IFN- γ capture antibody (BD Pharmingen) was incubated overnight at 4°C. The next day, the plates were washed 4 times with wash buffer (PBS containing 0.05% Tween 20), and incubated for 2 hours with blocking buffer (PBS with 10% heat-inactivated FBS). The wash step was repeated prior to adding standards and samples in duplicate to wells and the plates incubated overnight at 4°C (IFN- γ standards were diluted in AIM-V medium, Invitrogen). The detection limits for the IFN- γ standard (Pharmingen recombinant IFN- γ) was 4000 pg/ml and 31 pg/ml. The next day, the wash step was repeated and incubated with 100 μ l of the 1 μ g/ml biotinylated mouse anti-human IFN- γ antibody (BD Pharmingen) for 45 minutes. Wash step was repeated to remove unbound antibody and the plates incubated for 30 minutes with 100 μ l of 2.5 μ g/ml Avidin Peroxidase (Sigma-Aldrich). The final wash step was repeated prior to adding substrate solution, OPD tablets (Sigma-Aldrich) and incubate for 40 minutes in the dark to allow colour to develop. The colour development was stopped by adding 50 μ l of 1M H₂SO₄ (Merck). The absorbance was read on a Bio-Rad Benchmark Microplate Reader at 450nm and the data generated managed through Microplate Manager Software (version 5.2, Build 103, Bio-Rad Laboratories).

2. 3 Western Blots

2.3.1 Lysate preparation

Whole cell lysates of PBMCs were prepared in lysis buffer (20mM Tris-HCl pH 7.6, 150 mM sodium chloride, 1mM EDTA, 1% Triton X-100) containing Protease inhibitor cocktail tablets (Roche. Basel, Switzerland) and Phosphatase inhibitor cocktail 2 inhibitors (Sigma-Aldrich). Protein concentrations were determined using Bradford method, Quick Start Bradford Dye Reagent (Bio-Rad).

2.3.2 Protein Determination

For protein determination the Bradford method was used. Prior to use the dye reagent must reach room temperature as it is stored at 4°C. Bovine serum albumin (BSA) (Bio-Rad) standards were prepared in PBS according to the table supplied by manufacturer with a range of 2000 - 125µg/ml. Standards were added to a 96-well plate in duplicate and samples in triplicate. 250µl of the dye was added to all wells. The plate was incubated in the dark for ten minutes at room temperature and read at a wavelength of 595nm on the Bio-Rad iMark microplate reader.

2.3.3 General Western Blot procedure

An 8% SDS-Page gel was prepared (H₂O, 30% acrylamide mix (Sigma-Aldrich), 1.5M Tris-HCl pH 8.8, 10% SDS, 10% ammonium persulfate, Tetramethylethylenediamine (TEMED, Sigma-Aldrich)(Refer to Appendix A). The gel was loaded with 5µl Rainbow marker (RPN756V, Amersham, GE Healthcare, Wisconsin, USA). A total volume of 20µl sample and 5µl Laemmli buffer (125mM Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 2.5% β-mercaptoethanol, 0.1% bromophenol blue) was loaded per well, containing a final protein concentration of 20µg. The gel was run at 50V until the running front reached the resolving gel, the voltage was increased to 200V and the gel continued to run at the increased voltage for 45minutes. Once the gel was completed the gel was transferred to the blotting unit, the gel 'sandwich' was run at 100V for 45minutes. Following the blotting process the Hybond ECL Nitrocellulose membrane (Amersham) was placed in a TBS solution containing 5% fat free milk powder for overnight blocking at 4°C. After completion of the overnight blocking step, the primary antibody and secondary antibody were added to the membrane respectively in a predetermined dilution (see 2.3.4 and 2.3.5) for 60minutes on the shaker at room temperature. There were 3x 10 minute wash steps each with TBS and 0.1% Tween-20 that separated the primary and secondary antibody steps.

Detection of the banding pattern on the membrane was performed in the dark room through the addition of the chemiluminescence substance, ECL Advance Western Blotting Detection Kit (Amersham). Following the addition of the chemiluminescence substrate the Hyperfilm (Amersham) was exposed to the membrane for 20seconds prior to being developed by the Amersham Pharmacia Biotech Hyper processor (AM4).

UN-SCAN-IT Graph Digitizing Software version 5.1 from Silk Scientific Corporation was used to digitize and extract (x,y) data values from the various Western blots. The pixel density data obtained was plotted in GraphPad Prism[®] version 5.0 as a visual presentation of the banding pattern observed on the Western blots.

2.3.4 Glucocorticoid and Progesterone Receptors

Dilutions at which primary GR (H-300) and PR (C-19) rabbit polyclonal IgG antibodies were used was 1:1000, both purchased from Santa Cruz Biotechnology (Texas, USA). The ECL Rabbit IgG, horseradish peroxidase (HRP)-linked secondary antibody from donkey (Amersham) was used at a dilution of 1:5000.

2.3.5 Kinase (ERK 1/2, p38 MAPK, JNK)

The Western Blots for the kinase blots were done according to the table 2.1 listed below. The table below shows the dilution of the antibodies as well as the buffer in which it was diluted for use. All the antibodies in the table were purchased from Cell Signalling Technology (Massachusetts, USA).

Table 2.1: Primary and Secondary concentrations and solutions that were used in the kinase Western blots.

Kinase	Primary Antibody Dilution (Solution)	Secondary Antibody Dilution (Solution)
ERK 1/2 (phosphoprotein + total protein)	1: 1000, (5% w/v BSA, TBS/0,1% Tween20.)	1:4000, (TBS/ 0.01% Tween20.)
p38 (phosphoprotein + total protein)	1: 1000, (5% w/v BSA, TBS/0,1% Tween20.)	1:4000, (0.5% Milk powder, TBS/0.1% Tween20.)
JNK (phosphoprotein + total protein)	1: 500, (5% w/v BSA, TBS/0,1% Tween20.)	1:4000, (0.5% Milk powder, TBS/0.1% Tween20.)

2.3.6. Human Phospho-Kinase Array

A Human Phospho-Kinase Array kit, Proteome Profiler™ Array (RnD Systems, Minneapolis, USA) was purchased for the parallel determination of the relative levels of protein phosphorylation of the following kinases: p38 α , ERK 1/2, JNK 1/2/3, GSK-3 α/β , p53, EGF R, MSK 1/2, AMPK α 1, Akt 1/2/3, TOR, CREB, HSP27, AMPK α 2, β -catenin, p70S6, c-Jun, Src, Lyn, Lck, STAT2, STAT 5A/B, RSK 1/2/3, eNOS, Fyn, Fgr, STAT6, STAT 5b, STAT 3, p27, PLC- γ 1, Hck, Chk-2, FAK, PDGF R β , WINK1, PYK2 and Hsp60. Within the array the capture antibodies have been spotted in duplicate on nitrocellulose membranes. Cell lysate samples were diluted and mixed with a cocktail of biotinylated detection antibodies (supplied in kit). The sample and antibody mixture was incubated with the nitrocellulose membranes. Any protein that is present will bind to its associated capture antibody on the membrane. Streptavidin-Horseradish Peroxidase and chemiluminescent detection reagents were added, and the signal that was produced was in proportion to the amount of kinase that was bound. The chemiluminescence of the nitrocellulose membranes was detected in the same manner as a Western blot (section 2.3.3).

2.4 Receptor Expression

The chart below (figure 2.1) depicts the sample preparation process that was followed with the sample preparation from the stimulated PBMC samples to determine GR, PR, AR, mPR- α , mPR- β , mPR- γ and PGRMC1 expression levels. Real-Time PCR assay was run on the ABI Applied Biosystems 7900HT Fast Real-Time PCR System.

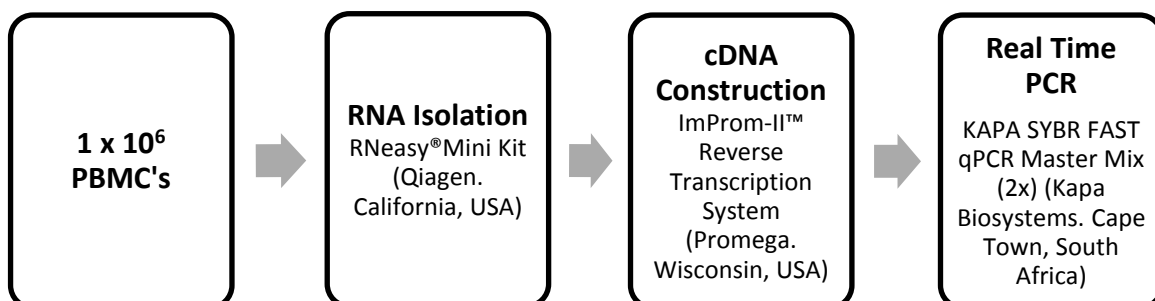


Figure 2.1: Sample process followed for the preparation of PBMC samples for the RT-PCR run. Detailed explanation of the procedures followed for the PBMC samples are described below:

2.4.1 RNA Isolations

The RNA was isolated from the PBMC samples using the RNeasy®Mini Kit (Qiagen). The standard protocol supplied by the manufacturer was optimized to produce the highest yield of RNA from the samples. Below is a brief outline of the protocol that was followed.

One million PBMCs per well was placed in a 24 well tissue culture plate and stimulants were added to AIM-V media and the cells were harvested at 1, 3 and 6 days post stimulation. The cells were harvested into a 1.5ml eppendorf tubes (Merck) and spun down at 1500rpm for 5 minutes, all supernatant was removed with aspiration. The cell pellet was disrupted by adding 350µl RLT Buffer (supplied in kit) and vortexed for 1 minute to homogenize the cell lysate and then placed on ice for 3 minutes. After the incubation on ice, 350µl of a 70% ethanol solution was added to the cell lysate and mixed well through pipetting up and down. The sample was transferred to an RNeasy spin column and centrifuged for 1 minute at 15 000 rpm, the flow through was discarded from the spin column. The spin column was washed by adding 700µl RW1 Buffer to the column and centrifuged for 45 seconds at 15 000 rpm, flow through was discarded carefully to avoid contact with the spin column. The spin column membrane was washed by 500µl RPE Buffer, centrifuged at 15 000 rpm for 45 seconds, and flow through discarded. This final wash step was repeated with a prolonged centrifugation time of 2 minutes, the longer centrifugation step serves to dry out the membrane before the elution step. The RNeasy spin column was transferred to a new 1.5ml collection tube, and 35µl RNase-free water was added directly to the spin column membrane and placed on ice for 3 minutes before centrifuging for 1 minute at 15 000rpm.

The final elution contains the isolated RNA sample. An additional DNase treatment step was required to remove DNA contaminants in the isolated RNA sample. A TURBO DNA-*free*™ DNase Treatment Ambion kit (Applied Biosystems, Life Technologies. California, USA) was purchased. The procedure followed is outlined below.

1µl of TURBO DNase was added to RNA sample and incubated at 37°C for 30 minutes. After the incubation period 2µl of DNase Inactivation Reagent was added and mixed well prior to incubation at room temperature for 5 minutes. The reagents were mixed occasionally by flicking the tube 2-3 times during the incubation period. The tube was centrifuged for 90 seconds at 10 000g. The DNase Inactivation Reagent forms a pellet at the bottom of the tube and the supernatant was transferred to a new tube.

The concentration of RNA in each sample was determined through nanodrop technology on the Nanodrop ND-100 machine (Inqaba Biotec. Pretoria, South Africa). A260/A280 ratio of 2 was considered a pure RNA sample.

2.4.2 Hotstart PCR

To verify that a pure RNA sample was isolated from the PBMCs, a Hotstart (Qiagen) PCR following manufactures protocol was performed using GAPDH primers (IDT. Iowa, USA). The PCR products were run on a SDS-PAGE gel at 100V for 1,5 hours. The gel was visualised using the Gel Doc system V-Box. The absence of bands within the gel apart from the positive control, indicate that the RNA sample was pure and does not contain DNA contamination.

2.4.3 cDNA library construction

The RNA isolated from the PBMC samples was used to construct cDNA to be used in Real Time PCR assays. A commercially available Promega kit ImProm-II™ Reverse Transcription System (Promega) was purchased.

The 5µl of experimental RNA solution used was composed of the following components; 4µl of target RNA and 1µl oligo-dT receptor primer. The RNA and primer mixture were placed in a preheated 70°C heat block for 5 minutes then placed on ice for 5 minutes. After which the tubes were spun down for 10 seconds in a microcentrifuge to collect all condensate.

Reverse Transcription reaction mixture was made up as described below (Table 2.2) and 15µl of the reaction mixture was added to reaction tubes on ice. The 5µl of experimental RNA was added to the 15µl RT reaction mixture to render a final 20µl working volume.

Table 2.2: Composition of the Reverse Transcription mixture used in the Promega ImProm-II kit.

Reverse Transcription Reaction Mixture	
Nuclease-Free H ₂ O	3.7µl
ImProm-II™ 5X Reaction Buffer	4.0µl
MgCl ₂ [6mM]	4.8µl
dNTP Mix	1.0µl
Recombinant RNasin® Ribonuclease Inhibitor	0.5µl
ImProm-II™ Reverse Transcriptase	1.0µl
Final Volume	15µl

The PCR tubes were placed in an ABI Applied Biosystems Veriti 96 well thermal cycler PCR machine that has been programmed with the following protocol: 5 minutes at 25°C, 42 minutes at 42°C and 15 minutes at 70°C.

The concentration of DNA in each sample was determined through nanodrop technology on the Nanodrop ND-100 machine (Inqaba Biotec. Pretoria, South Africa). A260/A280 ratio of 1.8 was considered a pure DNA sample.

2.4.4 Real Time-PCR

The conditions for the Real Time-PCR were adapted from published data (68). The samples used for qPCR analysis, were prepared according to the protocol supplied by Manufacturer of KAPA SYBR FAST qPCR kit Master Mix (2x) ABI Prism (Kapa Biosystems).

After optimization the protocol implemented for the SYBR Green Real-Time PCR assay was an initial denaturation step of 10 minutes at 95°C, the amplification step involved 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The final dissociation step was 1 cycle of 1 minute at 95°C, 2 minutes at 60°C and 15 seconds at 95°C.

The following sense and antisense (5' – 3') primer sequences were used for amplification during the RT-PCR runs:

PR, AAATCATTGCCAGGTTTTTCG and TGCCACATGGTAAGGCATAA;

PGRMC1, TATGGGGTCTTTGCTGGAAG and GCCCACGTGATGATACTTGA;

mPR-α, TCAGACCTGGCGCTTCTATT and AGGTGAAAGAGGCAAGGACA;

mPR-β, CACGAAGGACCCACAAAACCT and CAATCCCAAGCACCTAT;

mPR- γ , ACTATGGTGCCGTC AACCTC and CAGAGTCTGGGCTTCTGGAC;
GR, CCTAAGGACGGTCTGAAGAGC and GCCAAGTCTTGGCCCTCTAT;
AR, CCTGGCTTCCGGGCAACTTACA and GGACTTGTGCATGCCGTACTCA.

Gene- specific expression of the target gene sequence transcripts were normalized to the expression of housekeeping gene;

GAPDH, TGAACGGGAAGCTCACTGG and TCCACCACCCTGTTGCTGTA.

The receptor expression is depicted in the graphs as a fold change of the ΔC_t values. The ΔC_t values were calculated using the following formula; $\Delta C_t = 2^{(C_t(\text{Target}) - C_t(\text{Reference}))}$, the reference gene used here was GAPDH. The fold change was calculated by dividing the ΔC_t of the stimulatory condition (e.g. anti-CD3) with the ΔC_t of the US group.

2.5 Luminex Multiplex Assays

2.5.1 Cytokine Assay

Multianalyte profiling was performed on the Bio-Plex™ Suspension Array System (Bio-Rad Laboratories, California, USA). Microspheres used for calibration, as well as sheath fluid used during assay were purchased from Bio-Rad.

To determine the multiple cytokine expression levels of the PBMCs under different stimulatory conditions, namely anti-CD3 and BCG, a 13-plex cytokine kit (MPXHCYTO-60K-13, Merck-Millipore, Massachusetts, USA) was used to measure the following cytokines: Granulocyte colony-stimulating factor (G-CSF), IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-13 and TNF- α .

2.5.2 Phosphoprotein Signalling Assays

The cell lysates that were used for the phosphoprotein signalling kits were prepared as follows:

The harvested cells were pelleted, the supernatant removed and the cell pellet washed through re-suspension in ice cold tris buffered saline (TBS) and centrifuged. The supernatant was discarded and cell pellet re-suspended in 100 μ l ice cold 1x Milliplex MAP lysis buffer

containing freshly prepared protease inhibitors (Roche). Cell lysates are rocked gently at 4°C for 15 minutes, before the cell lysates are stored at -80°C for analysis on Luminex assays.

The signalling cascades were analysed by two different phosphoprotein multiplex signalling assays, 7-plex T Cell Receptor Signalling Kit: CD3 ϵ , CREB, ERK/MAPK 1/2, LAT, Lck, Syk, ZAP-70 (48-690. Merck-Millipore); and an 8-Plex Multi-pathway Signalling Kit: CREB, ERK/MAPK 1/2, I κ B α , JNK/SAPK1, STAT3, STAT5A/B, p38/SAPK2A/B, p70S6 kinase (48-680. Merck-Millipore). The TCR signalling assay measures the differences in the TCR signalling cascade under different stimulatory conditions. The Multi-pathway kit covers more general signalling cascades that can signal through the receptor tyrosine kinases and activates different signalling cascades other than the TCR pathway.

Manufacturer's instructions were followed to perform the Luminex assay. In brief, from reconstituted standard a standard curve ranging from 3.2 pg/ml to 10 000 pg/ml was prepared. The filter plate was pre-wet for ten minutes with assay buffer which was discarded by vacuum aspiration. Assay buffer was added to all sample wells and AIM-V medium (Invitrogen) to the standard wells. Standards and two quality controls included in the kits and samples were added in duplicate to the pre-wet plate followed by antibody coated fluorescent beads. The plate was incubated on an orbital shaker for one hour at room temperature. The wells were emptied by vacuum and the plate washed with Wash buffer (supplied in kit). The biotinylated detection antibody was added and the plate incubated for 30 minutes on the shaker. The Streptavidin-Phycoerythrin was added and the plate again incubated for 30 minutes. After washing the plate twice with wash buffer, sheath fluid was added to all the wells and placed on shaker for five minutes, after which the plate was read immediately on a Bio-plex platform (Bio PlexTM, Bio Rad Laboratories), using a 5PL regression curve.

Manufacturer's instructions were followed to perform the Luminex assay. The protocol was similar to the one described above with the following alterations. After addition of cell lysates the plate was placed on an orbital shaker overnight at 4°C. After the biotinylated detection antibody was added and the plate was incubated for 1 hour on the shaker. The Streptavidin-PE was added and the plate incubated for 15 minutes, followed by the addition of 25 μ l Amplification buffer for 15 minutes on the shaker in the dark.

2.6 Statistical Analysis

ELISA results were analysed in GraphPad Prism[®] version 5.0, and all graphs shown display the mean \pm standard error of the mean (SEM). Statistical analysis performed on all results was a one-way analysis of variance (ANOVA) with a Bonferroni post hoc test. Statistical analysis on the Luminex and RT-PCR results was performed with the assistance of Prof. Martin Kidd (Centre for Statistical Consultation, University of Stellenbosch) using Statistica version 11, Statsoft. An ANOVA followed by a Fisher's least significant difference (LSD) post hoc test was done. The vertical bars on graphs depict the 0.95 confidence intervals (CI). A p-value less than or equal to 0.05 was accepted to be significantly different.

Chapter 3: Results

3.1 Introduction

Steroid hormone binding receptors can be found on the plasma membrane, in the cytosol and also in the nucleus of target cells. The receptors are located intracellularly (cytoplasmic) and initiate signalling cascades for steroid hormones which lead to changes in gene expression, these genomic effects are not immediate and take hours to days. The glucocorticoid receptor (GR) is a ubiquitously expressed transcription factor that mediates the effects of GCs and regulates the genes that play a role in development, metabolism, and immune response (29). The GR induces or represses the expression of genes in response to GCs, mediating such processes as apoptosis and cell growth and differentiation. The progesterone receptor (PR) is a protein found inside cells, and is activated by the steroid hormone progesterone. The effects of progesterone are mediated by two functionally different isoforms of the progesterone receptor, PR-A and PR-B (51).

Figure 3.1 shows a diagram of the ability of the hormones to bind to the different hormone receptors, the binding of cortisol and MPA to the GR leads to the inhibition of transcription and the suppression of cytokine secretion.

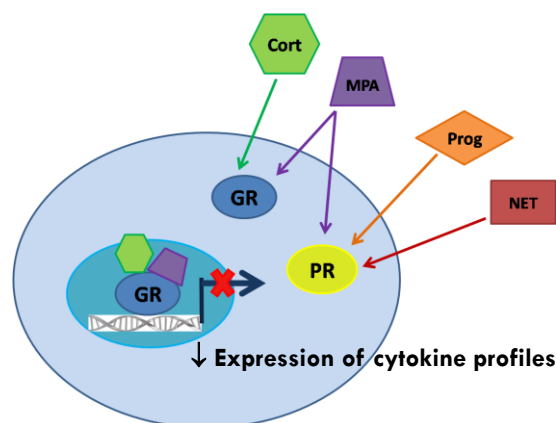


Figure 3.1: Schematic representation of the ability of the different hormones, cortisol, MPA, Progesterone and NET to interact with the glucocorticoid receptor (GR) and progesterone receptor (PR).

Cortisol (cort) can only bind to the GR. Both Progesterone (prog) and NET, only bind to the PR. Whereas, MPA, can bind to both the GR and the PR. Once the hormone binds to the receptor, it moves into the nucleus, where it binds to the DNA, and inhibits gene transcription, thus reducing the expression of cytokine profiles.

In my study the effect of the hormones cortisol, progesterone, MPA and NET on the secretion of cytokines and phosphorylation of signalling molecules was determined after stimulation with BCG and anti-CD3 respectively. PBMCs were stimulated with BCG to induce an antigen specific response, which occurs through recognition of the antigen bound to MHC on antigen presenting cells by the T-cell receptor (TCR) complex. This form of stimulation also leads to co-receptor activation and activation of more complex signal transduction cascade besides TCR signalling. Leading to cellular responses aimed at eliminating the encountered pathogens. Antigen presentation forms part of the acquired immune system. Whereas anti-CD3 stimulation induces a non-specific response, which only leads to TCR activation and signalling without co-receptor activation. The cells of the innate immune system recognize and react to encountered pathogens in a non-specific way, by recruiting immune cells to sites of infection through the production of cytokines.

Cytokines are a group of soluble proteins that act as regulators to control activities of individual cells and tissues. Cytokines can mediate the interactions between cells directly and are able to regulate processes taking place in the extracellular environment. Upon activation of a T-cell it can lead to different outcomes, it can alter the production of transcription gene products, T-cell differentiation, T-cell proliferation and cytokine secretion. Figure 3.2 is a graphic representation of the interaction between the GR and the TCR and the downstream signalling molecules involved. Refer to section 1.3.2 for the description of the components involved.

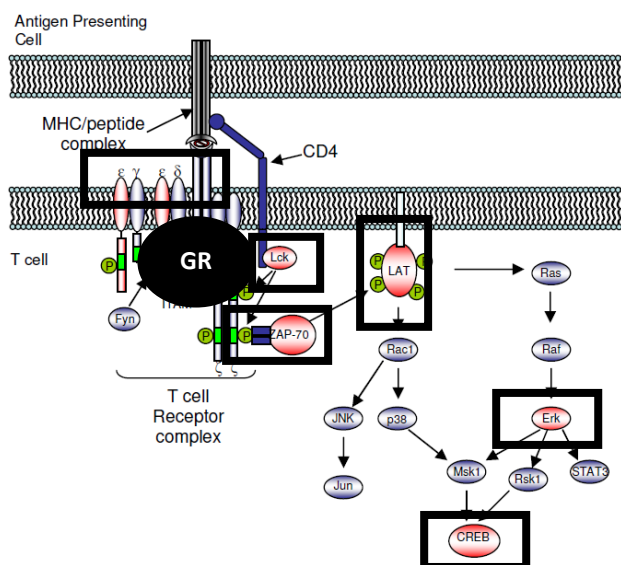


Figure 3.2: Schematic representation of the different kinases involved in the TCR signalling cascade upon T-cell activation, the blocks indicate the analytes analysed in the 7-plex Luminex panel.

The TCR signalling pathway is not the only means of signalling, cells can respond to their environment in a variety of different ways through intracellular signalling pathways. Signalling through Receptor Tyrosine kinases (RTK) often promote increased metabolism and cell growth. The activation of ERK/MAP kinase is one of the key Ser/Thr kinases activated via the RTK signalling pathway, promoting increased activity of p70 S6 kinase, Msk1, STAT3, CREB, and other signalling molecules. Other signalling pathways induced via stress can promote p38, JNK, and I κ B α signalling (figure 3.3). Refer to section 1.3.2.1 for a detailed explanation of the components involved in these pathways.

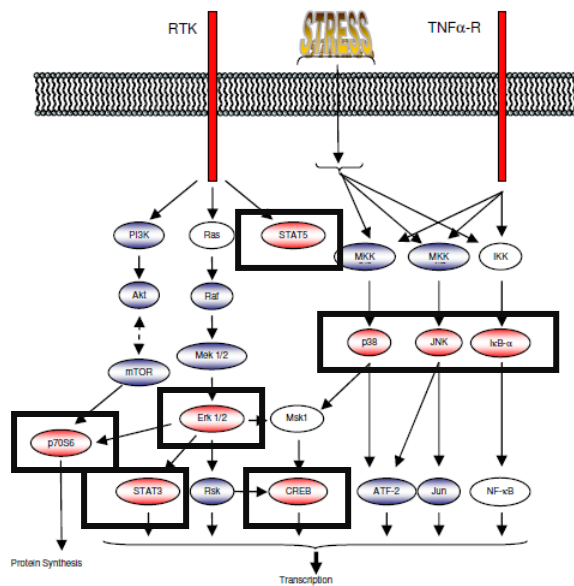


Figure 3.3: Schematic representation of the kinases and transcription factors analysed in the Multiple Signalling pathway kit with an indication of the pathway involved.

3.2 Experimental Design

Whole blood samples were collected from non-contraceptive users and PBMCs were isolated. The PBMCs were stimulated for 1, 3 and 6 days respectively with anti-CD3 (1 μ g/ml) and BCG at a multiplicity of infection (MOI) of 5:1. The isolated cells were stimulated with high concentrations (10 μ M) of MPA and NET as well as the endogenous hormones cortisol and progesterone. The effect of the addition of the hormones to the stimulated cells was observed by the alterations in the anti-CD3 induced and BCG induced cytokine secretion, receptor expression levels and phosphorylation of signalling molecules. The supernatants of the stimulated cells were harvested for ELISA and Luminex assays. The cells were harvested and the same cell lysates were used for both the 7-plex and 8-plex Luminex signalling assays.

PBMCs from the same patients were used for the cell stimulations from which RNA was isolated for the RT-PCR experiments performed.

3.3 Effect of hormones on cytokine secretion in human PBMCs

3.3.1 The effect of the hormones cortisol, MPA, progesterone and NET on BCG and anti-CD3 induced cytokine secretion in human PBMCs

A Multiplex bead array assay was used to assess the cytokine production from supernatants collected from stimulated PBMCs isolated from whole blood samples of woman not currently receiving any steroid treatment or using any hormone based contraception method. The Luminex panel revealed that alterations occurred in the secretion profiles of the different cytokines under the different stimulations.

The results of three cytokines namely, IL-2, IL-4 and IL-8 have not been reported on in section 3.3.1. They were excluded as the detected levels for IL-2 and IL-4 were below the detectable ranges specified within the manufacture's guidelines. High background was detected for the IL-8 bead, thus we deemed that the results were not reliable and was excluded from the results section.

i. IL-1 α

Cortisol and MPA significantly suppressed the anti-CD3 induced production of IL-1 α for both day 3 and day 6 time points (figure 3.4 a, c). Although not statistically significant it can be seen that the anti-CD3 induced production of IL-1 α was suppressed in the progesterone and NET stimulations for both time points. The cortisol and MPA stimulations showed suppression of the anti-CD3 induced IL-1 α production compared to progesterone and NET stimulations which did not suppress the IL-1 α production. The addition of cortisol significantly suppressed the BCG induced production of IL-1 α for both day 3 and day 6 time points (figure 3.4 b, d). The BCG induced production of IL-1 α was suppressed by MPA, progesterone and NET stimulations for both time points, however only MPA on day 6 reached a level of statistical significance. The IL-1 α production induced by BCG was significantly inhibited by cortisol when compared to MPA. Both cortisol and MPA showed more inhibition on the IL-1 α BCG induced production when compared to progesterone and NET stimulations.

Thus it can be seen that MPA mimics the glucocorticoid activity of cortisol while NET mimics progesterone.

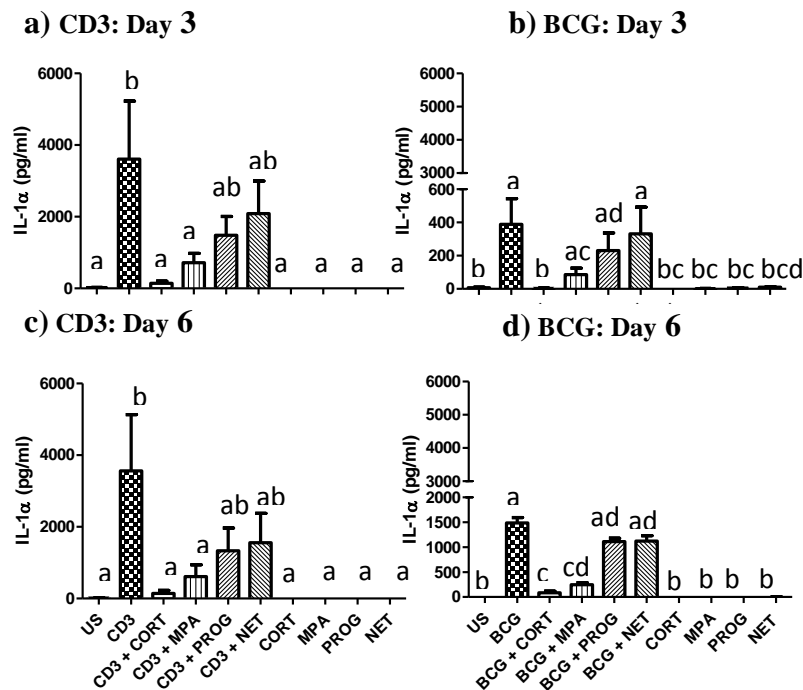


Figure 3.4: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced IL-1 α secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG (b&d) or $1 \mu\text{g/ml}$ anti-CD3 (a&c) respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. Unstimulated (US), BCG, BCG + all four hormone groups, $n=2$. US, anti-CD3, $n=2$. Anti-CD3 + all four hormone groups, $n=3$. Cortisol, MPA, progesterone and NET, $n=1$. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher least significant difference (LSD) post hoc test and presented as mean \pm SEM. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different.

ii. IL-1 β

The cortisol and MPA stimulations significantly inhibited the anti-CD3 induced production of IL-1 β for day 3 (figure 3.5a). The anti-CD3 induced production of IL-1 β was inhibited by progesterone and NET stimulations for both time points levels were not statistically significant (figure 3.5b). Cortisol and MPA stimulations showed inhibition of the IL-1 β production induced by anti-CD3, progesterone and NET stimulations did not inhibit the IL-1 β to the same extent.

At both day 3 and day 6 time points cortisol significantly suppressed the BCG induced production of IL-1 β (figure 3.5 b, d). The BCG induced production of IL-1 β was inhibited in the MPA, progesterone and NET stimulations for both the time points, although on day 6 a

level of statistical significance was reached in MPA stimulation. The IL-1 β BCG induced production was suppressed by cortisol and MPA stimulations when compared to progesterone and NET stimulations which did not inhibit the BCG induced production of IL-1 β to the same extent. Therefore it can be concluded that cortisol and MPA has a suppressive effect on the anti-CD3 induced production of IL-1 β more than progesterone and NET stimulations. MPA mimics the effect of cortisol, more than that of progesterone.

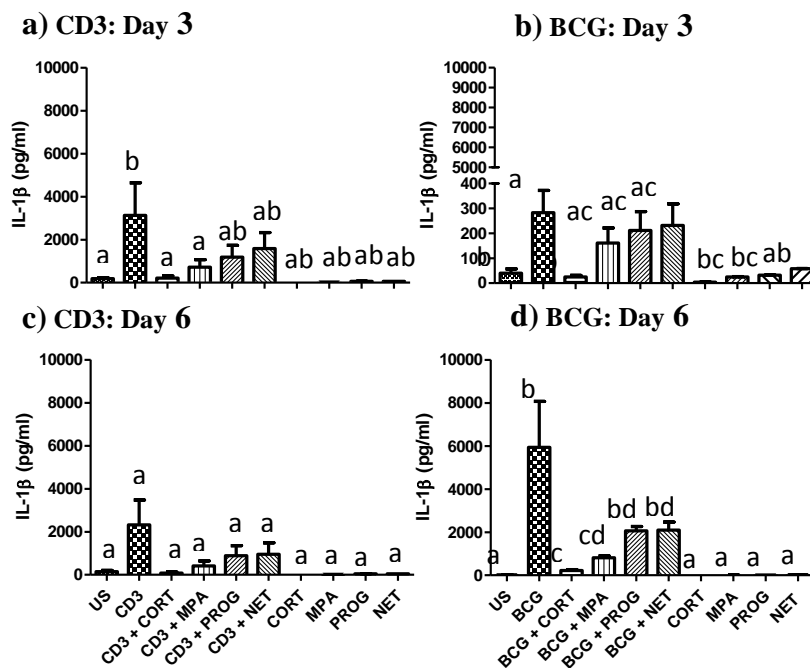


Figure 3.5: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced IL-1 β secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG (b&d) or 1 μ g/ml anti-CD3 (a&c) respectively, in the presence and absence of 10 μ M of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. US, BCG, BCG + all four hormone groups, n=2. US, anti-CD3, n=2. Anti-CD3 + all four hormone groups, n=3. Cortisol, MPA, progesterone and NET, n=1. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD post hoc test and presented as mean \pm SEM. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different.

iii. IL-6

At both day 3 and day 6 (figure 3.6 a, c), cortisol and MPA significantly inhibited anti-CD3 induced IL-6 expression. Progesterone and NET stimulations did not exhibit an inhibitory effect on the anti-CD3 induced IL-6 production.

For both day 3 and day 6 time points the BCG induced IL-6 production was significantly suppressed by cortisol and MPA at both time points (figure 3.6 b, d). The IL-6 production induced by BCG was not inhibited by progesterone and NET stimulations.

Therefore cortisol and MPA have a suppressive effect on the BCG and anti-CD3 induced production of IL-6. However, progesterone and NET no suppressive effect on the BCG and anti-CD3 induced IL-6 production.

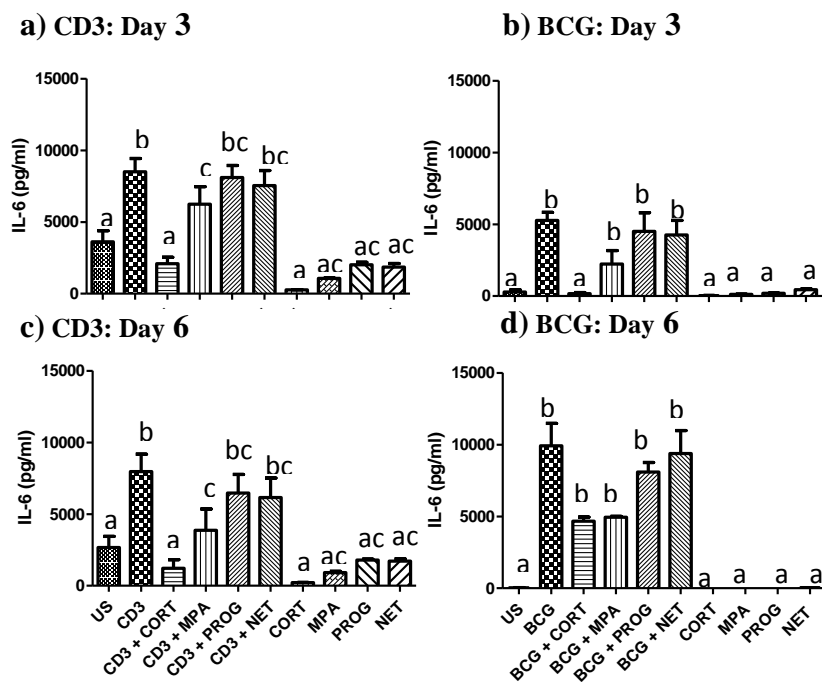


Figure 3.6: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced IL-6 secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M. bovis* BCG (b&d) or $1 \mu\text{g/ml}$ anti-CD3 (a&c) respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. US, BCG, BCG + all four hormone groups, $n=2$. US, anti-CD3, $n=2$. Anti-CD3 + all four hormone groups, $n=3$. Cortisol, MPA, progesterone and NET, $n=1$. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD post hoc test and presented as mean \pm SEM. The letters a, b and c indicate statistical significance, where values with the same letter are not significantly different.

iv. IL-10

The anti-CD3 induced production of IL-10 was inhibited by cortisol, MPA, progesterone and NET (figure 3.7 a, c). The cortisol and NET inhibition on the anti-CD3 induced IL-10 secretion reached a significant level at day 3.

The BCG induced IL-10 production was significantly suppressed by cortisol at day 3 and day 6 time points (figure 3.7 b, d). MPA significantly suppressed the BCG induced secretion of IL-10 in a similar way to cortisol at day 6. The BCG induced IL-10 secretion of progesterone and NET stimulations were higher than those of cortisol and MPA stimulations at day 6.

From the findings it can be seen that in the BCG stimulations at the earlier time points the synthetic progestins mimic progesterone, as they do not show the same suppressive effect on the production of IL-10 as is seen with cortisol.

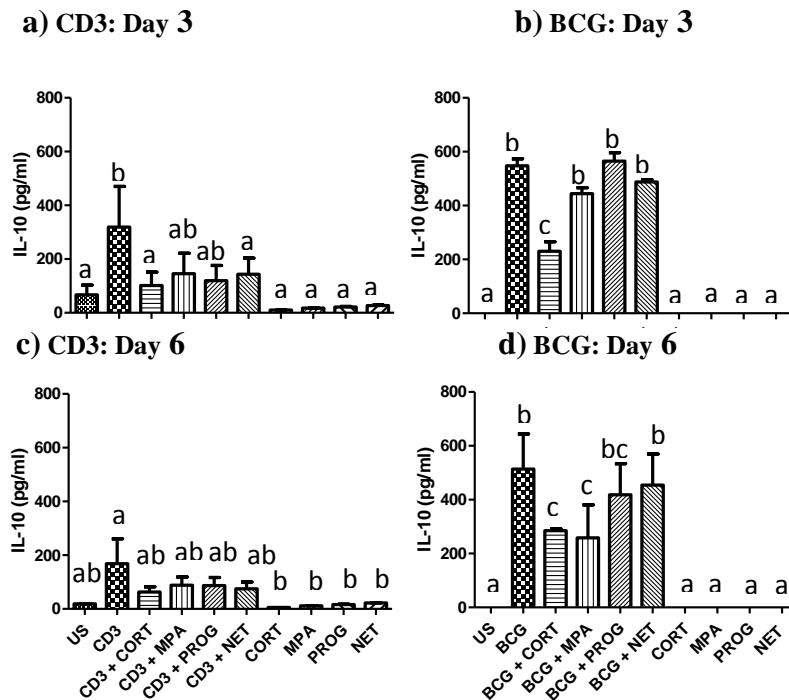


Figure 3.7: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced IL-10 secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG (b&d) or $1 \mu\text{g/ml}$ anti-CD3 (a&c) respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. US, BCG, BCG + all four hormone groups, $n=2$. US, anti-CD3, $n=2$. Anti-CD3 + all four hormone groups, $n=3$. Cortisol, MPA, progesterone and NET, $n=1$. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD post hoc test and presented as mean \pm SEM. The letters a, b and c indicate statistical significance, where values with the same letter are not significantly different.

v. IL-12p40

The anti-CD3 stimulations on day 3 and day 6 time points had very low production of IL12p40 (figure 3.8 a, c). It could be said that the detectable levels of IL-12p40 were too low to be detected. However, the pattern of inhibition remains consistent even at the low detection levels.

The BCG induced IL-12p40 secretion was significantly inhibited by cortisol and MPA stimulation at both the day 3 and day 6 time points (figure 3.8 b, d). The BCG induced secretion of IL-12p40 was significantly suppressed by progesterone and NET stimulations for day 3, however the level of inhibition did not reach a level of statistical significance on day 6. Cortisol and MPA showed significantly higher inhibition levels of BCG induced production of IL-12p40 in comparison with progesterone and NET stimulations. From the BCG stimulation it can be seen that IL-12p40 was mainly affected by the cortisol and MPA addition.

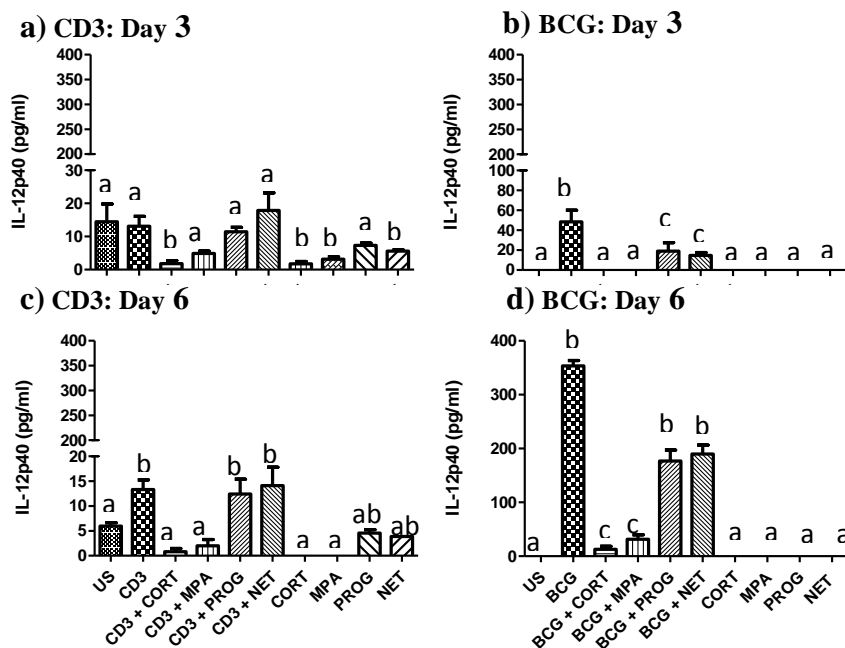


Figure 3.8: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced IL-12p40 secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG (b&d) or $1 \mu\text{g/ml}$ anti-CD3 (a&c) respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. US, BCG, BCG + all four hormone groups, n=2. US, anti-CD3, n=2. Anti-CD3 + all four hormone groups, n=3. Cortisol, MPA, progesterone and NET, n=1. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD post hoc test and presented as mean \pm SEM. The letters a, b and c indicate statistical significance, where values with the same letter are not significantly different.

vi. IL-12p70

The anti-CD3 induced IL-12p70 secretion was significantly suppressed by cortisol and MPA day 3 (figure 3.9a). The detectable levels of IL-12p70 were very low for day 6 post stimulation (figure 3.9c).

Cortisol and MPA stimulations significantly inhibited the BCG induced production of IL-12p70 for both day 3 and day 6 time points (figure 3.9 b, d). The production of the BCG induced IL-12p70 was suppressed in the progesterone and NET stimulations for both the time points, however the difference in IL-12p70 inhibition did not reach a level of statistical significance for day 6. Inhibition of IL-12p70 by cortisol and MPA was significantly lower than the progesterone and NET inhibition.

It can be seen that in the case of IL-12p70 secretion that MPA mimics cortisol with the inhibition seen in both the BCG induced and anti-CD3 induced stimulations.

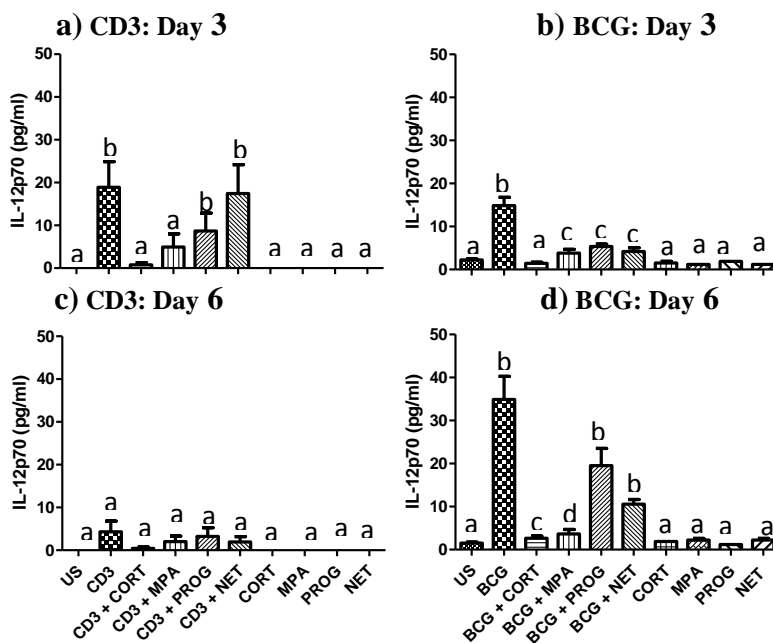


Figure 3.9: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced IL-12p70 secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG (b&d) or $1 \mu\text{g/ml}$ anti-CD3 (a&c) respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. US, BCG, BCG + all four hormone groups, $n=2$. US, anti-CD3, $n=2$. Anti-CD3 + all four hormone groups, $n=3$. Cortisol, MPA, progesterone and NET, $n=1$. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD post hoc test and presented as mean \pm SEM. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different.

vii. IL-13

Cortisol and MPA stimulations significantly inhibited the anti-CD3 induced secretion of IL-13 for both the day 3 and day 6 time points (figure 3.10 a, c). Although the anti-CD3 induced IL-13 inhibition by progesterone is statistically significant it can be seen that the production

of IL-13 was suppressed by NET as well for both time points. However, cortisol and MPA stimulations showed higher suppression levels of the anti-CD3 induced IL-13 production than was seen with progesterone and NET.

The addition of cortisol and MPA to BCG infected cells significantly suppressed the production of IL-13 when compared to BCG for both the day 3 and day 6 time points (figure 3.10 b, d). The inhibition of cortisol and MPA on the BCG induced secretion of IL-13 was significantly lower compared to the level of inhibition of IL-13 induced secretion of progesterone and NET stimulations. Therefore it can be seen that the addition of cortisol and MPA suppresses the secretion of IL-13 to a greater extent than can be observed with the addition of progesterone and NET to the stimulations. This strengthens the notion that MPA mimics cortisol rather than progesterone.

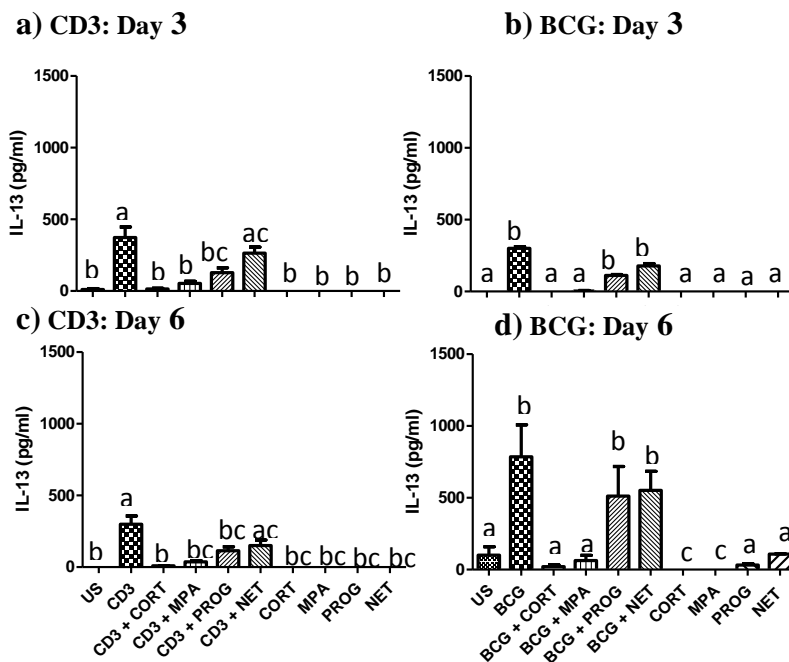


Figure 3.10: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced IL-13 secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG (b&d) or $1 \mu\text{g/ml}$ anti-CD3 (a&c) respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. Unstimulated (US), BCG, BCG + all four hormone groups, n=2. US, anti-CD3, n=2. Anti-CD3 + all four hormone groups, n=3. Cortisol, MPA, progesterone and NET, n=1. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD post hoc test and presented as mean \pm SEM. The letters a, b and c indicate statistical significance, where values with the same letter are not significantly different.

viii. IFN- γ

Cortisol and MPA suppressed the anti-CD3 induced production of IFN- γ at both the day 3 and day 6 time points (figure 3.11 a, c). It can be seen that the anti-CD3 induced production of IFN- γ was suppressed in both progesterone and NET stimulations for both time points. The cortisol and MPA stimulations showed higher suppression levels of the anti-CD3 induced IFN- γ production at day 6 than the progesterone and NET stimulations.

The addition of cortisol to BCG significantly suppressed the production of IFN- γ at both the time points (figure 3.11 b, d). The production of IFN- γ was suppressed by the addition of MPA, progesterone and NET to BCG stimulations for both the time points, however the difference in IFN- γ did not reach a level of statistical significance. The suppression of BCG induced IFN- γ production by cortisol showed significant levels of inhibition compared to the progesterone and NET.

Therefore the addition of cortisol and MPA suppresses the secretion of IFN- γ to a greater extent than the addition of progesterone and NET to the stimulations.

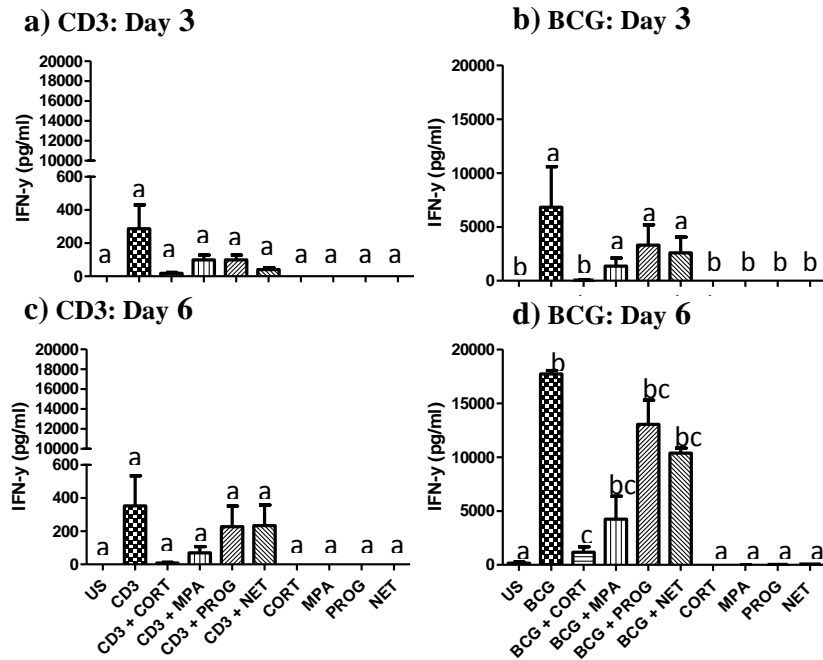


Figure 3.11: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced IFN γ secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M. bovis* BCG (b&d) or $1 \mu\text{g/ml}$ anti-CD3 (a&c) respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. US, BCG, BCG + all four hormone groups, $n=2$. US, anti-CD3, $n=2$. Anti-CD3 + all four hormone groups, $n=3$. Cortisol, MPA, progesterone and NET, $n=1$. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD post hoc test and presented as mean \pm SEM. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different.

ix. TNF- α

The production of TNF- α was also suppressed with the addition of cortisol and MPA to the anti-CD3 stimulations (figure 3.12 a, c). The level of inhibition on anti-CD3 induced TNF- α secretion reached statistical significance for cortisol for both day 3 and day 6, however MPA was only significant at day 3. The anti-CD3 induced TNF- α secretion was significantly suppressed by progesterone and NET at day 3.

The BCG induced production of TNF- α was significantly inhibited by the addition of cortisol and MPA for both day 3 and day 6 time points (figure 3.12 b, d). The TNF- α production induced by BCG was significantly inhibited by cortisol and MPA when compared to the BCG induced secretion of TNF- α by progesterone and NET stimulations. The effect of cortisol and MPA thus can be said to have a greater inhibitory effect on the secretion of TNF- α than the inhibitory effect observed with the addition of progesterone and NET to the BCG and anti-

CD3 stimulations. Again MPA appears to have more glucocorticoid activity compared to progestogenic activity.

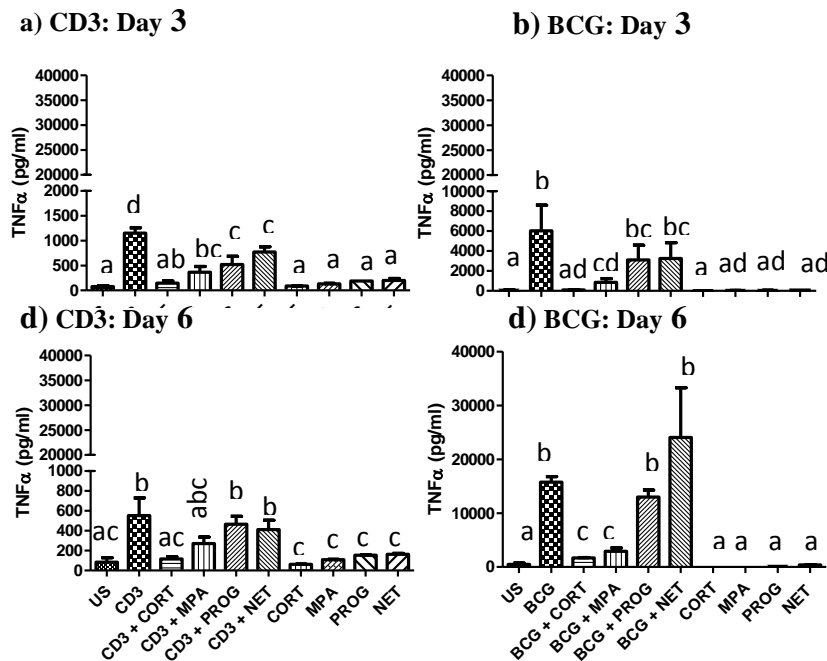


Figure 3.12: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced TNF- α secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG (b&d) or $1 \mu\text{g/ml}$ anti-CD3 (a&c) respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. US, BCG, BCG + all four hormone groups, $n=2$. US, anti-CD3, $n=2$. Anti-CD3 + all four hormone groups, $n=3$. Cortisol, MPA, progesterone and NET, $n=1$. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD post hoc test and presented as mean \pm SEM. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different.

x. G-CSF

The anti-CD3 induced G-CSF secretions pattern for cortisol, MPA, progesterone and NET for both day 3 and day 6 time points (figure 3.13 a, c) showed elevated levels but were not significant.

Cortisol inhibited the BCG induced production of G-CSF, whereas MPA, progesterone and NET did not exhibit the same inhibitory effect on the G-CSF secretion at day 3 (figure 3.13b). At day 6 the BCG induced G-CSF secretion was enhanced by the addition of cortisol and MPA, but not the addition of progesterone and NET (figure 3.13d). The secretion pattern

of G-CSF did not show the distinct pattern seen throughout this section, where cortisol and MPA inhibit the cytokine production.

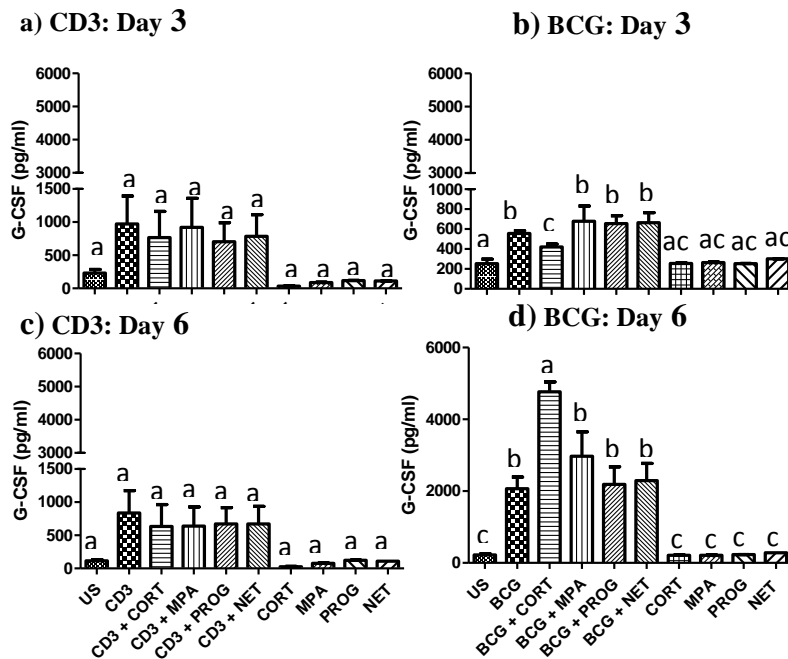


Figure 3.13: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced G-CSF secretion.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG (b&d) or $1 \mu\text{g/ml}$ anti-CD3 (a&c) respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The cytokine concentration was measured in culture supernatant, collected 3 and 6 days post-stimulation, with a 13-plex cytokine Luminex panel. US, BCG, BCG + all four hormone groups, $n=2$. US, anti-CD3, $n=2$. Anti-CD3 + all four hormone groups, $n=3$. Cortisol, MPA, progesterone and NET, $n=1$. All were performed in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD post hoc test and presented as mean \pm SEM. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different.

Summary:

The cytokine data described within this secretion shows that with regard to cytokine production, MPA mimics the endogenous hormone cortisol by suppressing the cytokine production. Whereas NET seems to mimic the effects of progesterone, thus does not have the same degree of cytokine suppression as is seen with MPA. These observations could suggest that MPA has glucocorticoid properties that are not present with NET.

3.4 GR and PR protein expression in PBMCs

In section 3.3 it was shown that certain cytokine secretion profiles were inhibited by the addition of cortisol and MPA to BCG and anti-CD3 stimulations. In order to determine through which steroid receptors the hormones mediate their action, we aimed to measure the levels of steroid receptors in PBMCs. We used two different approaches:

- a) Western blot for protein receptors where antibodies were available to us for the GR and PR.
- b) RT-PCR for the mRNA levels of the GR, PR, AR, mPR α , mPR β , mPR γ and PGRMC1.

The receptor expression was determined using Western Blot analysis of neat cell lysates. The expression of the two receptors within the PBMCs will provide insight into whether there are receptors available within our cells for hormone interaction. Figure 3.1 shows a schematic representation of the hormones ability to interact with the receptors.

i. Glucocorticoid receptor and progesterone receptor expression in PBMCs

The GR (H-300) antibody used in our experiments is recommended for detection of GR α (MW: 95 kDa) and GR β (MW: 90 kDa). The PR (C-19) antibody used in our experiments is recommended for detection of PR-A (MW: 81kDa) and PR-B (MW: 116 kDa).

The PBMC cells expressed the GR receptor (figure 3.14a). The band was detected at approximately 97kDa, this could be accounted to the close proximity of the two GR isoforms (GR α , 95 kDa and GR β , 90 kDa), thus too close together to be clearly distinguished as two separate bands. The presence of the GR receptors within the cells confirms that the hormones used within our stimulations could mediate their effects via the GR.

The level of PR expression was not detectable by Western blot in the PBMCs (figure 3.14b). This could potentially be attributed to, too low PR numbers to be picked up at protein level, but could be detectable at RNA level.

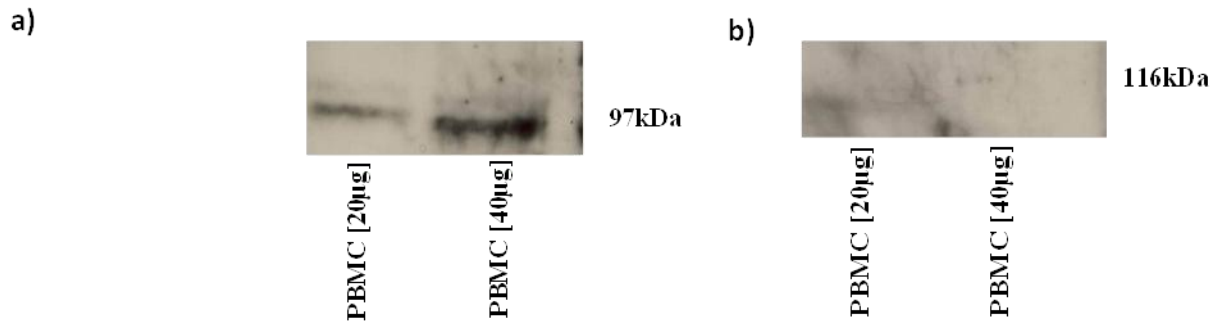


Figure 3.14: Western Blot representing (a) GR (b) PR banding pattern in PBMC cells.

a) 20µg and 40µg PBMC cell lysates were subjected to SDS-PAGE and proteins transferred to hybond ECL membranes. Blot probed with a 1:1000 of the GR-reactive and PR-reactive rabbit polyclonal antibodies respectively. Followed by the incubation with 1:5000 of the Rabbit IgG HRP-linked secondary antibody. Blots were visualized by treatment with chemiluminescent reagents followed by exposure to light-sensitive film. Samples yielded a single prominent band at approximately 97 kDa (GR) and 116kDa (PR).

The absence of detection of the classical PR by Western blot in PBMCs could be due to the low detectable protein levels and has previously been reported (58)(68). This signifies that the effects of progesterone and NET are possibly mediated through membrane bound progesterone receptors such as mPR α , mPR β , mPR γ and PGRMC1. Antibodies to these receptors are not commercially available. Therefore we performed RT-PCR to ascertain the presence of those receptors in PBMCs. The presence of the GR within the PBMC cells supports the cytokine data seen in section 3.1 where inhibition of cytokine production was observed. This could potentially be due to cortisol and MPA hormones binding to the GR

3.5 The effect of the hormone cortisol, MPA, progesterone and NET on BCG and anti-CD3 induced alterations in receptor mRNA levels in human PBMCs

Our aim was to confirm the Western blot results by Real Time PCR to see whether the GR and PR mRNA levels correspond with what was seen in the Western blot. Additionally we wanted to evaluate expression levels for other steroid receptors such as AR, mPR α , mPR β , mPR γ and PGRMC1 for which no commercial antibodies are available. As well as determine the effect of BCG and anti-CD3 stimulations on the mRNA levels of the various receptors.

To determine the effect that the hormones, cortisol, progesterone, MPA and NET have on the expression of steroid receptors after stimulation with BCG and anti-CD3 respectively. A Real Time PCR assay was used to determine the receptor expression from PBMCs isolated from

whole blood samples of woman not currently receiving any steroid treatment or using any hormone based contraception method. The Real Time PCR revealed that alterations occurred in the receptor expression profiles of the different steroid receptors under the different stimulatory conditions.

Results were normalized relative to expression in unstimulated (US) cells and are expressed as fold change from basal levels (=1) and this is indicated on the graphs by means of a reference line on the vertical y-axis. All the stimulatory conditions are explained in reference to the US sample, a fold change value above 1 will indicate an increase in the expression of the receptor. A value below 1 will indicate a decrease in the receptor expression compared to basal levels.

i. GR

At day 1 post anti-CD3 stimulations no significant observations were made for the GR expression (figure 3.15a). The day 3 stimulations (figure 3.15c) reveal that MPA treatment induced significantly higher GR levels when added together with anti-CD3 compared to progesterone and NET. The progesterone only stimulation had significantly higher GR expression when compared to progesterone and NET stimulations in the presence of anti-CD3. The addition of cortisol to cells stimulated with anti-CD3 resulted in higher expression levels of GR in comparison with progesterone and NET GR expression levels. Although a difference in expression was observed it was not statistically significant. At day 6 post stimulation the MPA stimulation led to significantly higher levels of GR in comparison with anti-CD3 stimulations that included the addition of cortisol, MPA, progesterone and NET, as well as the NET only stimulation (figure 3.15e).

The stimulations with BCG for day 1, 3 and 6 had expression levels below the basal levels. Day 1 post BCG stimulation the addition of MPA and progesterone had significantly higher GR expression levels when compared to the addition of cortisol and NET (figure 3.15b). The addition of MPA to BCG stimulation had significantly higher GR expression when compared to the hormone only stimulations of cortisol, MPA, progesterone and NET. The addition of progesterone to the BCG stimulation produced significantly higher GR expression when compared to the addition of cortisol to BCG. The hormone only stimulations cortisol, MPA, progesterone and NET showed significantly lower GR expression when compared to the BCG induced progesterone and MPA GR expression levels. Day 3 post stimulation time

point shows no significant differences for the BCG stimulations (figure 3.15d). The changes in GR expression for day 6 post stimulation shows that the addition of progesterone to BCG produced significantly higher GR expression levels when compared to the addition of cortisol and MPA to BCG (figure 3.15f). The hormone only stimulations cortisol, MPA, progesterone and NET, displayed significantly lower receptor expression when compared to the addition of progesterone to BCG stimulation GR expression levels.

The BCG stimulations all had expression levels below the basal levels indicating decreased GR expression levels. The anti-CD3 stimulations revealed that the addition of MPA or MPA by itself increased the GR expression levels of the stimulated cells.

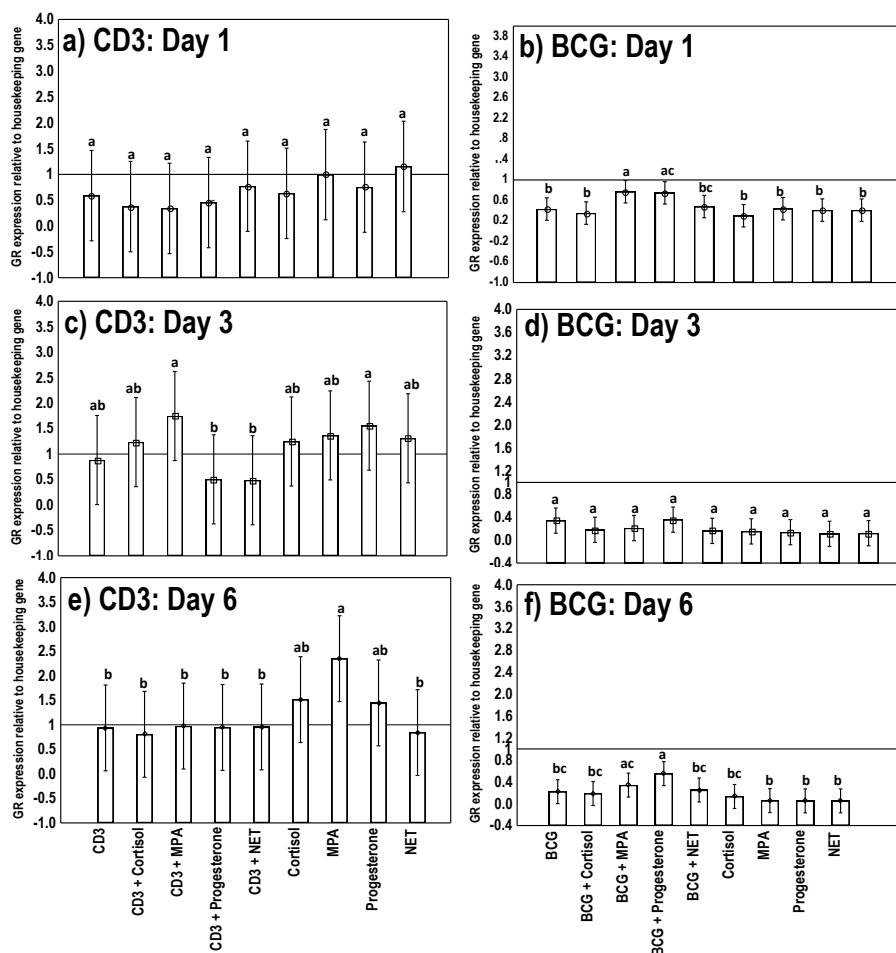


Figure 3.15: The effect of cortisol, MPA, progesterone and NET on BCG and anti-CD3-induced GR expression.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The receptor expression was measured in the cell lysates harvested on day 3 and 6 post stimulation, using real time RT-PCR based on the expression of GAPDH. $n=2$ for all stimulation conditions, experiments were run in triplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b and c indicates statistical significance, where values with the same letter are not significantly different from each other. A P value of 0.05 was regarded as significantly different.

ii. PR

Post anti-CD3 stimulation at day 1, revealed no significant changes in the PR expression levels (figure 3.16a). The changes in the PR expression for day 3 (figure 3.16c) showed that the progesterone only stimulation had a PR expression level that was significantly higher than the PR expression of the anti-CD3 stimulation with added cortisol, MPA, progesterone, NET, as well as the hormone only stimulations cortisol, MPA and NET. For the anti-CD3 stimulations on day 6 post stimulation there were no statistically significant differences in PR expression observed (figure 3.16e).

From the PR expression for the BCG day 1 post stimulation (figure 3.16b), the addition of MPA to BCG showed significantly higher PR expression levels when compared to the BCG induced cortisol and NET levels. The hormone only stimulations cortisol, MPA, progesterone and NET had PR expression levels that significantly lower than the MPA levels in the BCG induced stimulations. Day 3 post stimulation with BCG revealed no statistically significant differences in the expression levels of PR (figure 3.16d). Post stimulation with BCG on day 6 the addition of cortisol to BCG had PR expression levels that were significantly lower than was seen for the addition of MPA, progesterone and NET to BCG stimulation. The addition of cortisol to BCG had significantly lower PR expression than the hormone only groups as well namely cortisol, MPA, progesterone and NET (figure 3.16f).

The progesterone only group had increased PR expression the in anti-CD3 stimulations. From the BCG stimulations the addition of MPA and progesterone increased the PR expression, whereas the addition of cortisol to BCG inhibited the expression levels of PR in the cells.

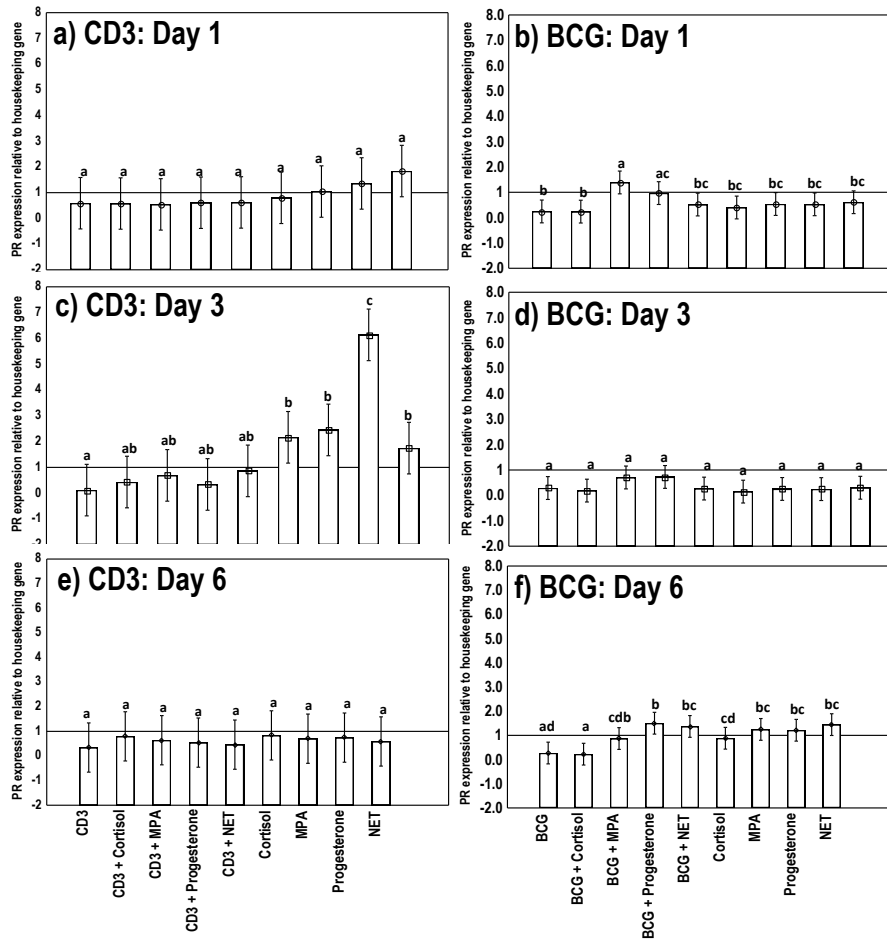


Figure 3.16: The effect of cortisol, MPA, progesterone and NET on BCG and anti-CD3-induced PR expression.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The receptor expression was measured in the cell lysates harvested on day 3 and 6 post stimulation, using real time RT-PCR based on the expression of GAPDH. $n=2$ for all stimulations within the panel. With all experiments performed in triplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicates statistical significance, where values with the same letter are not significantly different from each other. A P value of 0.05 was regarded as significantly different.

iii. AR

The AR expression for the anti-CD3 day 1 stimulations revealed no statistically significant results (figure 3.17a). Day 3 post stimulation the MPA only stimulation had significantly higher AR expression when compared to the addition of the hormones cortisol, MPA, progesterone, NET to anti-CD3 stimulation, as well as the cortisol only stimulation (figure 3.17c). The stimulation with progesterone only had significantly higher AR expression when compared to the addition of progesterone to the anti-CD3 stimulation. The addition of MPA, progesterone and NET to anti-CD3 stimulation produced significantly lower AR expression levels in comparison with NET only. The cortisol only stimulation had significantly higher expression levels of AR when compared to the anti-CD3 induced progesterone receptor expression and the NET only stimulation. Day 6 (figure 3.17e) the cortisol only stimulation had significantly higher AR expression levels when compared to the anti-CD3 stimulated progesterone treated cells, and the NET only treated cells.

On day 1 post stimulation for BCG the addition of cortisol to BCG and the progesterone only and NET only stimulations have significantly higher expression levels of AR when compared to the stimulations where MPA, progesterone and NET were added to the BCG stimulation, as well as the cortisol, MPA and NET only stimulations (figure 3.17a). Day 3 post BCG stimulation the addition of cortisol and MPA to BCG stimulations expressed significantly higher AR levels when compared to the addition of progesterone and NET to BCG stimulation, as well as the cortisol, MPA, progesterone and NET only stimulations (figure 3.17c). The BCG day 6 stimulations reveal that the addition of progesterone to BCG had a significantly higher AR expression level when compared to the addition of cortisol to the BCG stimulation as well the cortisol only stimulation (figure 3.17e).

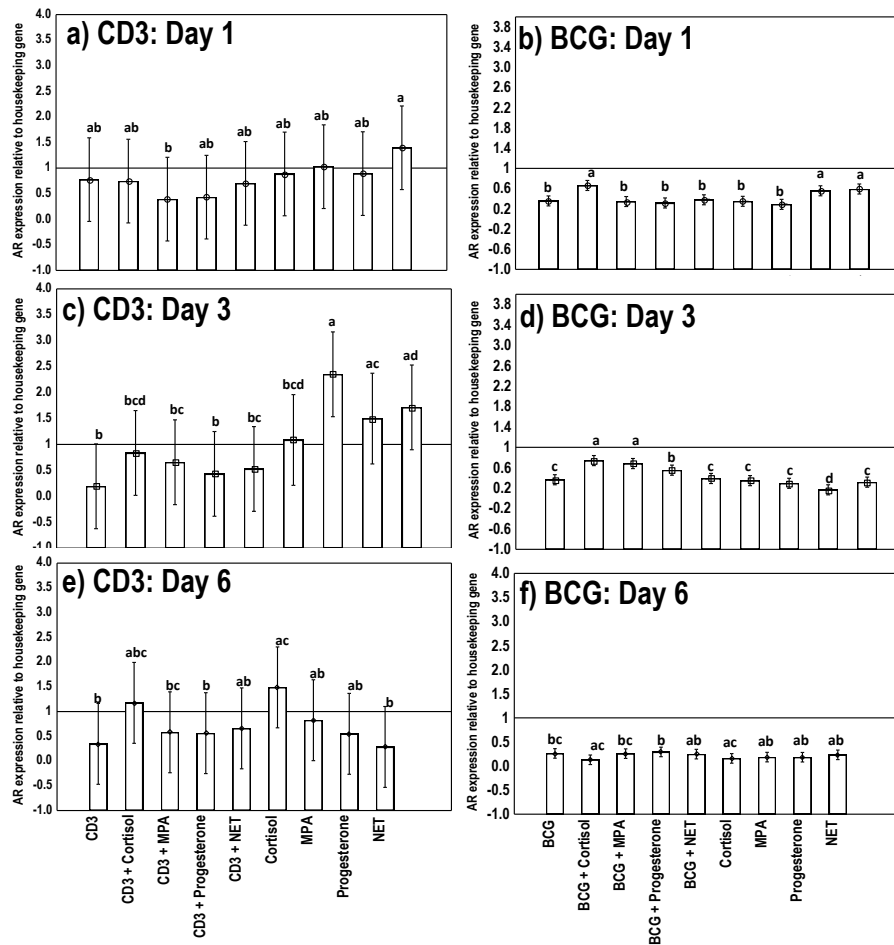


Figure 3.17: The effect of cortisol, MPA, progesterone and NET on BCG and anti-CD3-induced AR expression.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M. bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The receptor expression was measured in the cell lysates harvested on day 3 and 6 post stimulation, using real time RT-PCR based on the expression of GAPDH. $n=2$ for all stimulations within the panel. With all experiments performed in triplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicates statistical significance, where values with the same letter are not significantly different from each other. A P value of 0.05 was regarded as significantly different.

iv. mPR- α

For the day 1 anti-CD3 stimulations the progesterone only stimulation had significantly higher mPR- α expression levels when compared to the addition of cortisol, MPA, progesterone and NET to the anti-CD3 stimulations as well as MPA only stimulation (figure 3.18a). On day 3 post stimulation the MPA only stimulation had mPR- α expression levels that were significantly higher than the expression levels of the anti-CD3 stimulations where cortisol, MPA, progesterone and NET were added (figure 3.18c). At day 6 post stimulation

the progesterone only stimulation had mPR- α expression levels that were significantly higher than the stimulations where MPA and NET was added to anti-CD3 (figure 3.18e).

Day 1 the changes in mPR- α expression levels for BCG stimulations with the addition of MPA showed significantly higher mPR- α expression levels when compared to the addition of cortisol, progesterone and NET to BCG stimulated cells as well as the cortisol, MPA, progesterone and NET only stimulations (figure 3.18b). The addition of cortisol to the BCG stimulated cells had significantly lower mPR- α expression levels when compared to the addition of progesterone to BCG stimulated cells as well as the cortisol and progesterone only stimulations. The BCG stimulated cells on day 3 post stimulation showed that the addition of MPA had significantly higher mPR- α expression when compared to the addition of cortisol and NET to BCG stimulations (figure 3.18d). On day 6 post stimulation the addition of MPA expressed significantly higher mPR- α in the cells in comparison with the cortisol only stimulation (figure 3.18f).

The additions of MPA and progesterone only stimulations lead to an increase in the mPR- α expression levels in the PBMCs. The addition of MPA and progesterone led to an increase in the BCG induced mPR- α expression.

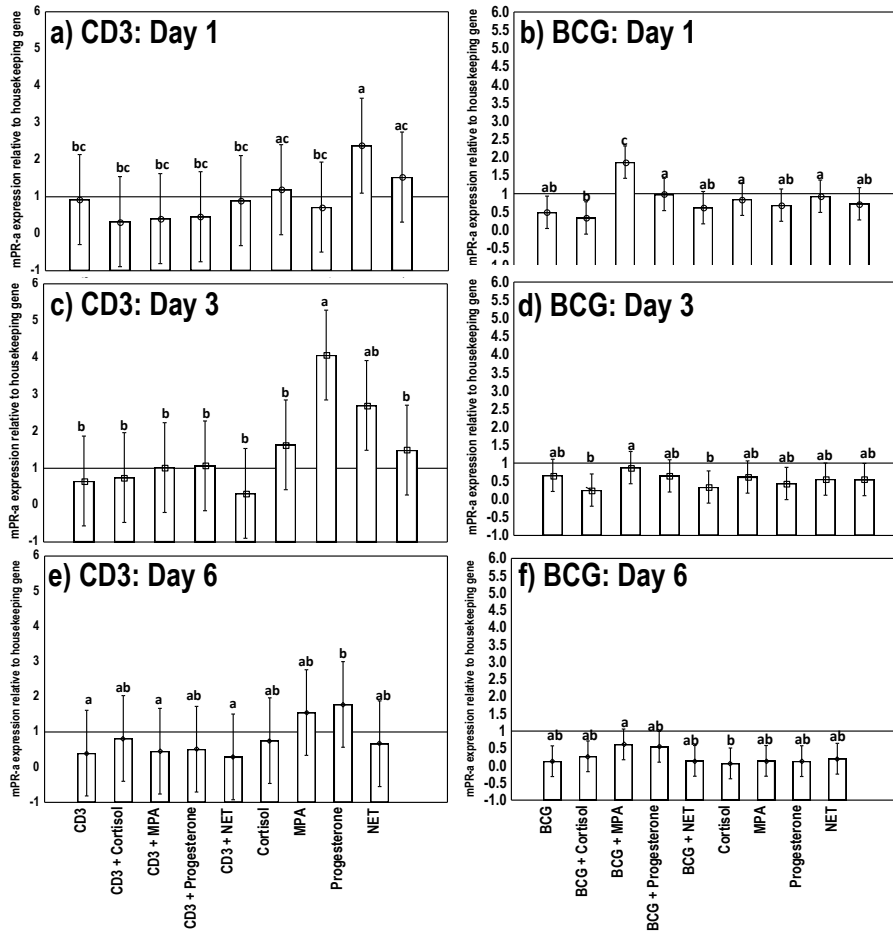


Figure 3.18: The effect of cortisol, MPA, progesterone and NET on BCG and anti-CD3-induced mPR- α expression.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The receptor expression was measured in the cell lysates harvested on day 3 and 6 post stimulation, using real time RT-PCR based on the expression of GAPDH. $n=2$ for all stimulations within the panel. With all experiments performed in triplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b and c indicates statistical significance, where values with the same letter are not significantly different from each other. A P value of 0.05 was regarded as significantly different.

v. mPR- β

At day 1 post stimulation the differences observed in the anti-CD3 stimulations did not reach a level of statistical differences for any of the stimulations (figure 3.19a). For the stimulations at day 3 the mPR- β expression levels of the cortisol only stimulation had significantly higher expression levels in comparison with the anti-CD3 stimulations were cortisol, MPA, progesterone and NET was added to the stimulation along with the MPA, progesterone and NET only stimulations (figure 3.19c). Day 6 post stimulation with anti-CD3 showed that the MPA only stimulation expressed significantly higher levels of mPR- β when compared to

anti-CD3 stimulations with the addition of the hormones cortisol, MPA, progesterone and NET including the cortisol only stimulation (figure 3.19e).

From the day 1 BCG stimulations addition of MPA to the BCG stimulation had significantly increased the mPR- β expression when compared to the BCG stimulation where cortisol, MPA and NET were added to the stimulations, together with the cortisol, MPA, progesterone and NET only stimulations (figure 3.19b). On day 3 the addition of progesterone to the BCG stimulation had significantly higher mPR- β expression levels when compared to addition of cortisol to the BCG stimulation (figure 3.19d). On day 6 stimulations show that the addition of MPA to the BCG stimulation expressed significantly higher mPR- β levels when compared to the addition of cortisol and NET to the BCG stimulation as well as the cortisol only stimulation (figure 3.19f).

The addition of MPA and progesterone to BCG stimulated cells resulted in an increase in the expression levels of the mPR- β . This was also seen in the MPA and progesterone only stimulations in the anti-CD3 stimulated cells.

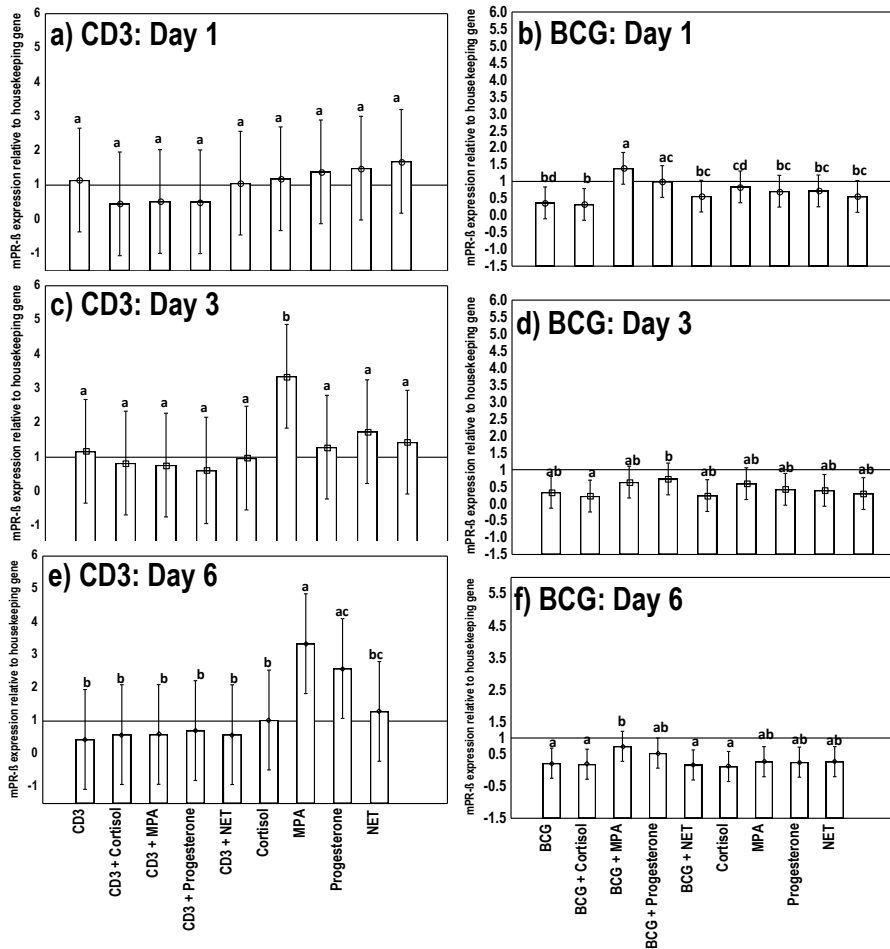


Figure 3.19: The effect of cortisol, MPA, progesterone and NET on BCG and anti-CD3-induced mPR-β expression.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The receptor expression was measured in the cell lysates harvested on day 3 and 6 post stimulation, using real time RT-PCR based on the expression of GAPDH. $n=2$ for all stimulations within the panel. With all experiments performed in triplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b and c indicates statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

vi. mPR-γ

On day 1 MPA significantly increased anti-CD3 mediated mPR-γ expression in comparison with the addition of cortisol to the anti-CD3 stimulation along with the cortisol only stimulation (figure 3.20a). The MPA, progesterone and NET only stimulations expressed significantly higher mPR-γ levels when compared to the anti-CD3 induced MPA expression levels. On day 3 the observed changes in mPR-γ expression levels did not reach a level of statistical significance (figure 3.20c). On day 6 the MPA and progesterone only stimulations had significantly higher anti-CD3 mediated mPR-γ expression levels when compared the

anti-CD3 mediated expression for cortisol, MPA, progesterone and NET, as well as the cortisol and NET only stimulations (figure 3.20e).

On day 1 the addition of cortisol to the BCG stimulations expressed significantly lower mPR- γ compared to the BCG induced MPA and progesterone expression and MPA only expression levels. The MPA expressed significantly higher BCG induced mPR- γ levels when compared to BCG induced expression levels for cortisol and NET, along with the hormone only cortisol, MPA, progesterone and NET stimulations (figure 3.20b). On day 3 no statistically significant differences were observed (figure 3.20d). On day 6 the addition of MPA to the BCG stimulation expressed significantly higher mPR- γ in comparison with the addition of cortisol and NET to the BCG stimulations as well as the cortisol only stimulation (figure 3.20f).

The addition of MPA and progesterone to BCG stimulated cells, including the hormone only stimulations of MPA and progesterone increased the mPR- γ expression levels of the cells. The increase in mPR- γ expression level was not seen with the addition of cortisol to BCG s

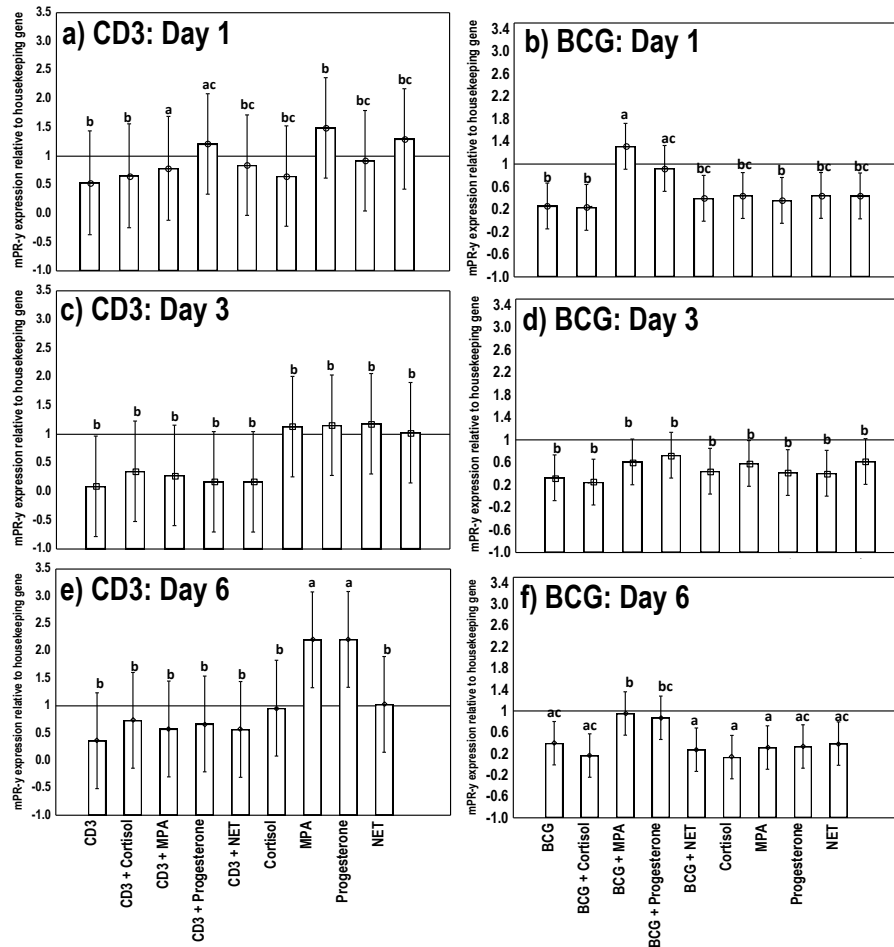


Figure 3.20: The effect of cortisol, MPA, progesterone and NET on BCG and anti-CD3-induced mPR- γ expression.

PBMCs (1×10^6 cells/well) were cultured and stimulated (with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The receptor expression was measured in the cell lysates harvested on day 3 and 6 post stimulation, using real time RT-PCR based on the expression of GAPDH. $n=2$ for all stimulations within the panel. With all experiments performed in triplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicates statistical significance, where values with the same letter are not significantly different from each other. A P value of 0.05 was regarded as significantly different.

vii. PGRMC1

On day 1 there were no statistically different observations for the anti-CD3 stimulations (figure 3.21a). On day 3 the progesterone only stimulation had significantly higher PGRMC1 expression when compared to the anti-CD3 mediated expression of cortisol, MPA, progesterone and NET along with the cortisol only stimulation (figure 3.21c). The MPA and NET only stimulations had significantly higher PGRMC1 expression levels when compared to the anti-CD3 mediated expression for cortisol, MPA and NET. On day 6 the cortisol only stimulation had significantly higher PGRMC1 expression levels when compared to the anti-

CD3 induced MPA, anti-CD3 induced progesterone and NET only PGRMC1 expression (figure 3.21e).

On day 1 the addition of MPA to the BCG stimulation had significantly higher PGRMC1 expression when compared to the BCG mediated cortisol and progesterone expression levels (figure 3.21b). On day 3 there were no statistically significant differences for the BCG stimulations (figure 3.21d). On day 6 the addition of progesterone to the BCG stimulations had significantly higher PGRMC1 expression levels in comparison with the BCG mediated expression levels of cortisol and NET, including the cortisol, MPA and progesterone only stimulations (figure 3.21f).

The addition of the hormones, cortisol, MPA and progesterone to anti-CD3 and BCG stimulations led to the increased expression levels of PGRMC1. This was also seen in the MPA and progesterone only stimulations.

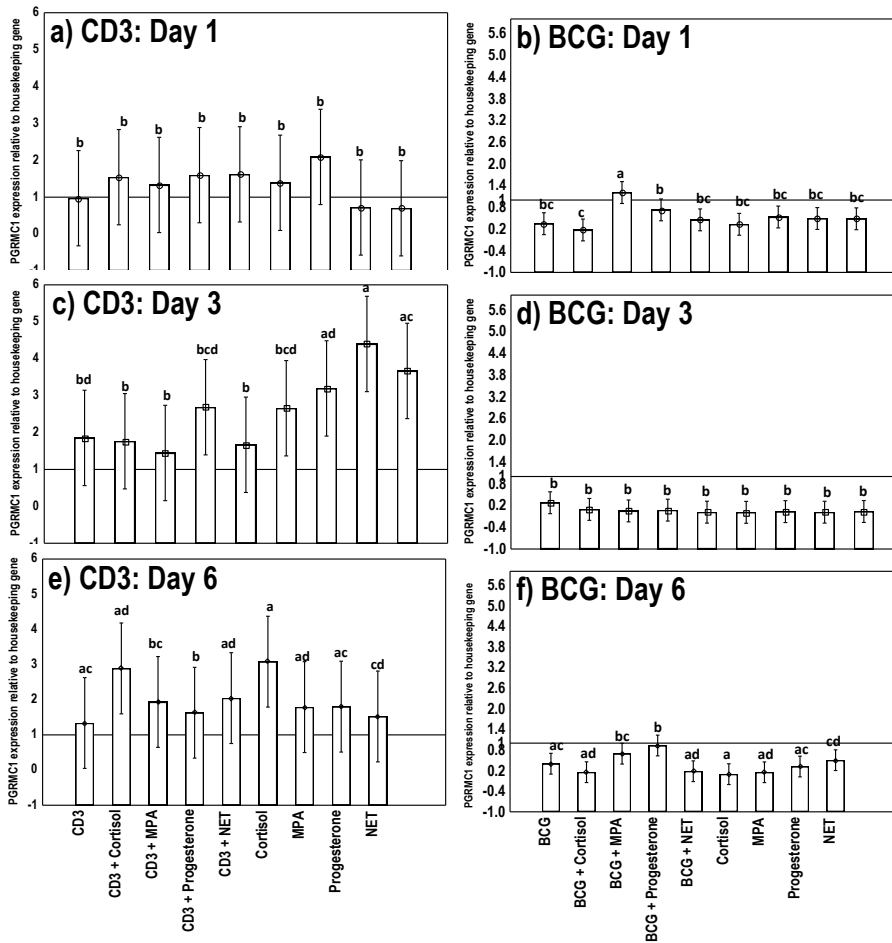


Figure 3.21: The effect of cortisol, MPA, progesterone and NET on BCG and anti-CD3-induced PGRMC1 expression.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The receptor expression was measured in the cell lysates harvested on day 3 and 6 post stimulation, using real time RT-PCR based on the expression of GAPDH. $n=2$ for all stimulations within the panel. With all experiments performed in triplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicates statistical significance, where values with the same letter are not significantly different from each other. A P value of 0.05 was regarded as significantly different.

Summary:

The receptors were detectable at mRNA level thus suggesting that they could be expressed at protein level to potentially interact with the hormones when introduced to the cells. The addition of the hormones MPA and progesterone to the cells increased the expression levels of the GR, AR, mPR- α , mPR- β , mPR- γ and PGRMC1, for the membrane receptors and PGRMC1 this occurred for both the anti-CD3 and BCG mediated expression. The expression levels of the PR were increased by the presence of progesterone only in both anti-CD3 and

BCG. The addition of MPA to the BCG stimulations increased the mRNA expression of PR. In the anti-CD3 mediated expression of the AR the presence of NET increased the expression of AR, this was also seen in the NET only stimulation.

The limitation of the RT-PCR receptor expression experiments was that the same patients were not used for the anti-CD3 and BCG stimulations, thus inter-patient variability could affect the results. It would be more preferable to isolate PBMCs from the same patients for both the stimulation assays, anti-CD3 and BCG, to minimize the effect that patient variability would have. Thus a direct comparison can be drawn between the stimulatory conditions.

3.6 BCG and anti-CD3 induced activation of signalling molecules in human PBMCs

The aim of this investigation was to determine what signalling molecules are affected by the BCG and anti-CD3 stimulations. Over and above what the addition of the hormone will be on the signalling molecules. To meet these aim three different techniques were used:

- a) A phospho-kinase array kit, which determines protein levels.
- b) Western blot for the total and phosphorylated protein for the following kinases p38, JNK and ERK 1/2.
- c) Luminex technology that determines phosphoprotein levels for CD3 ϵ , Lck, LAT, ZAP-70, ERK 1/2, CREB, STAT5, p38, JNK, I κ B α , p70S6 and STAT3.

3.6.1 Human Phospho-kinase array in stimulated PBMCs

The human phospho-kinase array is based on the principles of Western blot technology. The following signalling molecules were measured in the PBMC lysates within the array: p38 α , ERK 1/2, JNK 1/2/3, GSK-3 α/β , p53, EGF R, MSK 1/2, AMPK α 1, Akt 1/2/3, TOR, CREB, HSP27, AMPK α 2, β -catenin, p70S6, c-Jun, Src, Lyn, Lck, STAT2, STAT 5A/B, RSK 1/2/3, eNOS, Fyn, Fgr, STAT6, STAT 5b, STAT 3, p27, PLC- γ 1, Hck, Chk-2, FAK, PDGF R β , WINK1, PYK2 and Hsp60. Each of the dots represents a kinase in duplicate. Each of the stimulations is run on two blots, blot A and blot B, as can be seen in figure 3.23. The two spots on the periphery of the blots indicated by the white blocks are the positive controls and used to orientate the blots so the kinases can be identified during analysis. Although differences can be visually attained from the blots, the Unscan-it software was not sensitive

enough to quantify the dots, due to the small size of the dots on the blot and a lot of background was included in the pixel density value generated by the software.

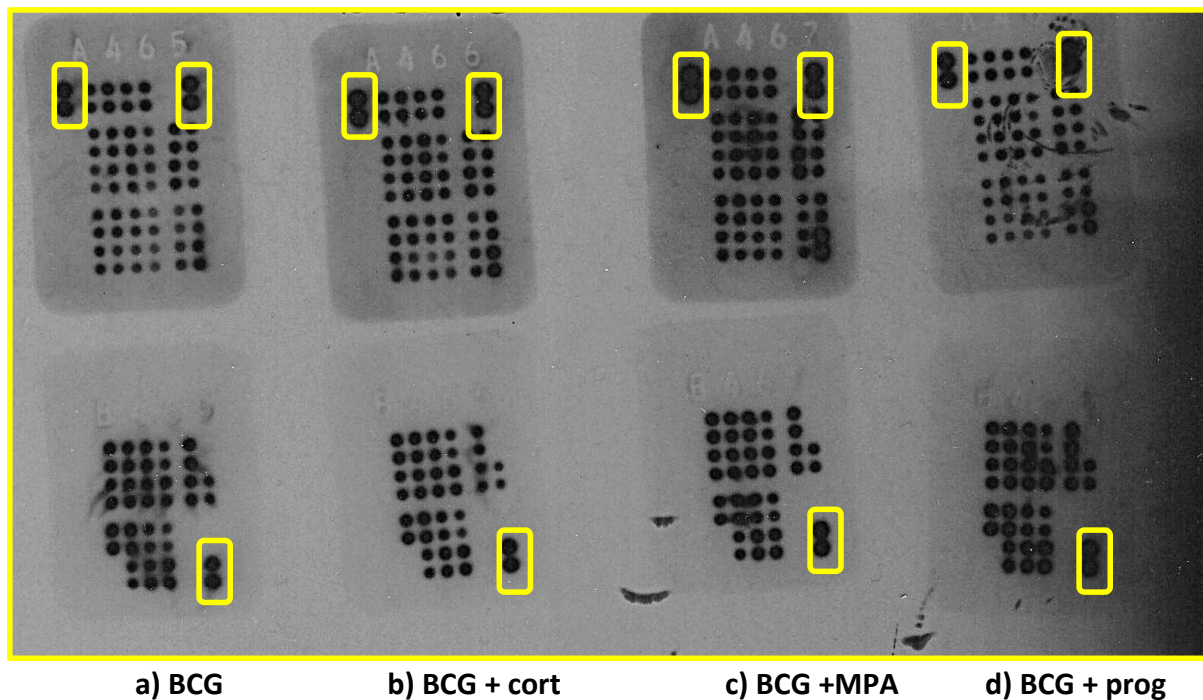


Figure 3.22: Four stimulation conditions were analysed using the Human Phospho-Kinase array a) BCG, b) BCG + cort, c) BCG + MPA and d) BCG + Prog.

Blots were blocked in buffer provided. Stimulated 1×10^6 PBMC cell lysates were incubated overnight with blots. Next day incubation with detection antibody cocktail and streptavidin-HRP enzymes. Blots were visualized by treatment with chemiluminescent reagents followed by exposure to light-sensitive film.

As the Human Phospho-Kinase array technique was not sensitive enough to meet our aim of determining the differences in signalling molecules under different stimulations with the addition of hormones. Normal Western blot antibodies were acquired, to generate quantifiable data.

3.6.2 Kinase expression in stimulated PBMCs

The aim was to determine alterations in the expression of the kinases after stimulation with hormones. The active phospho-protein state as well as total expression of ERK 1/2, JNK and p38 was looked at within in cell lysates of PBMCs. Once a kinase becomes phosphorylated it becomes activated and can interact with downstream signalling molecules. When the phosphate is removed from the kinase, it returns to its inactive state.

The phospho-protein antibody was used to detect the amount of active kinase in PBMCs, which would then be compared to and normalised to the total amount of kinase protein within the cells. The same blot was first probed with the phospho-protein antibody, prior to being stripped and re-blotted with the total protein antibody. The phospho-protein was first blotted, so that any residual signal that remains after the stripping process will not affect the blot to such an extent as it would have been included in the total protein.

i. p38

The Western blot gels were quantified using software to assign pixel density values to the individual lanes on the blot. The graphs are depicted as the pixel density values generated from the phospho-protein where normalized against the total protein pixel density values. From the p38 Western blot data (figure 3.23A, B) it can be seen that the addition of cortisol to BCG led to a reduction in the phosphorylation of the p38 kinase (figure 3.23B). The addition of progesterone did not inhibit the BCG induced phosphorylation of p38, but increased the phosphorylation of the p38 kinase.

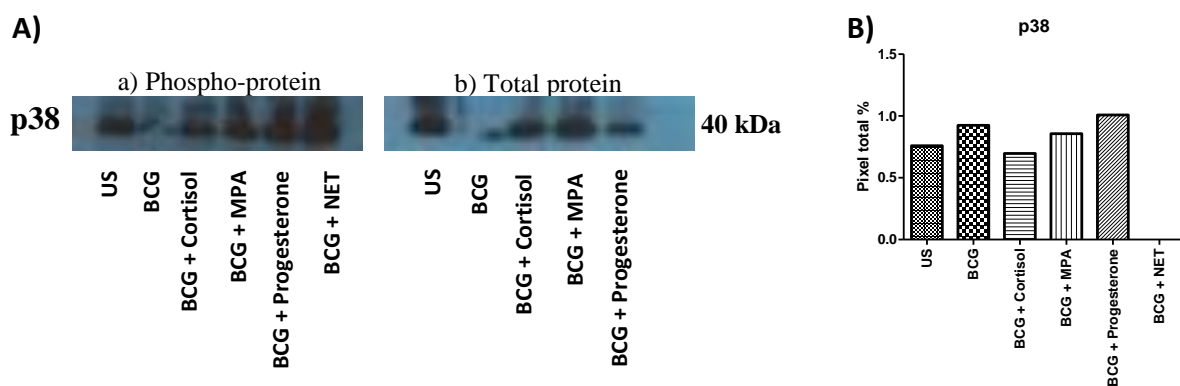


Figure 3.23: Western Blot representing the banding pattern of p38 a) phospho- and b) total protein in PBMC cells.

A) 20 μ g Stimulated PBMC cell lysates were subjected to SDS-PAGE and proteins transferred to hybond ECL membranes. Blot probed with a 1:1000 of the p38-reactive monoclonal antibody for phosphor- and total protein. Followed by incubation with 1:5000 of the Rabbit IgG HRP-linked secondary antibody. Blots were visualized by treatment with chemiluminescent reagents followed by exposure to light-sensitive film. Samples yielded a single prominent band at approximately 40 kDa. The data is representative of 2 independent experiments. B) A pixel density percentage value was assigned to the individual lanes in the Western blots using UN-SCAN-IT software. Graph shows the pixel density for the phosphor-protein blot divided by the total protein blot.

ii. JNK

From the Western blot data (figure 3.24A, B) the addition of MPA and NET increased the BCG induced phosphorylation of JNK. Whereas the BCG infected state and BCG induced cortisol levels JNK phosphorylation levels were lowered.

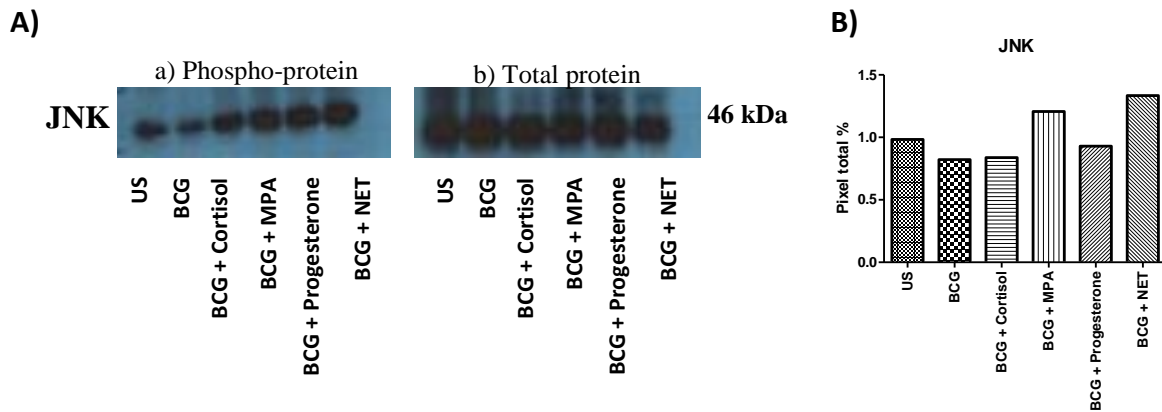


Figure 3.24: Western Blot representing the banding pattern of JNK a) phospho- and b) total protein in PBMC cells.

A) 20µg Stimulated PBMC cell lysates were subjected to SDS-PAGE and proteins transferred to hybond ECL membranes. Blot probed with a 1:1000 of the JNK-reactive monoclonal antibody for phosphor- and total protein. Followed by incubation with 1:5000 of the Rabbit IgG HRP-linked secondary antibody. Blots were visualized by treatment with chemiluminescent reagents followed by exposure to light-sensitive film. Samples yielded a single prominent band at approximately 46 kDa. The data is representative of 2 independent experiments. B) A pixel density percentage value was assigned to the individual lanes in the Western blots using UN-SCAN-IT software. Graph shows the pixel density for the phosphor-protein blot divided by the total protein blot.

iii. ERK 1/2

The Western blot data (figure 3.25A, B) shows that the addition of cortisol and MPA, increases the BCG induced phosphorylation of ERK 1/2. The total protein blot displays low levels of protein, this could possibly be attributed to the procedure used to strip the blot.

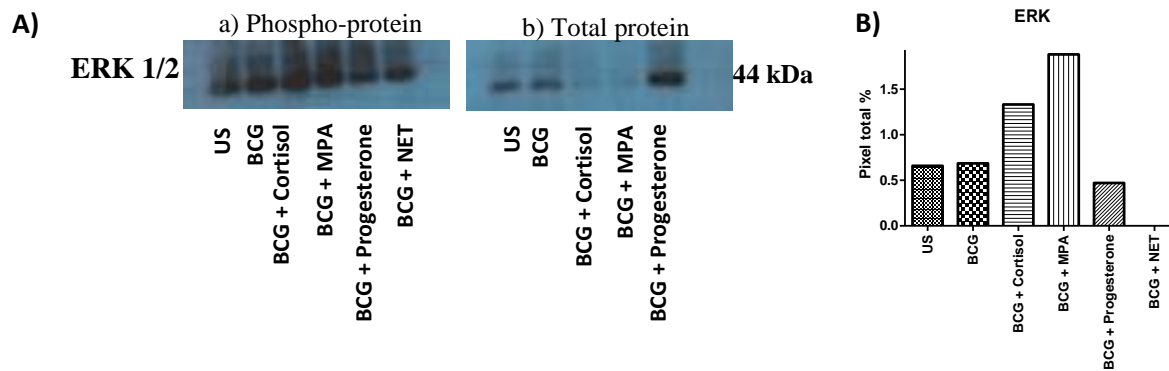


Figure 3.25: Western Blot representing the banding pattern of ERK 1/2 a) phospho- and b) total protein in PBMC cells.

A) 20 μ g Stimulated PBMC cell lysates were subjected to SDS-PAGE and proteins transferred to hybond ECL membranes. Blot probed with a 1:1000 of the ERK 1/2-reactive monoclonal antibody for phosphor- and total protein. Followed by incubation with 1:5000 of the Rabbit IgG HRP-linked secondary antibody. Blots were visualized by treatment with chemiluminescent reagents followed by exposure to light-sensitive film. Samples yielded a single prominent band at approximately 44 kDa. The data is representative of 2 independent experiments. B) A pixel density percentage value was assigned to the individual lanes in the Western blots using UN-SCAN-IT software. Graph shows the pixel density for the phosphor-protein blot divided by the total protein blot.

The Western blot kinase blots will not be able to detect subtle changes in kinase phosphorylation. There is also the possibility that in the stripping protocol some of the protein can be stripped off of the blot, or that the antibodies are not properly stripped and the total protein blot then gives a false representation of the amount of protein present. To meet the aims of determining the difference between signalling molecules with the addition of different hormones to anti-CD3 and BCG, a more sensitive method of detection is required. The introduction of a BCG infection in the PBMCs will induce an antigen specific response, with potential co-receptor activation and the activation of several signalling cascades. Stimulation with anti-CD3 will induce a non-specific response, which directs TCR activation and signalling without co-receptor activation. The Luminex technology was selected as it is a very sensitive technique and therefore will be able to detect subtle changes within the signalling molecules under the different stimulations. The added advantage of this technique is that multiple analytes can be tested per sample thus making direct comparison of stimulations possible. The kits that we selected for the two sections below describe the signally molecules of the non-specific TCR signalling (3.4.3), and a broader range of kinases and transcription factors that form part of multiple signalling cascades (3.4.4).

3.6.3 The effect of the hormone cortisol, MPA, progesterone and NET on BCG and anti-CD3 induced TCR signalling in human PBMCs

3.6.3.1 Anti-CD3- and anti-CD3+CD28-induced activation of kinases

To determine whether the kit was able to detect changes in TCR signalling within our sample cohort, a positive control sample was run on the kit. PBMCs from a patient were stimulated with anti-CD3, known to activate T-cell activation, as well as anti-CD3 with anti-CD28, known for its activation and proliferation of T-cells, the addition of CD28 amplifies the signal transduction. In both the anti-CD3 and anti-CD3 + anti-CD28 stimulations a very high level of Lck was detected. The high detectable levels of Lck and downstream, signalling molecule CREB, shows that the TCR was indeed activated, Lck is activated upon TCR activation and then leads to the activation of downstream signalling molecules. In the US sample there are high Lck present, but the downstream molecules were not phosphorylated.

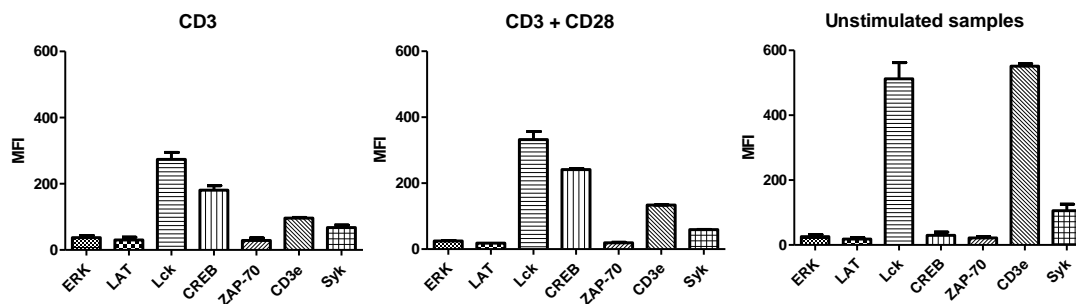


Figure 3.26: Mean Fluorescent Intensity (MFI) of the different kinases in the PBMCs.

PBMCs (1×10^6 cells/well) were cultured and stimulated with $1 \mu\text{g/ml}$ anti-CD3 or anti-CD3+anti-CD28 respectively. The concentration of the phosphorylation of the kinases was measured with a 7-plex Luminex panel. The data was presented as mean \pm SEM. ($n=1$, in duplicate).

The fact that detectable levels for all the kinases were found with the kit it was deemed to be a suitable for our research purposes. Although both the anti-CD3 and anti-CD3 + anti-CD28 could be used for T-cell activation, the anti-CD3 was chosen for further experiments as the anti-CD3 + anti-CD28 has the ability to activate more than one pathway through co-activation, thus in order to try and minimize the activation of numerous signalling pathways anti-CD3 was used as a stimulant.

To determine the effect that the hormones, cortisol, progesterone, MPA and NET would have on the signalling pathways after stimulation with BCG and anti-CD3 respectively, multiplex bead array assays were used to assess the phosphoprotein signalling molecule production from PBMCs isolated from whole blood samples of woman not currently receiving any steroid treatment or using any hormone based contraception method. The Luminex panels revealed

that alterations occurred in the phosphoprotein profiles of the different signalling molecules under the different stimulatory conditions.

3.6.3.2 Observations made on day 1 post anti-CD3 stimulation.

The T-cell signalling was looked at when PBMCs were stimulated with anti-CD3 antibody which is a known T-cell activator under different stimulatory conditions in the presence or absence of hormones (Cortisol, Progesterone, MPA and NET).

Identical samples were run on both the 7-plex and 8-plex Luminex panels, the sample aliquot was thawed once and loaded onto the two plates respectively.

The inclusion of day 1 in the anti-CD3 stimulation panel acted as a screening tool to see whether differences were observed in kinase activation under the different stimulations, and whether further investigation could be considered. Usually kinase activation is rapid within minutes. However, our time points were used to co-incide with the cytokine data collection time points. The supernatants from the cells were used for cytokine determination. Thus the possibility of detecting the early effects is negligible, but the genomic effects could be detected.

There were no statistically significant changes observed in the day 1 anti-CD3 stimulations. The time points, day 3 and day 6, were included to determine whether the changes in kinase activation were only detectable at the later time points.

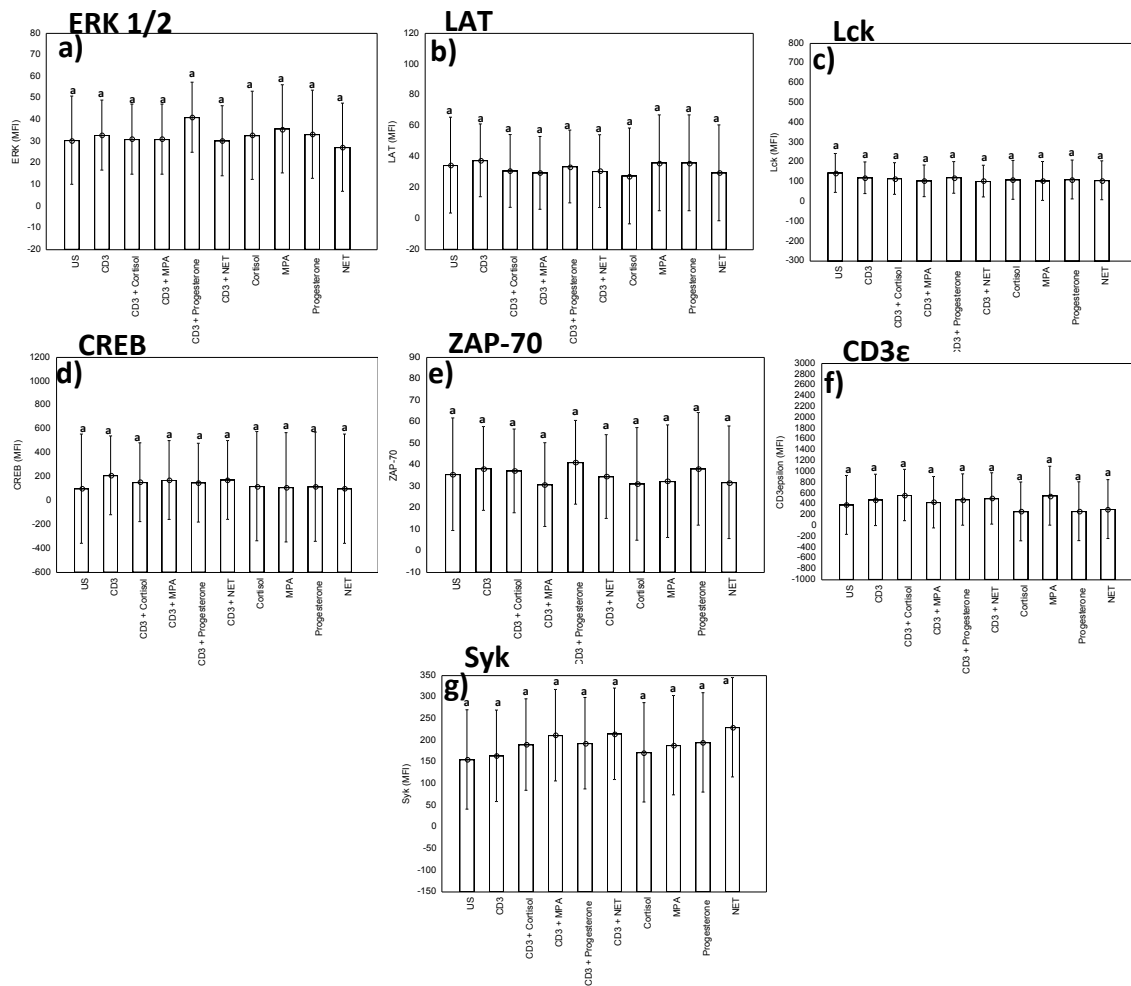


Figure 3.26: The effect of cortisol, MPA, progesterone and NET within anti-CD3-induced responses on TCR signalling a) ERK 1/2, b) LAT, c) Lck, d) ZAP70, e) CD3ε, f) CREB and g) Syk phosphoprotein levels on day 1 post stimulation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 1 post stimulation, via a 7-plex TCR Luminex panel. US, anti-CD3 and anti-anti-CD3 + all four hormones, $n=2$. Cortisol, MPA, progesterone and NET, $n=1$. All experiments were run in duplicate. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letter a indicates statistical significance, where values with the same letter are not significantly different from each other. A P value of 0.05 was regarded as significantly different.

3.6.3.3 Observations made on day 3 and day 6 post BCG and anti-CD3 stimulation.

i. CD3ε

On day 3 (figure 3.27b) the addition of cortisol, MPA, progesterone and NET to BCG stimulations showed a decrease in phosphorylated CD3ε levels. On day 6 the addition of cortisol, MPA, progesterone and NET to BCG stimulations showed an increase in phosphorylated CD3ε (figure 3.27d).

Six days post stimulation with anti-CD3 revealed that NET had significantly lower anti-CD3 induced levels of phosphorylated CD3 ϵ (figure 3.27c). MPA stimulation had significantly higher anti-CD3 induced CD3 ϵ phosphorylation levels when compared to cortisol and NET stimulations.

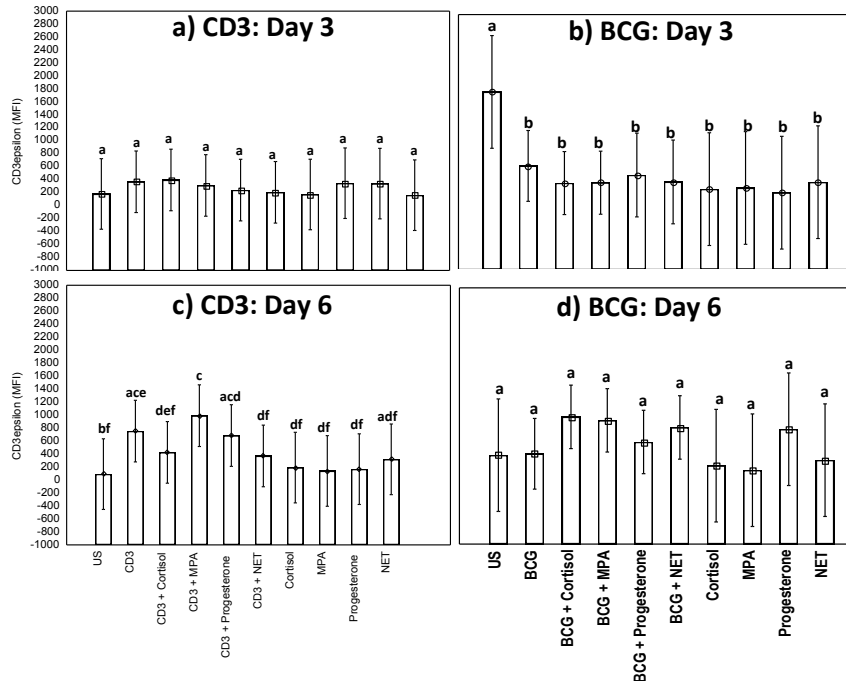


Figure 3.27: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on CD3 ϵ phosphorylation levels.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M. bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 7-plex TCR Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

ii. Lck

On day 3 for anti-CD3 stimulation (figure 3.28a) the addition of cortisol shows a decrease in detectable levels, though not significant, of phosphorylated Lck. Cortisol only stimulation produced Lck levels that were significantly lower than the anti-CD3 induced cortisol, MPA, progesterone and NET stimulations Lck levels. On day 6 with anti-CD3 (figure 3.28c) it can be seen that the anti-CD3 induced stimulation decreased the level of Lck phosphorylation significantly for cortisol, MPA, progesterone, NET, as well as cortisol only, MPA only, progesterone only and NET only stimulations. Cortisol, MPA and progesterone only

stimulations have significantly lower phosphorylated Lck levels when compared to the anti-CD3 induced MPA and progesterone Lck levels.

On day 3 (figure 3.28b) there was a decrease in Lck phosphorylation levels of the BCG induced cortisol and MPA levels, however the difference did not reach a level of significance. The phosphorylation of Lck in progesterone and NET in BCG stimulated cells, were higher than the Lck level displayed by the BCG induced Lck phosphorylation in cortisol and MPA stimulated cells.

On day 6 (figure 3.28d) the progesterone only stimulation had significantly lower Lck phosphorylation than NET only.

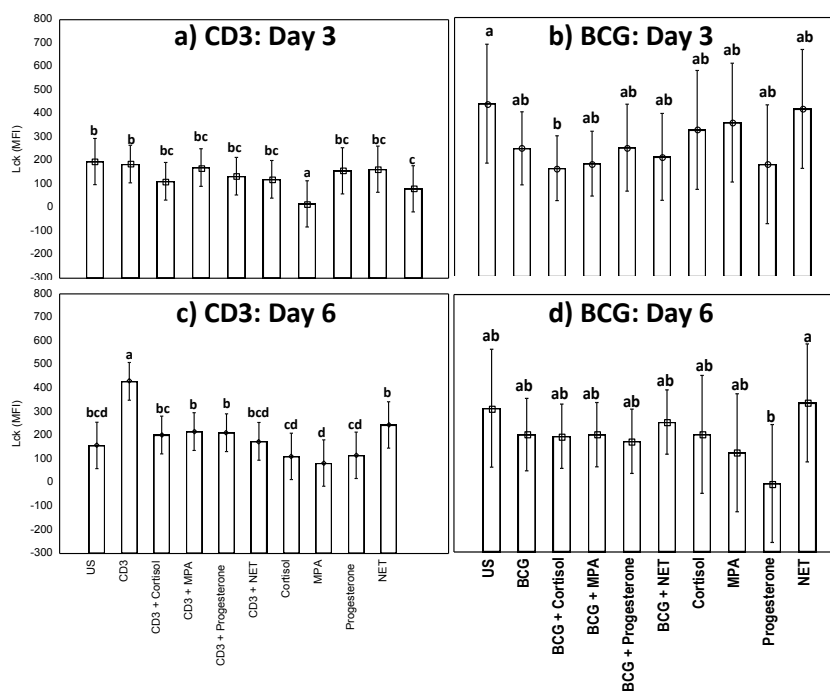


Figure 3.28: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on Lck phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 7-plex TCR Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

iii. ZAP-70

Day 3 (figure 3.29a) revealed no changes that reached a level of statistical significance. On day 6 (figure 3.29c) the anti-CD3 induced NET stimulation showed lower levels of phosphorylated ZAP-70, although not statistically significant.

On day 3 (figure 3.29b) no observations reached a level of statistical significance. The BCG stimulated cells had significantly lower levels of phosphorylated ZAP-70 when compared to the BCG infected cells treated with NET stimulation levels. On day 6 (figure 3.29d) the changes in ZAP-70 levels did not reach significant levels for any of the observations made at day 6 post stimulation.

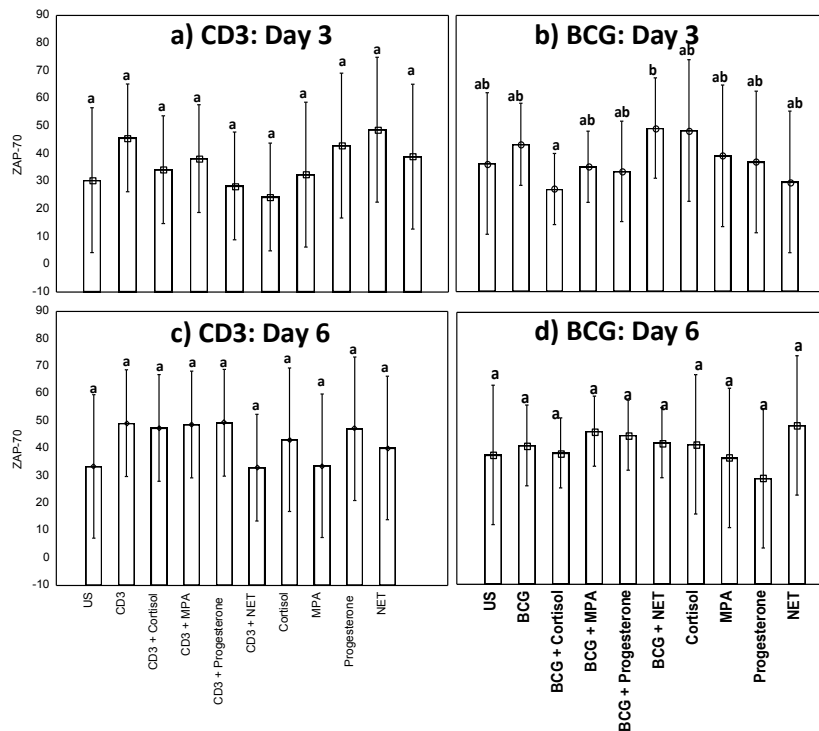


Figure 3.29: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on ZAP70 phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 7-plex TCR Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, and b indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

iv. LAT

On day 3 (figure 3.30a) the anti-CD3 induced levels of phosphorylated LAT were decreased by cortisol, MPA, progesterone and NET, a level of statistical significance was only observed for anti-CD3 induced cortisol and NET stimulations. The anti-CD3 stimulated cells treated with cortisol and NET had lower levels of LAT phosphorylation when compared to anti-CD3 stimulated MPA and progesterone treated cells. In the hormone only stimulated groups, progesterone treated cells had higher levels of phosphorylated LAT compared to cortisol, MPA and NET stimulations. On day 6 (figure 3.30c) the anti-CD3 induced levels of LAT phosphorylation were higher than the cortisol, MPA and NET treated cells.

On day 3 (figure 3.30b) the BCG stimulated cells decreased the phosphorylated LAT levels when compared to cortisol, MPA and progesterone treated cells. However from the hormone treated cells, only the BCG induced lowering of cortisol LAT levels were significantly lower. On day 6 (figure 3.30d) the addition of MPA to BCG stimulation showed higher levels of LAT phosphorylation when compared to the addition of cortisol, progesterone and NET to BCG stimulations. The BCG induced cortisol LAT phosphorylation level was lower than the BCG induced MPA, progesterone and NET phosphorylated LAT levels. However, none of the observed differences reached a level of statistical significance.

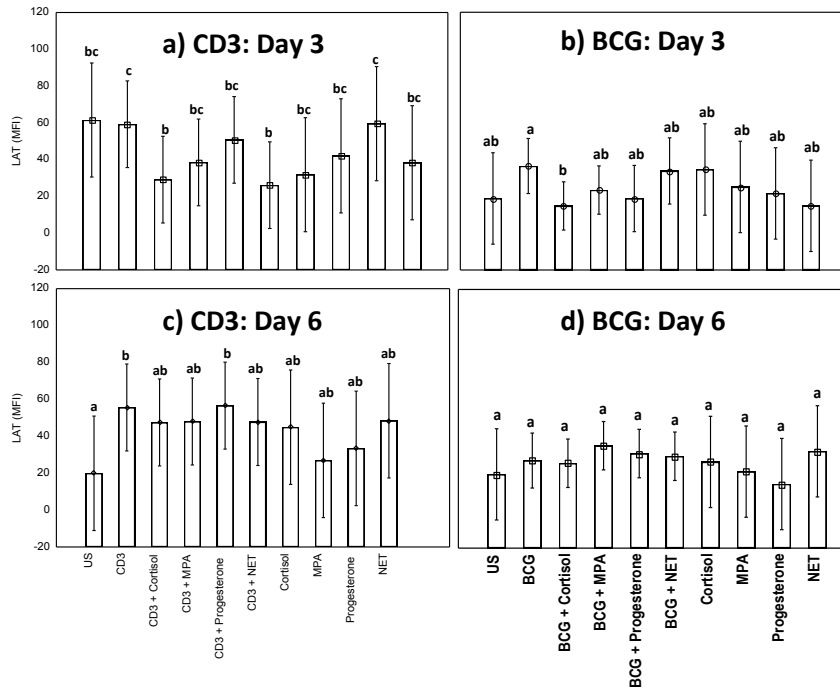


Figure 3.30: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on LAT phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or 1 μ g/ml anti-CD3 respectively, in the presence and absence of 10 μ M of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 7-plex TCR Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, and b indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

v. CREB

Day 3 (figure 3.31a) shows the detectable levels of phosphorylated CREB are decreased in anti-CD3 stimulated cells in the cortisol, MPA, progesterone and NET treated cells. Only anti-CD3 stimulated induced phosphorylation of CREB in NET treated cells reached significant difference. The anti-CD3 induced CREB phosphorylation levels of cells treated with cortisol and progesterone were increased in comparison with the CREB phosphorylation induced by anti-CD3 in cells treated with MPA. The anti-CD3 induced NET phosphorylated CREB levels were inhibited when compared to anti-CD3 induced levels of cells treated with cortisol, MPA and progesterone. On day 6 (figure 3.31c) none of the observations reached a level of statistical significance.

On day 3 and day 6 (figure 3.31b, d) the BCG stimulations showed very low levels of detectable phosphorylated CREB. The range of MFI detected for CREB did not exceed 100. This equals one tenth of the detectable range achieved by the anti-CD3 stimulations, which

had a detectable range reaching 1000 MFI. This occurrence of BCG failing to activate CREB can also be seen in the 8-plex Multiple signalling panel CREB graph shown in figure 3.39b, d.

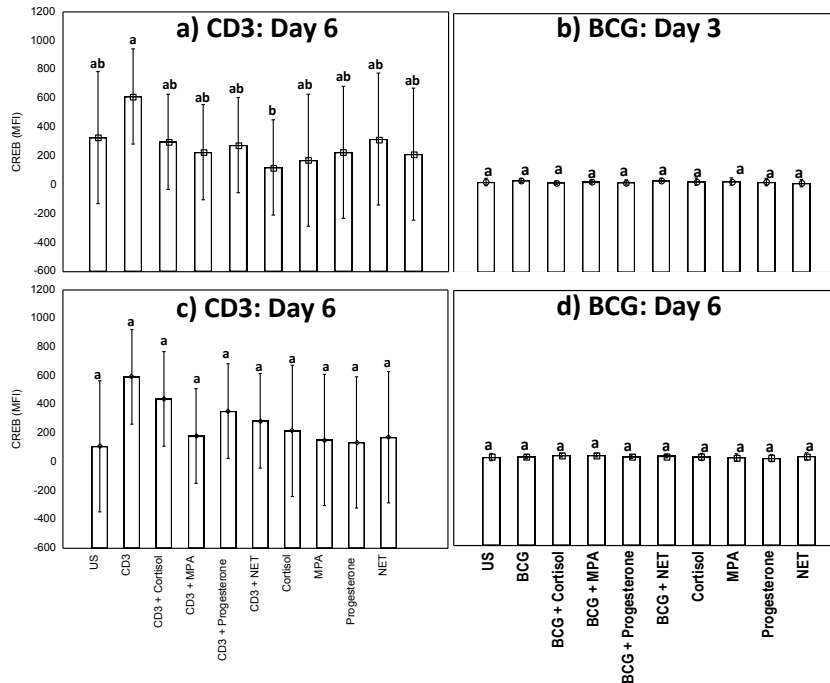


Figure 3.31: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on CREB phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 7-plex TCR Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, and b indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

vi. ERK/MAP kinase 1/2

On day 3 (figure 3.32a) the level of phosphorylated ERK 1/2 is increased in the anti-CD3 stimulated cells treated with cortisol compared to the anti-CD3 induced, MPA, progesterone and NET treated. This however, does not reach a level of statistical significance. The addition of NET, to anti-CD3 stimulated cells decreased the levels of ERK 1/2 phosphorylation when compared to the addition of cortisol, MPA and progesterone. On day 6 (figure 3.32c) ERK/MAP kinase 1/2 of anti-CD3 stimulated cells. The anti-CD3 induced NET ERK 1/2 levels showed the highest inhibition of phosphorylated ERK 1/2 when compared to the anti-anti-CD3 induced levels of cortisol, MPA, and progesterone stimulations. With the anti-CD3

induced level of ERK 1/2 for NET being significantly lower than the anti-CD3 induced levels of MPA and progesterone stimulations.

No observations of statistical significance were reached for the day 3 (figure 3.32b) nor day 6 (figure 3.32d) for the BCG stimulations.

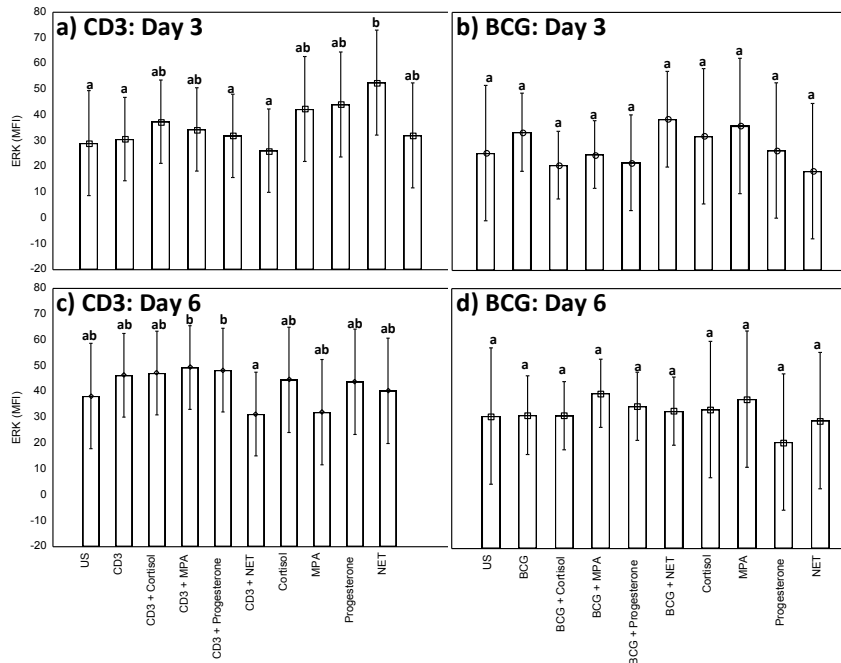


Figure 3.32: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on ERK 1/2 phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 7-plex TCR Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, and b indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

vii. SYK

On day 3 (figure 3.33a) anti-CD3 induced level of phosphorylated Syk in cortisol treated cells had significantly higher levels when compared MPA, progesterone and NET treated cells. The addition of NET to CD3 stimulation displayed significantly lower levels of phosphorylated Syk in comparison with the addition of cortisol, MPA and progesterone to anti-CD3 stimulations. On day 6 (figure 3.33c) the addition of cortisol to anti-CD3 stimulations produced significantly higher Syk phosphorylation levels when compared to the addition of progesterone, and NET stimulations. The addition of MPA, progesterone and

NET to anti-CD3 stimulations led to the inhibition of phosphorylated Syk levels however the differences are not statistically significant.

There were no statistically significant differences observed for the day 3 time point (figure 3.33b), for any of the stimulations within the BCG panel. On day 6 (figure 3.33d) the addition of cortisol, MPA, progesterone and NET to BG stimulations had increased the phosphorylated Syk levels.

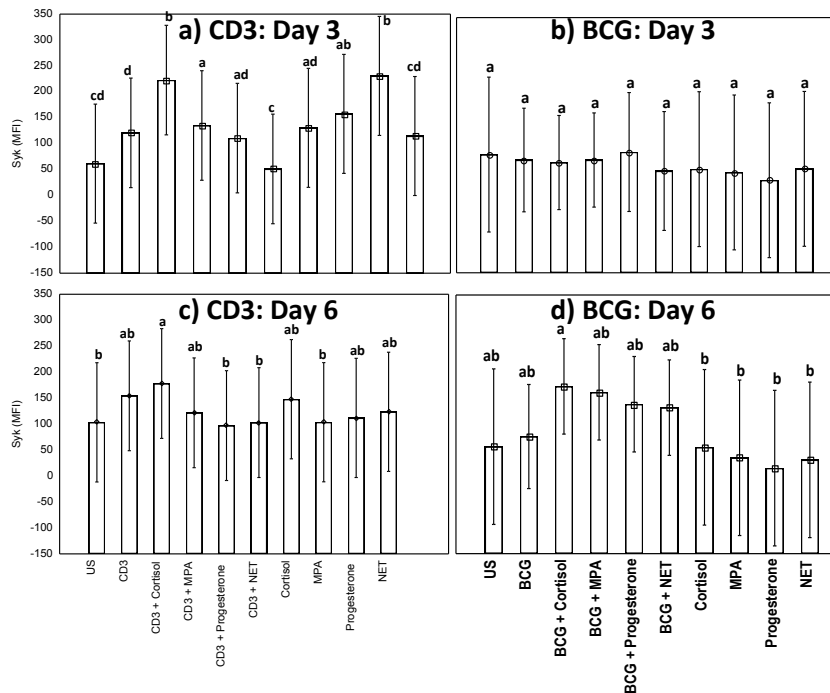


Figure 3.33: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on Syk phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 7-plex TCR Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, and b indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

Summary:

The addition of the anti-CD3 stimulus led to an increase in phosphorylation from basal levels in the following signalling molecules CD3 ϵ , Lck, ZAP-70, LAT, CREB and Syk. The introduction of BCG infection led to a decrease in phosphorylation of the early TCR signalling molecules namely CD3 ϵ and Lck. The addition of cortisol to the stimulations led to the inhibition of phosphorylation on certain kinases, Lck, ZAP-70, LAT, ERK 1/2. The

addition of cortisol, MPA, progesterone and NET caused the inhibition of anti-CD3 induced phosphorylation of the transcription factor CREB. The addition of MPA, progesterone and NET in stimulations caused the inhibition of ERK 1/2 phosphorylation. Thus for the signalling molecules it cannot be clearly determined whether the hormones MPA and NET mimic cortisol or progesterone as they shift between exhibiting proestrogenic and glucocorticoid activity.

3.6.4 The effect of the hormones cortisol, MPA, progesterone and NET on BCG and anti-CD3 induced signalling pathways in PBMCs

The beads in the Luminex kit used to analyse the signalling pathways, detects the changes in phosphorylated p70S6 at threonine 412, phosphorylated STAT5A/B at tyrosine 694 and 699, STAT3 at serine 727, phosphorylated p38 MAP kinase at threonine 180 and tyrosine 182 and JNK at threonine 183 and tyrosine 185. The bead used to detect the changes in the phosphorylated I κ B α at serine 32.

3.6.4.1 Observations made on day 1 post anti-CD3 stimulation.

The changes in the phosphoprotein expression of numerous cell signalling molecules are depicted in figure 3.34 (a-h), these kinases do not belong to a singular pathway but can play a role in different signalling cascades.

The changes in ERK 1/2 (figure 3.34a), no levels of significant differences were observed for the day 1 time point.

No differences were observed for STAT3 (figure 3.34b).

The changes in the level of JNK expression (figure 3.34c), was suppressed by cortisol in the anti-CD3 stimulated cells. MPA and NET stimulated cells had no inhibitory effect on the production of JNK when compared to cortisol treated cells. Progesterone treated cells had no effect on the anti-CD3 induced JNK phosphorylation.

Cortisol treated cells produced significantly lower levels of p70S6 (figure 3.34d) when compared to the anti-CD3 induced responses of the MPA and NET treated cells. The MPA and NET only stimulated cells produced more p70S6 than the cortisol and progesterone only stimulated cells.

The I κ B α levels for the anti-CD3 induced stimulations (figure 3.34e) produced more I κ B α than was produced by the four hormone only stimulations. None of the observed differences reached a level of significant difference.

The changes in STAT5A/B levels (figure 3.34f), the treatment of cells with cortisol inhibited the anti-CD3 induced production of STAT5A/B. MPA, progesterone and NET enhanced the anti-CD3 induced production of STAT5A/B when compared to the suppressive effect of cortisol. All four hormone only treated cells produced significantly lower levels of STAT5A/B in comparison with anti-CD3 stimulated cells. Stimulation with anti-CD3 in cells results in the activation of STAT5A/B.

No changes in the phosphorylation of CREB was seen at day 1 (figure 3.34g).

Cortisol suppressed the anti-CD3 induced phosphorylation of p38 (figure 3.34h). In the anti-CD3 stimulated cells the cortisol treatment had a greater suppressive effect when compared to the inhibition levels of MPA, progesterone and NET treated cells. Cortisol only had significantly lower p38 than all the anti-CD3 stimulated cells with hormone treatment.

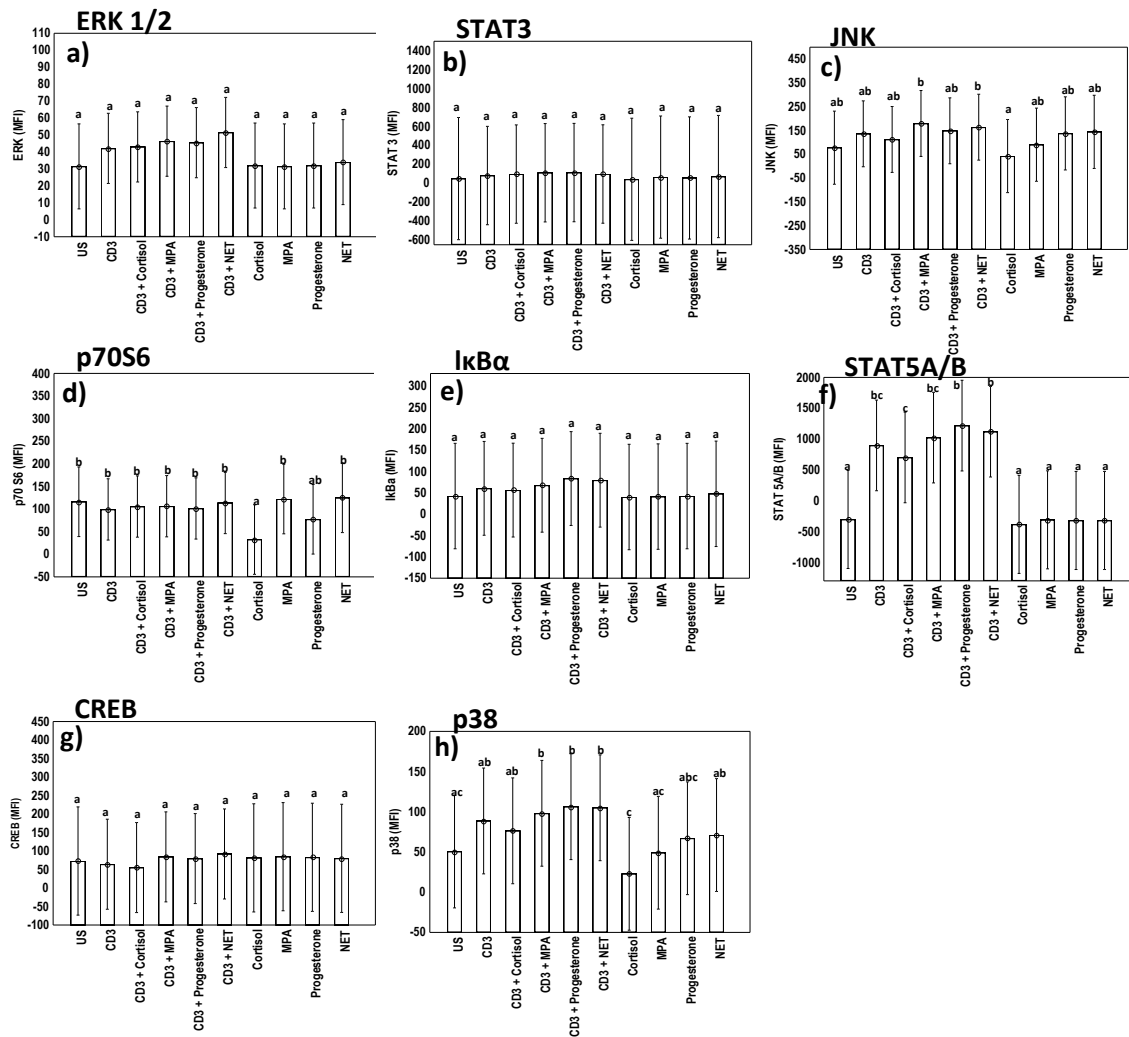


Figure 3.34: The effect of cortisol, MPA, progesterone and NET on anti-CD3-induced on a) ERK 1/2, b) STAT3, c) JNK, d) p70S6, e) IκBα, f) STAT5A/B, g) CREB and h) p38 MAP kinase phosphorylation on day 1 post stimulation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 1 post stimulation, via a 7-plex TCR Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b and c indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

3.6.4.2 Observations made on day 3 and day 6 post BCG and anti-CD3 stimulation.

i. STAT5A/B

Day 3 (figure 3.35a) cortisol and MPA enhanced the anti-CD3 induced phosphorylation of STAT5A/B, however the progesterone and NET exhibited a suppressive effect on the STAT5A/B phosphorylation levels. Day 6 did not display a noticeable change between the different stimulatory conditions (figure 3.35c).

Day 3 (figure 3.35b) showed that cortisol, MPA, progesterone and NET significantly inhibited the BCG induced phosphorylated STAT5A/B levels. On day 6 (figure 3.35d) the BCG induced stimulation shows a significantly higher STAT5A/B level was produced when compared to cortisol, MPA and progesterone only stimulations.

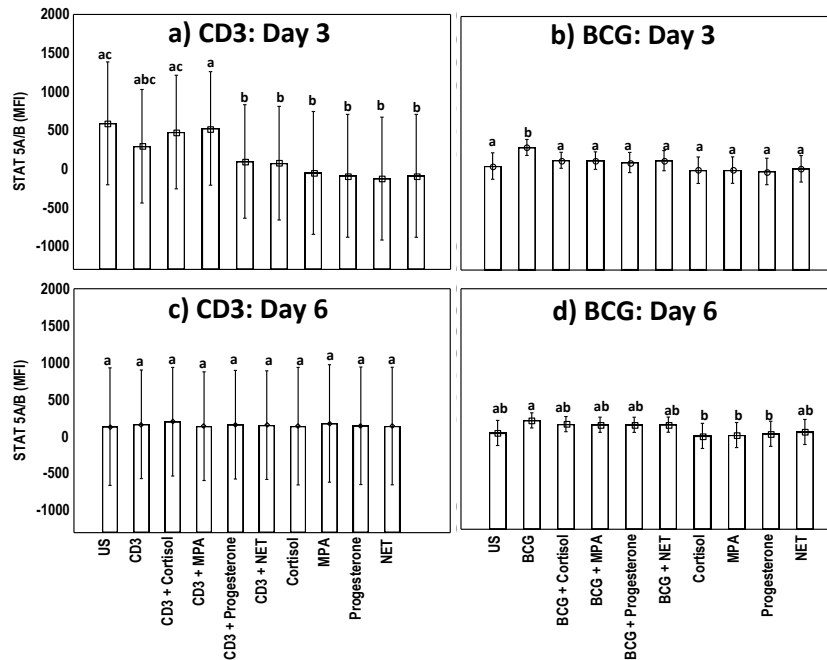


Figure 3.35: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on STAT5A/B phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 8-plex Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b and c indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

ii. ERK/MAPK 1/2

On day 3 (figure 3.36a), cortisol treatment enhanced the phosphorylation of ERK 1/2 in anti-CD3 stimulated cells. The NET treated cells significantly inhibited the phosphorylation of ERK 1/2 compared to MPA and progesterone treated anti-CD3 stimulated cells. On day 6 (figure 3.36c), cortisol treatment enhanced the phosphorylation of ERK 1/2 in anti-CD3 stimulated cells. MPA treated cells had significantly lower in the phosphorylated ERK 1/2 when compared to the cortisol treated anti-CD3 stimulated cells.

Day 3 (figure 3.36b) no statistically significant differences were observed.

On day 6 (figure 3.36d), cortisol, MPA, progesterone and NET treatment suppressed the phosphorylation of ERK 1/2 in BCG stimulated cells. The cortisol only stimulation produced significantly lower levels of phosphorylated ERK 1/2 when compared to the BCG induced effect on cortisol, progesterone, NET and progesterone only stimulations.

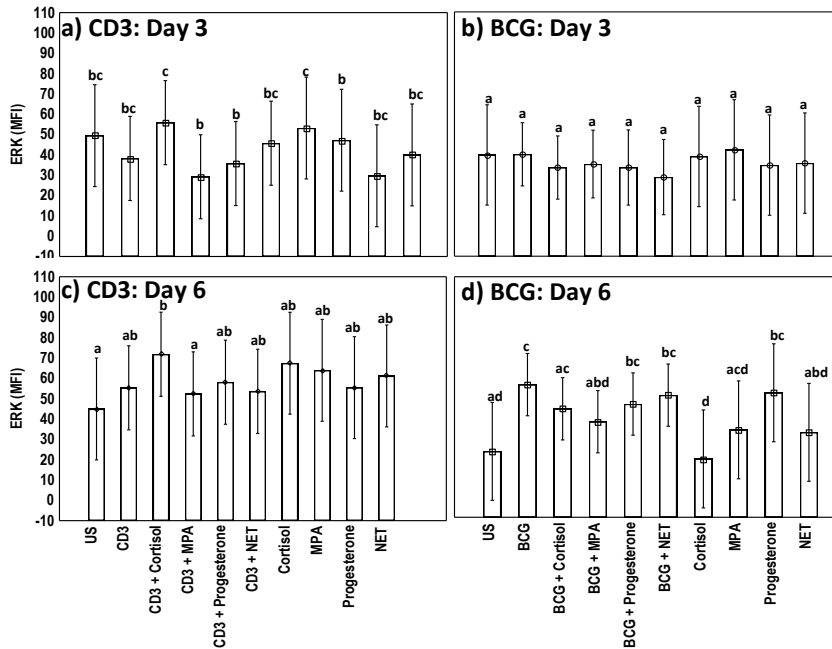


Figure 3.36: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on ERK 1/2 phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 8-plex Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

iii. p70S6 kinase

On day 3 (figure 3.37a), cortisol treatment enhanced the anti-CD3 mediated production of phosphorylated p70S6 levels in comparison with the effect of MPA and progesterone. The addition of NET to anti-CD3 stimulated cells enhanced the phosphorylation of p70S6 production, when compared to the inhibitory effect of cortisol, MPA and progesterone treated cells. Cortisol only had a significantly higher level of p70S6 when compared to all the other stimulatory conditions. On day 6 (figure 3.37c), MPA stimulation had significantly higher levels of phosphorylated p70S6 when compared all the other stimulatory conditions. Cortisol

treatment significantly enhanced the anti-CD3 induced p70S6 phosphorylation levels when compared to the anti-CD3 stimulated MPA treated cells.

On day 3 (figure 3.37b) stimulations for BCG had no distinguishable pattern and failed to reach any significant differences. On day 6 (figure 3.37d) BCG stimulated cells had significantly higher levels of phosphorylated p70S6 when compared to the cortisol, MPA, progesterone and NET only stimulations.

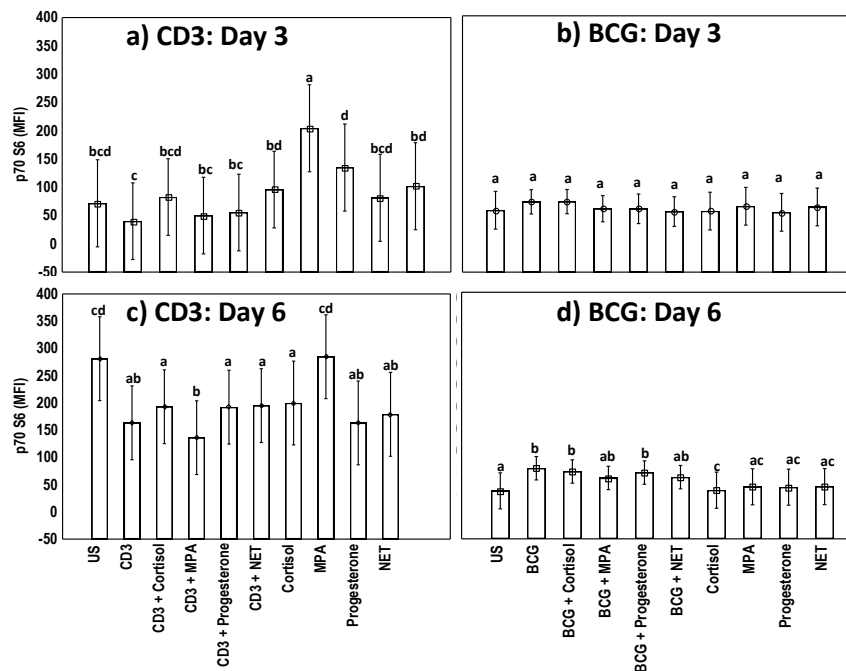


Figure 3.37: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on p70S6 phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 8-plex Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

iv. STAT3

Day 3 (figure 3.38a) post anti-CD3 stimulation there was no difference among any of the conditions. On day 6 (figure 3.38c), the treatment of anti-CD3 stimulated cells with cortisol, MPA, progesterone and NET significantly inhibited the phosphorylation of STAT3.

On day 3 (figure 3.38b) post BCG stimulation no distinct pattern could be identified for the different stimulatory conditions and none reached a level of significance. Day 6 (figure

3.38d) the BCG stimulated cells treated with cortisol, MPA, progesterone and NET had lower detectable levels of phosphorylated STAT3.

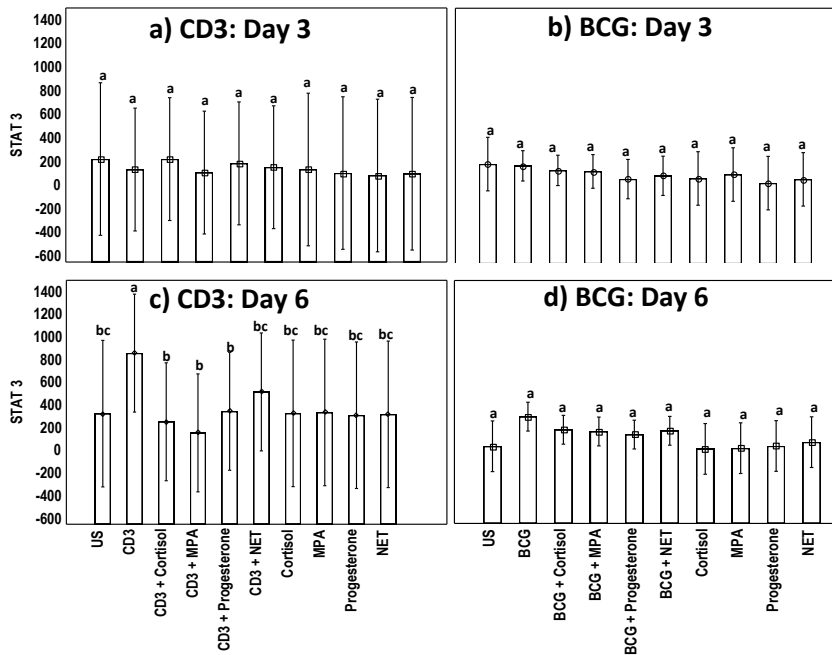


Figure 3.38: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on STAT3 phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M. bovis* BCG or $1 \mu\text{g/ml}$ anti-CD3 respectively, in the presence and absence of $10 \mu\text{M}$ of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 8-plex Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b and c indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

v. CREB

On day 3 (figure 3.39a) no levels of statistical significance were observed. Day 6 (figure 3.39c) the phosphorylation of CREB in the anti-CD3 stimulated cells was significantly inhibited by MPA treatment. The inhibition of MPA treatment on the anti-CD3 stimulated phosphorylation of CREB was significantly lower than in the cortisol, progesterone and NET treated cells.

Day 6 (figure 3.39d) the BCG stimulated cells had significantly higher CREB phosphorylation than the other stimulatory conditions, except for the MPA and progesterone treated BCG stimulated cells.

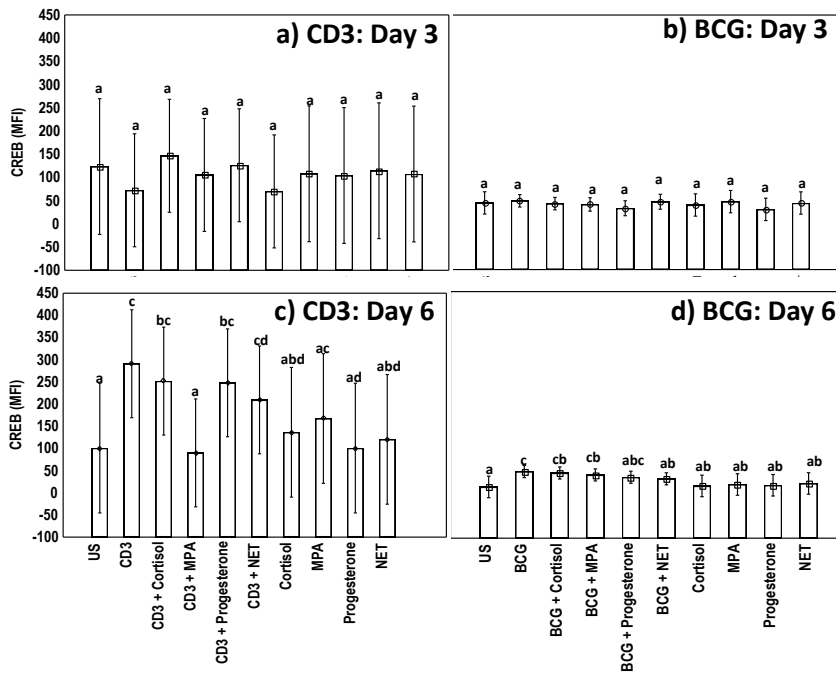


Figure 3.39: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on CREB phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or 1 μ g/ml anti-CD3 respectively, in the presence and absence of 10 μ M of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 8-plex Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

vi. p38 MAP kinase

Day 3 (figure 3.40 a) the anti-CD3 induced effect of p38 phosphorylation was enhanced by the cortisol treatment as it had higher phosphorylated p38 when compared to the anti-CD3 stimulated MPA and progesterone treated cells. In the hormone only stimulations the progesterone only treated cells had lower p38 phosphorylation compared to the cortisol, MPA and NET only treated cells. None of the differences that were observed reached a level that was significantly different. On day 6 (figure 3.40c) the anti-CD3 stimulated cortisol treated cells had significantly higher phosphorylated p38 when compared to anti-CD3 stimulated cells. The addition of cortisol enhanced the anti-CD3 induced effect on p38 when compared to the p38 phosphorylation of anti-CD3 stimulated cells where MPA, progesterone and NET was added to the cells.

Day 3 (figure 3.40b) no significant differences were seen in the levels of phosphorylated p38. Day 6 (figure 3.40d) the addition of cortisol, MPA, progesterone and NET to BCG stimulated cells significantly suppressed the p38 phosphorylation.

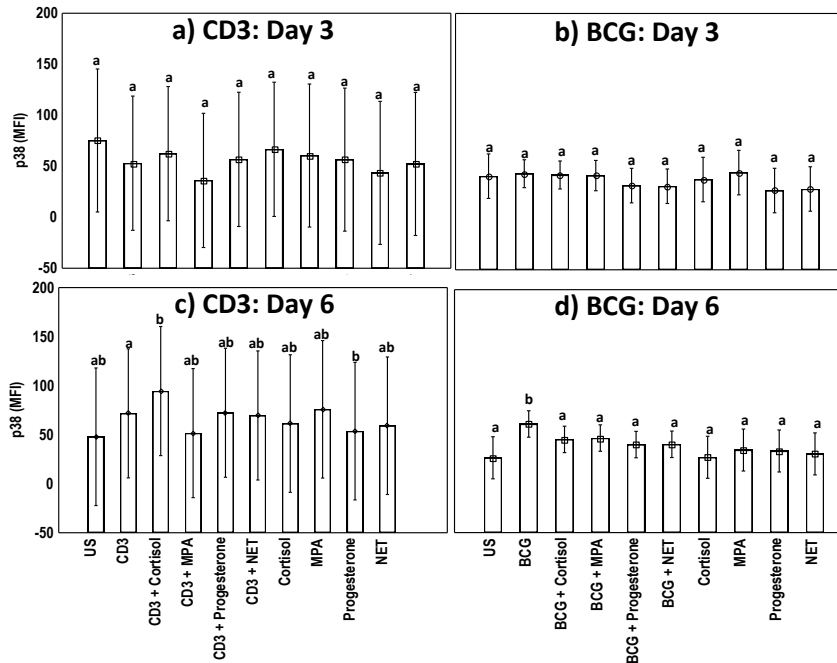


Figure 3.40: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on p38 MAP kinase phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or 1 μ g/ml anti-CD3 respectively, in the presence and absence of 10 μ M of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 8-plex Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, and b indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

vii. JNK

Day 3 (figure 3.41a) no significant differences were seen for the anti-CD3 induced effects on phosphorylation of JNK. Day 6 (figure 3.41c) MPA significantly suppressed the JNK phosphorylation in anti-CD3 stimulated cells when compared to the effect of cortisol and NET treatment on anti-CD3 stimulated US cells. The four hormone only stimulations produced overall less JNK than the anti-CD3 induced effects.

On day 3 (figure 3.41b), no significant differences were seen for the BCG induced effects on phosphorylation of JNK. Day 6 (figure 3.41d) the level of phosphorylated JNK was significantly suppressed by the addition of cortisol, MPA, progesterone and NET to BCG

stimulated cells. The hormone only stimulations had significantly lower JNK phosphorylation when compared to the BCG induced effects in stimulated cells that were comparable to the US value.

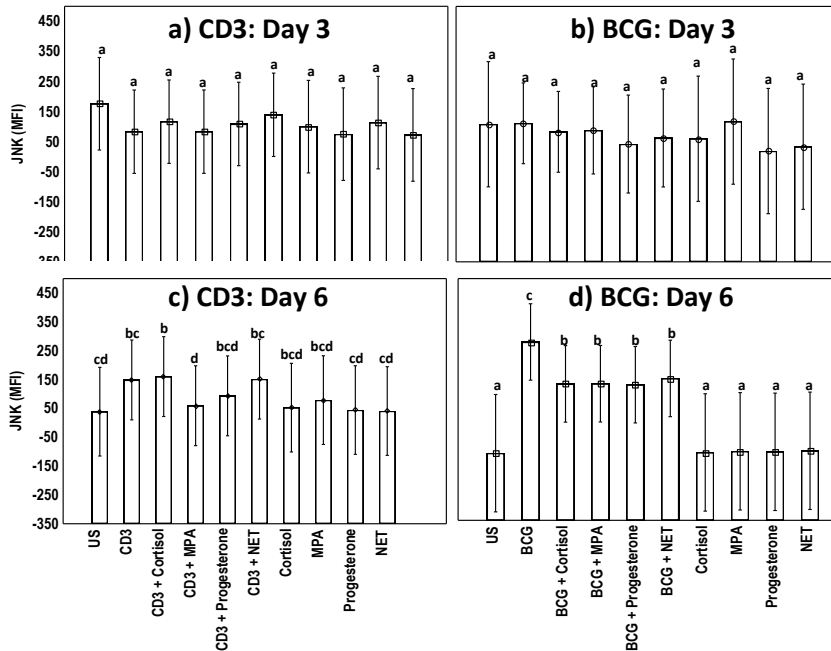


Figure 3.41: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on JNK phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or 1 μ g/ml anti-CD3 respectively, in the presence and absence of 10 μ M of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 8-plex Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

viii. $\text{I}\kappa\text{B}\alpha$

Day 3 (figure 3.42a) no statistically significant differences were observed for this time point.

Day 6 (figure 3.42c) the addition of MPA and progesterone to the anti-CD3 stimulations led to the significant suppression of the $\text{I}\kappa\text{B}\alpha$ phosphorylation levels when compared to anti-CD3 induced enhancement seen by cortisol.

Post BCG stimulation for both the day 3 and day 6 time points (figure 3.42b, d) no significant observations can be made regarding the observed levels of phosphorylated $\text{I}\kappa\text{B}\alpha$.

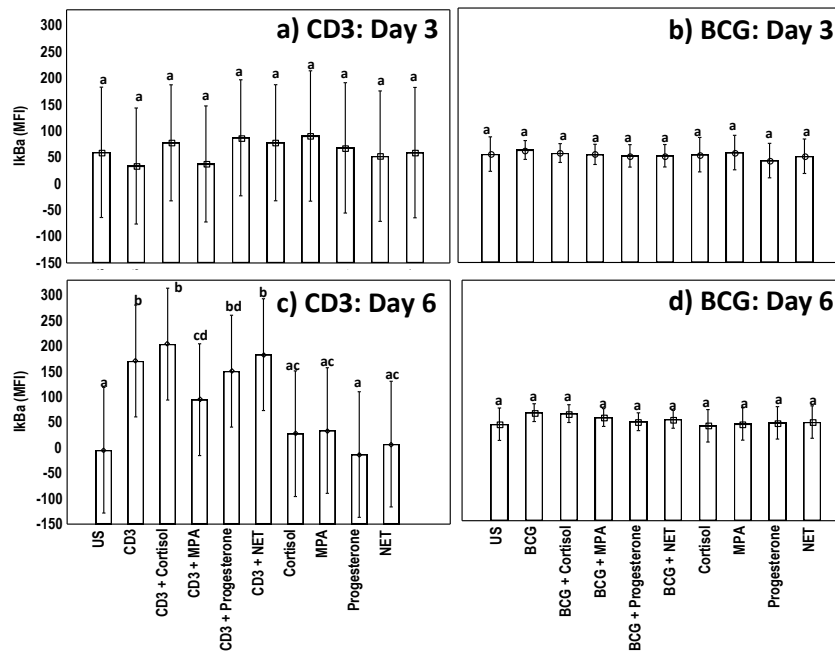


Figure 3.42: The effect of cortisol, MPA, progesterone and NET on BCG- and anti-CD3-induced on IκBα phosphorylation.

PBMCs (1×10^6 cells/well) were cultured and stimulated with a MOI of 5:1 *M.bovis* BCG or 1 μg/ml anti-CD3 respectively, in the presence and absence of 10 μM of the hormones, cortisol, MPA, progesterone and NET. The kinase expression was measured in the cell lysates harvested on day 3 and day 6 post stimulation, via a 8-plex Luminex panel. The data was analyzed using a one-way ANOVA with a Fisher LSD *post hoc* test and presented as least-squares means and 95% confidence intervals. The letters a, b, c and d indicate statistical significance, where values with the same letter are not significantly different from each other. A *P* value of 0.05 was regarded as significantly different.

Summary:

The introduction of BCG infection led to the increase in the phosphorylation and activation of STAT5A/B, ERK 1/2, p70S6, STAT3, p38 and JNK signalling molecules. Stimulation with anti-CD3 caused the inhibition of phosphorylation of p70S6, but led to the increase in phosphorylation for STAT3, p38, JNK and IκBα molecules. The addition of hormones led to the inhibition of phosphorylation for the kinases and transcription factors. The addition of cortisol, MPA, progesterone and NET led to the suppression of STAT5A/B, ERK 1/2, STAT3, CREB, p38 and JNK phosphorylation. The addition of hormones to led to the increase in p70S6 phosphorylation in the presence of MPA, progesterone and NET. IκBα phosphorylation of the anti-CD3 induced production was increased in the presence of cortisol, progesterone and NET but suppressed in the presence of MPA, progesterone and NET only. For the signalling pathways the synthetic progestins exhibit actions that mimic the glucocorticoid activity seen in cortisol, however at times the synthetic progestins exerts progestogenic activity seen in progesterone treated cells.

Chapter 4: Discussion

4.1 Effect of hormones on cytokine secretion in BCG infected PBMCs

The aim of the project was to investigate the immune modulatory properties of the synthetic progestins MPA versus NET. Both of these synthetic progestins were designed to mimic the endogenous hormone progesterone, but only MPA possesses selective glucocorticoid activity (52). To achieve this aim PBMCs from non-hormone contraceptive users were stimulated with BCG and CD3 in the presence of high concentrations (10^{-5} M) of cortisol, MPA, progesterone and NET. The effects of MPA and NET were compared to the endogenous hormones cortisol and progesterone, as MPA binds with a high affinity to and activates both the glucocorticoid and progesterone receptors. The inhibitory effect of glucocorticoids on inflammatory proteins is proposed to be primarily mediated through trans-repression. The decrease in transcription through the occurrence of trans-repression has been reported for a number of cytokines and chemokines which include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-11, IL-12, IL-13, IL-16, IL-17, IL-18, TNF α and GM-CSF, IL-8, MIP-1 α , MCP-1, MCP-3, MCP-4 and eotaxin (31). This study shows that the presence of the hormones, cortisol and MPA, altered the BCG induced and CD3 induced cytokine secretions of IL-1 α , IL-1 β , IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IFN- γ , and TNF- α . Progesterone and NET did not show the same degree of inhibition in cytokine secretion as was seen with cortisol and MPA. The presence of cortisol, MPA, progesterone and NET enhanced the secretion of G-CSF, possibly through trans-activation via a GRE. These results suggest that MPA has the potential to alter cytokine expression to the same extent as cortisol, whereas NET seems to mimic progesterone rather than cortisol.

From my stimulations it was seen that cortisol and MPA in both the BCG induced and CD3 induced cytokine secretions at both the day 3 and day 6 time points showed inhibition of cytokine secretions for IL-1 α , IL-1 β , IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IFN- γ and TNF- α . This has the potential to affect the immune response of MPA users during infectious diseases, as the inhibition of cytokines secretion during infection could prove to be detrimental to the host, as cytokines play a key role in the immune response.

The inhibitory effect that glucocorticoids exhibit on cytokine secretion has been shown previously in the following cytokines IL-2, IFN γ , IL-1 β , IL-8, TNF α , IL-4, IL-5, IL-6, IL-10,

IL-13, IL-17 as well as G-MCSF, where the addition of DEX inhibited the PHA induced cytokine secretion in whole blood supernatants (69). The inhibition of IL-1 α , IL-17, IL-6, IL-2, IFN- γ , IL-5, GM-CSF, MIP-1 β secretion by the hormones, cortisol and MPA but not progesterone, in BCG induced stimulations was shown previously (67). It has been shown that DEX and MPA leads to GR-mediated trans-repression of the IL-2 promoter in human lymphocytes (58). The effect of DEX and MPA in human lymphocytes stimulated with phorbol ester and ionomycin, displayed that DEX and MPA inhibited the phorbol ester and ionomycin induced IL-6 and IL-2 production, whereas progesterone only partially inhibited the secretion of IL-2 and IL-6 (58). The inhibitory effect of MPA is therefore just as strong as DEX whereas progesterone had little immunosuppressive effect compared to that of MPA.

MPA was synthesised to resemble progesterone, however it possess selective GC properties, thus it not only binds with a high affinity to the PR but also to the GR (70). NET has negligible binding affinity for the GR in comparison with MPA (70). The binding affinity of MPA to the GR could be the reason why it inhibits the induced cytokine production of IL-1 α , IL-1 β , IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IFN- γ , and TNF- α , as was also observed for cortisol in my results. In my experiments the secretion of G-CSF was enhanced in PBMCs stimulated with BCG, together with cortisol when compared to the BCG only response. This result confirms previous observation (67). In progesterone and MPA stimulated endometrial stromal cells, the enhancing effect in G-CSF production was observed previously (71).

In my study an inhibition of the BCG and CD3 induced cytokine production by cortisol and MPA in IFN- γ , IL-12, IL-1, IL-6, IL-10 and TNF- α was seen. The inhibition of these cytokines can be detrimental to the wellbeing of MPA users. The following studies show the role of the different cytokines within the immune response.

i. IFN- γ

The part that IFN- γ plays with regard to M.tb infection is the ability of IFN- γ to activate the macrophages capability to inhibit the growth of mycobacteria (3). IFN- γ stimulates the release of TNF- α which assists in the inhibition of the mycobacterium, by the production of ROS from the IFN- γ activated macrophages (21). It has been found that individuals who present with lower levels of IFN- γ and IL-12 have a larger risk of acquiring active disease (4). A study done on patients who had been radiographically classified as presenting with severe clinical disease exhibited low levels of circulating IFN- γ in peripheral blood that was

associated with a severe state of disease (20). Within my study results I see that the secretion of IFN- γ is inhibited by cortisol and MPA in both the CD3 and BCG stimulated cells. The inhibition on the secretion of IFN- γ is not seen with progesterone and NET. This indicates that in the case of IFN- γ , MPA possesses the immunosuppressive effects similar to cortisol. This suppression of IFN- γ in response to mycobacteria could render women using MPA more susceptible to mycobacterial diseases including TB.

ii. IL-12

IL-12 plays a role in creating and maintaining the host's protective immune response (14). It has been shown that IL-12 plays an important role in TB, as individuals with a congenital defect in IL-12p40 or in the β 1 subunit of the IL-12 receptor, have an increased susceptibility to mycobacterial infections (14).

A study was done in a murine model to determine the importance of IL-12 within the context of M.tb infection (16). Administration of IL-12 therapy to CD4^{-/-} mice after M.tb infection, revealed a reduced bacterial burden in the lungs of the mice along with an increase in the overall survival. This alludes to the fact that IL-12 is beneficial in infectious disease models that require a Th-1 immune response (16). The model showed that the administration of IL-12 therapy increased the expression of IFN- γ at an early time point, 10 days post M.tb infection. However after 1 month there was a notable reduction in the frequency and number of IFN- γ producing T-cells present in the lungs. This led to the hypothesis that the initial peak in IFN- γ production from the exogenous IL-12 may be the cause of the overall enhanced outcome of the CD4^{-/-} mice (16).

In another murine study, an IL-12p40^{-/-} mouse model infected with M.tb was used to determine the capability of the mice to control the bacilli and determine the effects of the knock out on the immune response (17). The absence of IL-12p40 resulted in the uninhibited growth of M.tb bacilli in all target organs after a systemic infection. The growth correlated with a reduction in the expression of mRNA of IFN- γ , this was also reflected in the decrease in nonspecific as well as antigen specific IFN- γ protein production. The lack of IFN- γ production was linked to the delay in producing antigen specific T-cell activation within the mice (17). They concluded that IL-12 plays a key role in the generation of antigen-specific T cells that are able to produce IFN- γ . From the experiments conducted they projected that the initial interaction of the bacilli with the host induces IFN- γ production. The amount of IFN- γ

that is produced is dependent on IL-12 and TNF- α . A large production of IFN- γ is necessary to induce the expression of the IL-12R β 2 chain on naïve T cells. The expression of the receptor on the naïve T cells is needed for the T cells to act in response to the IL-12 and thus become antigen-specific IFN- γ producing cells (17). A study conducted in individuals with genetic defects in their IL-12/IL-23/IFN- γ system, found that the system deficiencies selectively enhanced their susceptibility to mycobacteria. This led to the conclusion that the IL-12/IL-23/IFN- γ system plays an important role in immunity against mycobacteria (72). In my results the BCG induced secretion of IL-12p40 and IL-12p70 were inhibited in by the addition of cortisol and MPA in the stimulations. The inhibition was not seen with NET. Thus women using MPA might have an increased susceptibility to M.tb infection, as a result of the inhibition of IL-12.

iii. IL-1

IL-1 is essential for the control of M.tb infection, however the regulation *in vivo* of the two IL-1 isoforms, IL-1 α and IL-1 β , and their exact role are not clearly defined (73). Guler *et al.* studied the role of IL-1 α and IL-1 β in chronic infection with M.tb and spontaneous reactivation of it in a mouse model. Blocking IL-1 α , but not IL-1 β , leads to an increased susceptibility to chronic infection with M.tb (74). When neutralizing IL-1 α or IL-1 β alone, there was no observed increase in the reactivation of latent tuberculosis in the mice. The generation of antibodies neutralizing IL-1 α and IL-1 β simultaneously led to an observed increase in the lung bacilli count when compared to the immunized control group. These results suggested that IL-1 α is the prime mediator of the IL-1RI-dependent and protective innate immune responses to M.tb in mice (74). During my study the addition of cortisol and MPA led to the significant inhibition of both IL-1 α and IL-1 β in the BCG and CD3 stimulated cells. The GC inhibiting effect on IL-1 production has been shown previously in whole blood where the addition of DEX resulted in the inhibition of LPS induced IL-1 production (75). In PBMCs stimulated with BCG at both time points, cortisol significantly inhibited the secretion of IL-1 β (67). This confirms my results. Thus it can be speculated that the use of MPA with the associated decrease in IL-1 could potentially lead to the increased susceptibility to M.tb infection and poor bacilli control as was seen in the murine model (74).

iv. IL-6

It is known that monocytes respond to *M.tb* stimulation by producing IL-6, TNF- α , IL-1 β and IL-10. However, the addition of glucocorticoids inhibits the production of IL-1, IL-6, IL-10, IL-13, IFN γ , and TNF α (69). IL-6 is also critical for immunity against *M.tb*. The increased susceptibility to *M.tb* of the IL-6 deficient mice seemed to be associated with a deficient IFN γ production during the early phase of infection. A correlation has been found in TB pleural patients showing a positive association between levels of IL-6 and IL-1 β with IFN γ concentrations in their peripheral and pleural compartments respectively (76). The inhibition of the cytokine production in my experiments was observed for both time points, the BCG induced and CD3 induced IL-6 secretion was inhibited by cortisol and MPA addition. Progesterone and NET had negligible inhibition on the BCG and CD3 induced IL-6 secretion. The lack of IL-6 with MPA users can potentially increase susceptibility to *M.tb* infection, this fits with the decrease in IL-1 also associated with MPA.

v. IL-10

IL-10 is produced by macrophages and T lymphocytes during *M.tb* infection, and is considered to primarily be an inhibitory cytokine, important for limiting inflammatory responses which could result in immunopathology. IL-10 down-regulates effector functions of monocytes/macrophages and DCs and reduces inflammatory responses by inhibiting the expression of MHC class II molecules, phagocytosis, the production of NO and reduces the production of the pro-inflammatory cytokines IL1 β , IL-8, IL-12, IFN γ and TNF α . It has been shown previously that mice deficient in IL-10 display unregulated inflammatory responses when exposed to LPS. This reveals the key role of IL-10 in keeping a balance in the immune system and in protecting the host from excessive immunopathology (77). Another study showed that an increase in IL-10 levels appears to support the mycobacterial survival in the host. Mice with defective IL-10 exhibit an increase in the anti-mycobacterial immunity (78). Beamer *et al.* conducted a mouse study that found that IL-10 reduces the protective response to *M.tb* in the CBA mouse strain, in which IL-10 is produced by phagocytes in the interior of the pulmonary lesion, where a reduction in the TNF and IL-12p40 expression can be observed (79). This was also seen in human PBMCs where it was shown that IL-10 inhibits the expression of IL-12p40 in PBMCs at transcriptional level. Thus IL-10 restrains clearance of the pathogen causing chronic infection (80). The inhibition of the cytokine production was seen in my study at both time points, for the BCG induced and CD3 induced IL-10 secretion

by cortisol and MPA addition. Thus the decrease in IL-10 secretion by MPA suggests that MPA has an overall inhibitory effect and affects Th-1 and Th-2 responses.

vi. TNF- α

Lymphocytes, endothelial cells and the monocytes and macrophages are all target cells for the immunosuppressive and anti-inflammatory actions of the GCs. The immunosuppressive effects on the monocytes and macrophages include the inhibition of IL-1 β and TNF- α secretion (81). Joyce *et al.* showed that stimulation with DEX led to the inhibition of TNF- α and IL-1 β secretion from monocytes as well as suppressed the secretion of sTNF-R75, sTNF-R55 and IL-1ra (81). TNF- α blocking has dramatic effects on the progression of tuberculosis in experimental models. Neutralization of TNF- α in murine models results in tuberculosis reactivation (82). Studies have also revealed that TNF- α is expressed in *M.tb* infected tissues during the latent phase of infection, suggesting a contribution, with other cytokines like IFN- γ , in the control of the bacteria (83). The immunosuppressive effect of cortisol and MPA on the BCG induced production of TNF- α , but not progesterone has been shown in PBMCs (67). This confirms the inhibition of TNF- α secretion by cortisol and MPA in the BCG induced and CD3 induced stimulations seen during my study. Progesterone and NET showed negligible inhibition of TNF- α in the BCG induced stimulations, this confirms the result seen by Kleynhans *et al.* During mycobacterial infection the TNF- α plays a key part in the host protective response by activating macrophages along with IFN- γ , TNF- α is mostly involved in the bacterial control and the formation of granuloma lesions (84). The inhibition of TNF- α in MPA users will be detrimental upon contact with infectious diseases, as the lack of TNF- α will prevent the macrophages from functioning optimally.

4.2 Effect of hormones on receptor expression levels in BCG infected PBMCs

The aim was to investigate the presence of the various receptors GR, PR, AR, mPR- α , mPR- β , mPR- γ and PGRMC1 in the PBMCs, to determine whether they can be potential targets for the binding of the endogenous hormones as well as the synthetic progestins. The role of GCs in the immune system is to modulate a wide variety of immune cell functions, the expression of cytokines, adhesion molecule expression, immune cell trafficking and the maturation, differentiation and production of inflammatory mediators (85). One general action of GCs is the suppression of inflammatory responses.

Glucocorticoids can diffuse across cell membranes where they are able to bind to GRs located in the cytoplasm. The GR binds to the chaperone molecules such as Hsp90, resulting in no signal transmission (27). Once ligand has bound to the GR, the GR changes its conformation and it will dissociate from the chaperone molecules revealing the nuclear localization signal of the receptor. The ligand-receptor complexes dimerize, and translocate to the nucleus where they can bind to GREs in the promoter regions of GR regulated genes where they are capable of altering the expression of steroid-sensitive genes in various ways (31). Upon the binding of the receptor homodimer to the GREs an increase in gene transcription occurs, known as trans-activation. A monomeric ligand-receptor complex changes gene expression through the process of protein to protein interactions with transcription factors which is referred to as trans-repression (27). The GR is associated with the TCR complex by direct protein-protein interaction with Lck/Fyn. In the presence of a GC the GR changes its conformation and dissociates from the TCR complex thereby inhibiting the TCR signalling (33).

i. GR

GR protein was detected in normal human lymphocytes as well as GR mRNA levels in lymphocytes and in Jurkat cells (58). The authors wanted to determine whether the dissociative GC-like effects of MPA (i.e. strong trans-repression, weak trans-activation) in human lymphocytes could also be seen in the other steroid receptors like PR and AR. MPA treatment produced noticeable trans-repression of IL-2 gene expression similar to that of hydrocortisone and DEX in the human lymphocytes and in Jurkat cells. While the MPA-mediated trans-activation of pGL3-GRE-tk81-luciferase, a DEX-inducible GRE-dependent promoter, was found to be minimal. However, based on the expression studies the authors concluded that the observed effects of MPA were mediated by GR, as in human lymphocytes they only detected GR at protein level in the cells, but not PR or AR (58). Some reports of increased GR levels, have been linked to an increase in the efficacy of ligand. However, it is speculated that the increase in GR concentration will result in enhanced potency of GR-mediated transcription (86). In addition, a shift from non-cooperative to positive cooperative ligand-binding as GR levels increase has also been shown *in vitro*, which would suggest an increase in affinity. However, the relationship between GR levels and ligand-binding affinity is controversial with some studies have indicated that increased GR levels result in increased

affinity, while others find a decrease in affinity (86). My study results I was able to detect the GR at both mRNA level as well as protein level. The detectable mRNA of the CD3 stimulated cells treated with cortisol and MPA at day 3 were higher than those treated with progesterone and NET, indicating that cortisol and MPA up-regulate GR levels. Whether the up-regulation that is seen is due to the increase in GR levels or the efficacy of the cortisol and MPA as ligand, needs to be determined. The decrease in the cytokine expression suggests that the cortisol and MPA must have interacted with the GR to induce the immune modulatory effects that are seen in the inhibition of cytokine expression. This is also evident in the downstream signalling molecules that are also inhibited with the addition of cortisol and MPA to stimulated cells for example, Lck, ZAP-70, LAT, STAT3 and JNK all show decrease in phosphorylation levels.

ii. PR

Progesterone elicits a variety of functional effects on immune cell types representing multiple hematopoietic lineages including alteration of cytokine gene transcription, change in ion transport, and inhibition of nitric oxide production (87). PR expression within human lymphocytes, which would be a requirement for any PR-mediated effect, is debatable. No detectable PR at mRNA or protein levels was found within unstimulated or stimulated human lymphocytes. It was thought that if PR is expressed in the human lymphocytes the levels are too low to apply any significant effect (58). A comparative study conducted between infants and adults found that, mPR- α , and mPR- γ mRNA levels were present at higher levels in cord blood mononuclear cells (CBMCs) than in adult PBMCs, while PGRMC1 was found only in CBMCs. The mRNA levels of PR were not detected in CBMCs nor the adult PBMCs, thus suggesting that progesterone acts through membrane PRs in CBMCs and PBMCs (68). In contradiction with the findings of Bamberger *et al.* (58) and Giannoni *et al.* (68) others found the PR was detectable at protein level in the cells by Western blot (88) However, my findings can corroborate the abovementioned findings, as I also could not detect the PR at protein levels in the PBMCs. The level of expression is too low for the detection limit of the Western blot. The PR levels were however detectable at mRNA level, though the levels of detection were low. This suggests that the signalling via the PR is not necessarily through the nuclear receptor but rather through the membrane receptors.

iii. AR

Bamberger *et al.*, found that the lymphocytes expressed AR at mRNA level, but the AR protein expression was found to be below the detection limit of Western blot experiments. This led them to conclude that AR does not play a role in facilitating the effects of MPA in human lymphocytes, as its expression is below the detection limit of Western blot used. Their experiment done using dihydrotestosterone, a stronger AR agonist than MPA, had no effect on IL-2 promoter activity in the lymphocytes (58). From my study results it can be seen that the NET only stimulation had high expression level of AR at both day 1 and day 3. This increase in AR expression levels could be due to the progestin NET being structurally related testosterone (89). As testosterone has the ability to bind to the AR, the similarities in structure could be why the stimulation with NET displayed an increase in the expression of the AR.

iv. mPR's

Studies propose that progesterone acts on immune cells through a non-genomic mechanism, likely at or near the plasma membrane. Studies done in human peripheral blood cells and isolated T cells from female donors and Jurkat cells showed expression at mRNA level for mPR α and mPR β , but not mPR γ nor PR (46). Contradicting results were reported by Chien *et al.*, observing mRNA levels in peripheral blood cells from male donors for mPR α , mPR β , and mPR γ (90). In a study it was found that the radiolabeled progesterone bound to the cell membranes of T lymphocytes and Jurkat cells with an affinity suitable for steroid receptor binding (46). The addition of progesterone to Jurkat cells lead to the activation of an inhibitory G protein. Many of these effects occur in cells that seemingly lack PR, potentially implicating mPRs as the signal initiators. However, limited research put forward that progesterone binding to mPRs produces functional effects in immune cells similar to those in Jurkat T cells, where progesterone stimulates the activity of inhibitory G proteins (46). The mPR- α has shown no progestin induced signalling changes no cAMP decrease, no ERK 1/2 activation and calcium mobilization. However, G-protein coupling has been seen in myometrial cells (reviewed in (48)). Human mPRs appear to not signal through ERK1/2 but activate p38 MAPK. Though, this speculation was only based on the observation that treatment of cultured human myometrial cells with cell-impermeable progesterone-BSA led to rapid phosphorylation of p38, but not ERK1/2 (45). In my study I showed that the NET had high mRNA levels for mPR α , mPR β and mPR γ at day 1 and day 3. MPA had high

mRNA levels for mPR α and mPR β at day 3, mPR γ showed very high mRNA levels at day 6. Progesterone showed high mRNA levels for mPR α , mPR β and mPR γ throughout day 1,3 and 6. The CD3 and hormone stimulations, had overall lower expression for all three mPR mRNA levels. Whereas, the three hormones, MPA, progesterone and NET, on their own showed an increase in mRNA levels detected for the mPRs. This could indicate that the hormones by themselves can signal via the membrane receptors without the stimulatory condition required, such as BCG and CD3. Both of the signalling molecules speculated within the literature to play a role in the signalling of mPRs, ERK 1/2 and p38 were inhibited by MPA, progesterone and NET within my findings. The inhibition of these signalling molecules indicates a decrease in the phosphorylation of the signalling molecule and the kinase remaining inactive. This could imply that in blood cells the mPRs do not signal the same way as is speculated in the breast cancer and reproductive cell models.

v. PGRMC1

In a porcine model the expression of PGRMC1 was looked at in the liver cells. It was found that although progesterone bound with highest affinity among the steroids tested to the PGRMC1. From the EC₅₀ it could be seen that cortisol exhibited 4% of the observed binding affinity. The authors suggest that under physiological conditions in the liver cells the PGRMC1 binding could be occupied with cortisol rather than progesterone due to the higher circulating concentration of cortisol (91). It has been reported previously that the PGRMC1 possesses a GRE, however it does not have a PRE (92). It has been suggested previously that women who have pre-existing breast cancer cells overexpressing PGRMC1 have an increased risk of developing breast cancer when receiving hormone therapy composed of estrogen or estrogen and progesterone combined (89). The type of progestogen used within the treatment seems to play a role. Using a progestin that is related structurally to testosterone for example NET, seem to be more tumorigenic than Progesterone related derivatives like MPA, which are less likely to stimulate the development of precancerous cells to tumors. The authors obtained these results by using cells that expressed raised levels of exogenous PGRMC1 (89). It can be seen from my results that at day 3, that the levels of detectable mRNA for MPA, progesterone and NET are high, as well as the progesterone CD3 induced levels of mRNA for PGMRC1. This remains the case at day 6, however now it is no longer the progesterone CD3 induced levels that are high, but rather the cortisol CD3 induced levels, with cortisol having the largest mRNA level in comparison with MPA, NET and progesterone. As it has been

shown previously in breast cancer cells that the synthetic progestins do bind to PGRMC1, this could be the scenario within the PBMCs as well, that the hormones bind to the PGRMC1. The increase in the mRNA levels in the presence of cortisol could potentially be due to the GRE found in the PGRMC1, or could potentially be attributed to the fact that cortisol can bind to the PGRMC1 thus could possibly signal through this receptor.

4.3 Effect of hormones on phosphorylation of signalling molecules in BCG infected PBMCs

The aim of the study was to investigate the effect of the endogenous hormones, cortisol and progesterone, and synthetic progestins, MPA and NET, on the BCG induced and CD3 induced phosphorylation of signalling molecules. Kinases phosphorylate proteins and phosphatases dephosphorylate proteins. Many enzymes and receptors are turned "on" or "off" by the process of phosphorylation and de-phosphorylation. Reversible phosphorylation results in a conformational change in the structure seen in many enzymes and receptors, causing them to become activated or deactivated. Phosphorylation usually occurs on serine, threonine, tyrosine and histidine residues in the protein molecules (93).

i. Lck

Lck is a protein found within lymphocytes. Lck is a tyrosine kinase and a member of the Src family, Lck phosphorylates the tyrosine residues of some of the proteins involved in the intracellular signalling pathways of the lymphocytes. Harr *et al.*, has shown that Lck protects cells from glucocorticoid induced apoptosis. In the T cells it was found that the Lck was downregulated by DEX and thus inhibits TCR activation (94). It has also been shown that glucocorticoids rapidly inhibit Lck by a non-genomic mechanism involving the interaction between the TCR complex and the ligand bound GR (33). Differences in the phosphorylation patterns of DEX-treated and non-DEX-treated cells, showed there was a decrease in phosphorylation of Lck/Fyn substrates in the DEX-treated group as well as an impairment of recruitment of these substrates to the TCR (33). Harr *et al.*, was the first to show that DEX downregulate, Lck transcripts as well as protein levels in a GR-dependant way (94). Numerous proteins forming part of the TCR signalling pathway were downregulated by DEX, ZAP-70 protein levels were decreased 24hours post DEX treatment, they found LAT protein to be downregulated too. This led to the conclusion that glucocorticoids are capable

of inhibiting the TCR signalling pathway by negatively regulating proteins in the pathway (94). From my study it can be seen on day 3 and day 6 that the addition of hormones (cortisol, MPA, progesterone and NET) to BCG had lower Lck phosphorylation levels than compared to the US sample group. Day 3 the CD3 induced phosphorylation levels of cortisol and cortisol on its own were inhibited more than was seen by MPA, progesterone and NET. However, by day 6 the CD3 induced Lck phosphorylation was significantly inhibited by cortisol, MPA, progesterone and NET, as well as cortisol, MPA, progesterone and NET. The initial signalling data at Day 1 showed no differences amongst stimulations. My results do not reflect the rapid non-genomic inhibition of TCR as was seen by Lowenberg *et al.* as my time points that were selected were too late. The possibility of a genomic effect is plausible as the effects of inhibition of phosphorylation are seen on day 3 and day 6 for CD3 stimulations. However, absent at day 6 in the BCG stimulations. CD3 stimulations were done specifically to activate the TCR signalling cascade, whereas BCG was chosen as a nonspecific antigen. Thus the lowering of Lck in the hormones treated cells stimulated with CD3 can be a result of genomic interactions of the hormones.

ii. ZAP-70

ZAP-70 is a member of the protein-tyrosine kinase family, it is a protein that is expressed near the membrane of T cells and natural killer T cells. It forms part of the TCR, and plays an important role in T-cell signalling. In Jurkat cells short term DEX exposure led to the phosphorylation of ZAP-70, and enhanced the association between the ZAP-70 and GR as a result of non-genomic glucocorticoid effects. It was also observed that the DEX exposure decreased the CD3 induced phosphorylation of ZAP-70 (95). In P116 cells (ZAP-70-deficient Jurkat subclone) that were lentivirally-transfected with wild type or point-mutated ZAP-70 constructs were exposed to a short term, high dose DEX treatment. Led to decrease in ZAP-70 phosphorylation in the 315-tyrosine and 492- tyrosine ZAP-70 expressing cells. This alluded to the involvement of these two tyrosine residues of the ZAP-70 kinase to be involved in the non-genomic GC signalling (96). Studies in antigen-specific murine CD4⁺ T cells and primary human T cells and found that M.tb cell wall glycolipids, mannose capped lipoarabinomannan (ManLAM) inhibited ZAP-70. Phosphorylation of signalling proteins were inhibited both upstream (Lck) and downstream (LAT) of ZAP-70 were inhibited by ManLAM (97). From my study it can be seen that at day 3 both the BCG induced and CD3 induced ZAP-70 phosphorylation levels were lowered by cortisol, compared to day 1 where

there was no decrease in the ZAP-70 phosphorylation observed in the CD3 induced results. Although my experiments were not short term exposure but long term (3 day) exposure I saw the same effect as was seen in high dose short term DEX exposed P116 Jurkat cells. Cortisol decreased the phosphorylation of the ZAP-70 as well as its upstream and downstream, Lck and LAT, signalling molecules. The decrease in the phosphorylation of ZAP-70 was not seen by MPA but NET.

iii. LAT

LAT is a membrane adapter protein that links the early biochemical events with the downstream signalling cascades needed for the activation of the MAPK cascade and the activation of ERK (98). Boldizar *et al.*, showed that LAT displayed increased phosphorylation after short term DEX exposure, this suggests the involvement of LAT in transmission of the non-genomic effects associated with ZAP-70 and downstream targets (96). Point mutations induced in ZAP-70 determined the downstream signalling effects, none of which affected the phosphorylation of LAT, this led them to conclude that the LAT was not involved in the non-genomic signalling of ZAP-70 (96). LAT can not only be phosphorylated by the ZAP-70 kinase, but also by Lck and it might be possible that non-genomic GC effects of LAT are induced through those molecules. The short term DEX exposure results are in contradiction with my results were I saw that at day 3 the CD3 induced LAT phosphorylation levels were significantly decreased by the addition of cortisol. The same effect was seen for the addition of NET. The androgenic effect of NET seemed to mimic GC effect seen in cortisol. Whereas MPA and progesterone had higher phosphorylation of LAT, than was observed for cortisol and NET. Day 3 of the BCG stimulations showed a decrease in LAT phosphorylation by cortisol and progesterone and, higher LAT phosphorylation by the synthetic progestins.

iv. STAT3

Zhang *et al.* through their transfection studies found a central role of STAT3 in synergistically enhancing glucocorticoid signalling (99). They hypothesised that STAT3 could interact directly with the GR, thus improving the trans-activation of each component through its responsive element. Through their co-immunoprecipitation experiments they identified that the STAT3-GR complex could be detected only in extracts acquired from cells co-stimulated with IL-6 and DEX (99). They were able to demonstrate the formation of a

STAT3-GR complex which was able to bind to the IL-6RE. They concluded that STAT3 acts as a potent co-activator of GRE-mediated transcription, independent of IL-6RE binding. Their findings reveal an interaction between IL-6 and GC signalling pathways, and could suggest that STAT3 may modulate other GC actions (99).

The synergistic relationship between GR and STAT3 was also seen by Unterberger *et al* (100). They wanted to find the possible mechanism leading to the involvement of STAT3 in GC induction of IL-10 gene expression, and whether the GR was involved. They found that using the synthetic steroid RU486 which is a GR and PR antagonist, they could completely block the IL-10 mRNA synthesis. This led to the deduction that there is a possibility that the GR directly interacts with the STAT3. This synergistic action is mediated by interaction of the GR protein with the STAT3 protein. One possible way this is achieved is by the binding of the GR to STAT3 as this will stabilize its DNA binding and retention in the nucleus. This implies that STAT3, transferring between cytoplasm and nucleus, is confined by its connection to the GR. Another option is that the GR leads to enhanced serine phosphorylation of STAT3 with subsequent nuclear translocation of STAT3 homodimers. Such as non-genomic mechanisms of GCs acting via the GR has been shown in PI-3 kinase and Akt (100). In breast cancer cells it was shown that MPA is able to induce STAT3 phosphorylation at serine727 and that the c-Src and ERK 1/2 MAPK signalling pathway is involved in this phosphorylation event (101). MPA is also able to induce STAT3 phosphorylation at tyrosine705 through the activation of the c-Src/Jak signalling pathway leading to *in vivo* cyclin D1 up-regulation and breast cancer growth (101). We see that on day 6 there is a significant decrease in the CD3 induced STAT3 phosphorylation levels in the cortisol, MPA, progesterone and NET stimulations. However the cortisol and MPA showed lower phosphorylation levels when compared to progesterone and NET. This means that there is less activated STAT3, yet in my cytokine data I see that the CD3 induced secretion of IL-6 and IL-10 are inhibited at day 6, this could be due to the cortisol and MPA activation of GR. The decrease in the cytokine production could potentially be due to the interaction of the STAT3-GR complex, and as cortisol and MPA bind with higher affinity to the GR compared to progesterone and NET, this could be why we see lower STAT3 levels as it is bound to the GR.

v. JNK

JNK belongs to the MAPK family, and is responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. They also play a role in T cell differentiation and the cellular apoptosis pathway. It has been suggested that this signalling pathway contributes to inflammatory responses in humans (102). In lung epithelial cells rhinovirus infection attenuates GR α nuclear translocation in response to DEX stimulation and showed reduced corticosteroid sensitivity. They found that the rhinovirus infection resulted in a JNK-dependent increase in GR Ser226 phosphorylation, responsible for the shuttling of GR α . They found that JNK-1 isoform activation is involved in rhinovirus-induced corticosteroid insensitivity, due to the JNK activation, but not a result of the induction which occurs quickly after virus stimulation. JNK activation, rather than increased expression of JNK-1, is likely involved in the rhinovirus induced corticosteroid insensitivity observed under the experimental conditions (102). Knock down of GR in L929sA cells shows that a decline in GR protein can partially revert the negative impact of DEX on STS and TNF-stimulated JNK phosphorylation, suggesting GR dependence. On day 6 it can be seen that the BCG induced JNK phosphorylation was significantly lower with the addition of cortisol, MPA, progesterone and NET.

vi. I κ B α

Crinelli *et al.*, found in monocytic cell lines exposed to DEX showed an increase in the intracellular concentration of I κ B α mRNA and protein. They found that the induction of I κ B α was activated and deactivated very rapidly both at the mRNA and protein levels (103). They suggested that the down-regulation of NF- κ B by glucocorticoids can be exerted through the protein-protein interactions of NF- κ B subunits and the GR, or through the induced synthesis of I κ B α . They concluded that DEX was able to interfere with the activation of the NF- κ B pathway and modulate TNF- α mRNA synthesis (103). Although within my results we see that on day 6 there is an increase in the CD3 induced I κ B α levels with the addition of cortisol, however the addition of MPA significantly reduced the CD3 induced I κ B α levels. This could potentially mean that the addition of cortisol acted in the same way as DEX, but not MPA. Within my results you can also see the TNF- α CD3 induced secretion was inhibited by the addition of cortisol and MPA, so this does indicate the possibility of the interference of the NF- κ B pathway activation.

vii. CREB and p38

My study indicated that both the time points that the phosphorylation of CREB and p38 was inhibited by the addition of cortisol, MPA, progesterone and NET. This could potentially indicate that the lack of p38 phosphorylation led to the inhibition of the CREB phosphorylation. In the BCG induced stimulations there is no change between the day 3 and day 6 CREB or p38 induced profiles. Previous literature has shown that both DEX and 17 beta-estradiol benzoate (E2b) induce CREB activation in CCRF-CEM cells (104). The authors found that both the cAMP antagonist Rp-8-Br-cAMPS, and the p38 MAPK inhibitor SB 203580 effectively prevent CREB activation and Annexin 1 (ANXA1) synthesis induced by DEX, E2b or the cAMP analogue dBcAMP. These results indicate that cAMP and p38 are both required for DEX-induced and E2b-induced CREB activation and ANXA1 synthesis, and are also strong evidence that CREB activation is required for ANXA1 synthesis in these cells. Both hormones required longer incubation periods for maximal response, suggesting that cAMP has to be produced, in response to both steroids, before CREB activation can occur in CCRF-CEM cells. The DEX induced and E2b induced ANXA1 expression requires the activation of the transcription factor CREB, which in turn seems to be mediated by cAMP and the p38. CREB, may play important roles in mediating the anti-inflammatory actions of GC and estrogen hormones (104).

Conclusion

The aim of my investigation was to determine the receptor involvement and the downstream signalling molecules affected by the addition of cortisol, MPA, progesterone and NET. The final aim was to determine the cytokine levels to determine whether the synthetic hormones mimic the immunosuppressive effect of cortisol, or mimic progesterone which they were created to resemble.

My study indicates that the GR could be detected at protein level in the PBMCs, but the PR was not present at protein level. The mRNA levels within the PBMCs indicate that the cells have mRNA expression for GR, PR, AR, mPR- α , mPR- β , mPR- γ and PCRMC1 receptors. Cortisol that was added to the cell stimulations resulted in an increase in GR expression levels. This could indicate that the availability of the ligand, led to the increase in the expression of the receptor, or the affinity of the receptor increased for the ligand as was suggested earlier. The addition of MPA and progesterone to the PBMCs increased the

expression levels of the GR, AR, mPR- α , mPR- β , mPR- γ and PGRMC1. The increase in the PR related receptors with the stimulation of MPA and progesterone, could potentially indicate that these are the receptors of choice for the signalling to occur through. However, both the PR and endogenous GR are present in all the cells, thus we cannot speculate that the effect will only be through the mPRs and PGRMC1 as the GR is still available for MPA to signal through. As for the membrane receptors and PGRMC1 this occurred for both the CD3 and BCG mediated expression. The expression levels of the PR were increased by the presence of progesterone only in the CD3 stimulations.

The signalling molecules showed inhibition by the addition of the hormones. The addition of cortisol inhibited the phosphorylation of Lck, ZAP-70, LAT, and cortisol increased the inhibition in the p70S6, p38 at day 6 and I κ B α . Cortisol, MPA, progesterone and NET inhibited the phosphorylation of ERK 1/2, STAT3, CREB, p38 and JNK. The inhibition of the phosphorylation by the hormones could indicate that the effects seen are the result of the genomic effect, as the non-genomic effect is rapid and the effects will not be detectable at the later time points.

The cytokine data indicates that there were immune modulatory effects that occurred within the stimulations. The addition of cortisol and MPA led to the inhibition of cytokine secretion such as IL-1 α , IL-1 β , IL-6, IL-10, TNF- α , IL-12 and IL-13. The suppression of the cytokines leads to a suppressed immune response upon mycobacterial encounter. Thus women who are using MPA as opposed to NET will possibly be more susceptible to infection, have poor bacilli control. The suppression of IL-10 will lead to the inhibition of proinflammatory cytokines such as IFN- γ , TNF- α and IL-12, thus harmful to the host. The failure to activate the secretion of IFN- γ by TNF- α will cause a lowering of the host's protective response.

Although we could not see throughout the results that MPA always mimicked cortisol as we hypothesised from the cytokine data. The cytokines play a big role in determining the outcome of the hosts immune response, thus the inhibition of cytokines by MPA as is seen with cortisol suggests that MPA has more glucocorticoid activity than progestogenic activity. Whereas NET has negligible inhibition on cytokines, thus acts more like progesterone and is more beneficial to use than MPA. Women who are currently undergoing TB treatment, cannot use an estradiol containing contraceptive, thus MPA is recommend due to its progesterone properties. Thus, both synthetic progestins MPA and NET will be suitable to use during TB treatment. However, it should be recommended to women receiving TB

treatment to rather use NET as an alternative to MPA. As NET does not have the selective GC activity seen with MPA that results in an altered immune response to TB.

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Addendum A: Solution Recipes

1) 10x Running Buffer

288g Glycin

60,6g Tris

Final volume is 2,0 litres, with dH₂O.

2) 1x Running Buffer

10x Running buffer

0,1% SDS

Final volume is 2,0 litres.

3) Blotting Buffer

200ml Methanol

200ml 1x Running buffer

Final volume is 2,0 liters

4) 1x TBS Buffer

20mM Tris (pH7,6)

150mM NaCl

Final volume is 2,0 liters

5) TBS and Tween

1x TBS Buffer

0,1% Tween-20

Final volume is 1,0 litres

6) Lysis Buffer (Bradford)

10ml Lysis buffer

1x protease inhibitor tablet

100µl phosphatase inhibitor (1:100 dilution)

7) Western Blot Blocking Buffer

5% fat free milk powder

20ml TBS

8) Primary Antibody

5% fat free milk powder

20ml TBS

1:1000 dilution with primary antibody

9) Secondary Antibody

5% fat free milk powder

20ml TBS

1:5000 dilution with secondary antibody

10) 5x Laemmli Buffer

125mM Tris (pH6,8)

20% Glycerol

5% SDS

2,5% Beta-mercaptoethanol

0,1% Bromophenol blue

Final volume is 10ml.

11) Lysis Buffer

20mM Tris (pH7,6)

150mM NaCl

1mM EDTA

1% Trton X-100

Final volume is 100ml

12) Resolving gel: 8% SDS-Polyacrylamide Gel Electrophoresis

18,5ml H₂O

10,7ml 30% Acrylamide mix

10,0ml 1,5M Tris (pH8,8)

0,4ml 10% SDS

0,4ml 10% Ammonium persulfate

0,024ml TEMED

13) 5% Stacking gel: SDS-Polyacrylamide Gel Electrophoresis

6,8ml H₂O

1,7ml 30% Acrylamide mix

1,25ml 1,5M Tris (pH8,8)

0,1ml 10% SDS

0,1ml 10% Ammonium persulfate

0,01ml TEMED