Evaluation of micro RNA expression profiles during BCG infection in the presence and absence of endogenous and synthetic steroids and possible implications on the host immune response to the pathogen

By

Leani Thiart

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Supervisor: Dr Katharina Ronacher
Co-supervisor: Prof Gerhard Walzl
Co-supervisor: Dr Léanie Kleynhans

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Abstract

Individuals latently infected with *Mycobacterium tuberculosis (M.tb)* contain the infection without showing signs of illness. Steroid hormones such as glucocorticoids (GCs) however can lead to reactivation of TB. Currently two different injectable contraceptives are available in South Africa, medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET). MPA is able to bind to and activate the glucocorticoid receptor (GR) and possesses selective GC activity, a pharmacological characteristic absent in NET.

The aim of this study was to investigate the immune modulatory effects of the two contraceptives MPA and NET on immune responses to mycobacteria *in vitro* and *in vivo*.

Human peripheral blood mononuclear cells (PBMCs) were infected with BCG (*M. bovis* Bacille Calmette-Guérin) and treated with MPA, NET, progesterone or cortisol and cytokine expression was measured in order to determine whether the synthetic progestins mimic endogenous progesterone or the GC cortisol. MPA, but not NET suppressed the expression of IFN-γ, IL-1α, IL-1β, IL-2, IL-12p40 and IL-13 similarly to cortisol.

Furthermore expression of miRNAs, small double stranded RNA molecules that bind to complementary sequences in mRNAs of target genes and cause their degradation, was determined under the different experimental conditions. The expression of several miRNAs including miR-30c, miR-222, miR-454 and miR-331-3p were differentially influenced by MPA, cortisol and/or NET in PBMCs stimulated with BCG. For example, BCG induced the expression of miR-454 (p=0.003) which was then inhibited to basal levels by cortisol (p=0.008), MPA (p=0.002) and NET (p=0.002). These results demonstrate that steroid hormones including the contraceptives MPA and NET can modulate immune responses to mycobacteria at the miRNA level.

To determine the effect of MPA and NET on BCG-induced expression of miRNAs *in vivo* a mouse model was used. C57BL/6 mice were injected weekly with either MPA or NET using a dose equivalent to humans and then infected with BCG. Mice treated with MPA had a higher spleen bacterial load 21 days after infection compared to both NET treated and control mice (p=0.023). In whole blood, MPA and NET treatment suppressed the BCG-induced production of miR-100 and miR-509-3p to basal levels. In contrast to NET, MPA induced expression of miR-99a expression independent of BCG infection. In the lung, the site of disease, a total number of 22 out of 377 miRNAs tested were differentially expressed 21 days after infection.

The results of this study indicate that both synthetic progestins altered the immune response to BCG at the level of cytokine expression as well as the miRNA level. MPA was found to mimic cortisol by

inhibiting secretion of inflammatory cytokines whereas NET appeared to have more progestogenic properties. Each of the steroid hormones was observed to induce a characteristic miRNA expression profile, both *in vitro* as well as *in vivo,* although not identical. These results highlight that the two contraceptives – although used interchangeably by women in developing countries - are pharmacologically unique and differentially modulate immune responses to mycobacteria. These data support the urgent need for further research into the link between hormonal contraceptives and susceptibility to infectious diseases.

Abstrak

Individue wat latent met *Mycobacterium tuberculosis (M.tb) geïnfekteer is,* onderdruk die infeksie en wys geen simptome van die siekte nie. Steroïed hormone soos glukokortikoïede (GKe) kan egter tot die heraktivering van TB lei. Daar is tans twee verskillende inspuitbare voorbehoedmiddels beskikbaar in Suid-Afrika, medroksiprogesteroon-asetaat (MPA) en noretisteroon enantaat (NET). MPA is staat om aan die glukokortikoïed reseptor (GR) te bind en dit te aktiveer. MPA beskik ook selektiewe GK aktiwiteit, 'n farmakologiese eienskap wat afwesig is in NET.

Die doel van hierdie studie was om die immuun-regulerende effekte van die twee voorbehoedmiddels, MPA en NET, op immuunresponse teen mikobakterieë *in vitro* en *in vivo* te ondersoek.

Menslike perifêre bloed mononukleêre selle (PBMSe) is met BCG geïnfekteer en met MPA, NET, progesteroon of kortisol behandel. Sitokien uitdrukking was gemeet om vas te stel of die sintetiese progestiene, endogene progesteroon of die GK kortisol naboots. MPA, maar nie NET, onderdruk die produksie van IFN-γ, IL- 1α, IL- 1β, IL- 2, IL- 12p40 en IL- 13 soortgelyk aan kortisol.

Verder is uitdrukking van miRNAs, klein dubbelstring RNS molekules wat aan komplimentêre volgorde in mRNAs van teiken gene bind en wat hul degradering veroorsaak, bepaal in elk van die verskillende eksperimentele toestande. Die uitdrukking van verskeie miRNAs insluitende miR-30c, miR-222, miR-454 en miR-331-3p is differensieël beïnvloed deur MPA, kortisol en/of NET in PBMSe wat met BCG gestimuleer is. Byvoorbeeld, BCG veroorsaak die uitdrukking van miR-454 wat dan geïnhibeer word tot agtergrondvlakke deur kortisol, MPA en NET. Hierdie resultate toon dat steroïed hormone, insluitend die voorbehoedmiddels MPA en NET, die immuunrespons teen mikobakterieë op miRNA-vlak affekteer.

Om die effek van MPA en NET op BCG-geïnduseerde uitdrukking van miRNAs *in vivo* te bepaal, is ’n muismodel gebruik. C57BL/6 muise is weekliks met 'n dosis van MPA of NET, ekwivalent aan dosisse gebruik in die mens, ingespuit en met BCG geïnfekteer. Muise wat met MPA behandel is, het 'n hoër bakteriële lading in die milt 21 dae na infeksie in vergelyking met NET-behandelde muise en kontrole muise. In hul bloed, onderdruk MPA en NET behandeling die BCG-geïnduseerde produksie van miR-100 en miR-509-3p tot basale vlakke. In teenstelling met NET, induseer MPA die uitdrukking van miR-99a onafhanklik van BCG infeksie. In die long is 'n totaal van 23 miRNAs differensieël uitgedruk 21 dae na die infeksie.

Die resultate van hierdie studie dui daarop dat beide sintetiese progestien die immuunrespons teen BCG verander op sitokien sowel as miRNA vlak. MPA boots hoofsaaklik kortisol na deur inhibering van sitokien-produksie terwyl NET meer progesterone eienskappe het. Op miRNA vlak veroorsaak

elk van die steroïed hormone 'n kenmerkende miRNA uitdrukkingsprofiel, beide *in vitro* asook *in vivo*. Hierdie resultate beklemtoon dat die twee voorbehoedmiddels - alhoewel hulle afwisselend gebruik word deur vroue in ontwikkelende lande - farmakologies uniek is en differensieël die immuunrespons reguleer teen Mycobacterium. Hierdie data ondersteun die dringende behoefte aan verdere navorsing in verband met hormonale voorbehoedmiddels en vatbaarheid vir aansteeklike siektes.

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Abbreviations

ACTH	adrenocorticotropic hormone
Ago	argonaute
AID	activation-induced cytidine deaminase
AIDS	acquired immunodeficiency syndrome
ALL	acute lymphoblastic leukemia
ANOVA	analysis of variance
AP1	activator protein 1
APC	antigen presenting cell
AR	androgenic receptor
BCG	Bacille Calmette-Guérin
BIM	BCL-2 interacting mediator of cell death
BSL	Biosafety level
CDK6	cyclin dependant kinase 6
cDNA	complementary DNA
CFSR	colony-stimulating factor receptor
CFU	colony forming unit
CI	confidence interval
Cort	cortisol
CRH	corticotrophin-releasing hormone
DC	dendritic cell
Dex	dexamethasone
DNA	deoxyribonucleic acid
ds	double stranded
ELISA	enzyme-linked immunosorbent assay
ESAT	early secretory antigenic target
FCS	fetal calf serum
FOXO3	forkhead box O3

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
G-CSF	colony-stimulating factor
gDNA	genomic DNA
GFI1	growth factor independant 1
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HIV	human immunodeficiency virus
HO-1	heam oxygenase-1
HPA	hypothalamic-pituitary-adrenal
hsp	heat shock protein
IFN	interferon
IFN-γRα	IFN-gamma receptor alpha
IL	interleukin
i.m	intramuscular
i.p	intraperitoneal
IRAK1	IL-1R-associated kinase 1
KSRP	KH-type splicing regulatory protein
LAM	lipoarabinomannan
LS	least squares
ManLAM	mannosylated lpoarabinomannan
MHC	major histocompatibility complex
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MOI	multiplicity of infection
MPA	medroxyprogesterone acetate
M.bovis	*Mycobacterium bovis*
M.tb	*Mycobacterium tuberculosis*

NET	norethisterone enanthate
NFIA	nuclear factor I/A
NFκB	nuclear factor kappa B
NFκB1	NFκB subunit 1
nGRE	negative GRE
NK	natural killer
NOD	nucleotide-binding oligomerization domain
nt	nucleotide
OADC	oleic acid albumin dextrose catalase
OD	optic density
OPD	o-Phenylenediamine dihydrochloride
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFMC	pleural fluid mononuclear cell
PIM	Phoasphatidyk-*myo*-inositol mannoside
PR	progesterone receptor
Prog	progesterone
PRR	pattern recognition receptors
PTEN	phosphatase and tensin homologue
QC	quality control
RNA	ribonucleic acid
RT	room temperature
RUNX1	runt-related transcription factor 1
SB	sodium borate
SEM	standard error of the mean
SHIP1	SH2-domain-containing inositol-5-phosphatase 1

SHP2	SH2-domain-containing protein tyrosine phosphatase 2
SIRPα	signal-regulatory protein α
Sox	SRY-related HMG-box
SPF	specified pathogen free
STAT	signal transducer and activator of transcription
SUN-IRG	Stellenbosch University Immunology Research Group
TB	tuberculosis
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAF6	TNFR-associated factor 6
Treg	regulatory T cell
TST	tuberculin skin test
US	unstimulated
UTR	untranslated region
WHO	World Health Organisation
ZN	Zhiel-Neelsen

Chapter 1: Introduction

1.1 Tuberculosis

1.1.1 Epidemiology

Tuberculosis (TB) is a major world-wide health problem with approximately one third of the world's population latently infected with the causative bacteria, *Mycobacterium tuberculosis (M.tb)* (1) with 95% of deaths occurring in the developing world (2). Smear-positive tuberculosis that is not treated in human immunodeficiency virus (HIV) negative persons has 10-year fatality rate of between 53% and 86%, with a weighted mean of 70% (3). An estimated 8.6 million incident cases were reported globally, of which 0.40-0.6 million cases were reported in South Africa, during 2010 (1). The disease also resulted in 1.1 million deaths among diseased individuals who where HIV-negative with an additional 0.35 million deaths among people who were HIV-positive during 2010 (1). It is estimated that 1.4 million people died of TB in 2011 of which 500 000 were women and around 990 000 were HIV negative (2).The World Health Organisation (WHO) estimates that South Africa has a TB incidence of approximately 1000 cases per 100 000 population and further estimates that it could be as high as 1 190 per 100 000(Figure 1.1) (1).
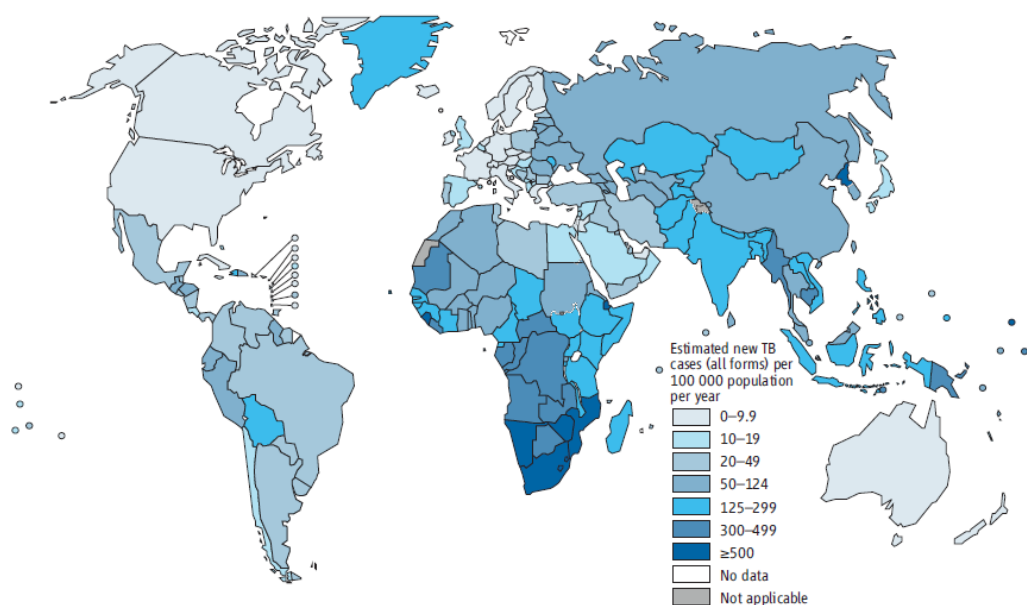
Figure 1.1 WHO incidence rate per 100 000 population per year (1,1)**.**

1.1.2 Mycobacterium tuberculosis infection

M.tb is transmitted when aerosols containing the bacteria are inhaled into the pulmonary alveoli, where an initial nonspecific inflammatory response is launched (4). In the majority of immune-competent individuals the bacilli are either killed or kept from actively proliferating and spreading. About 10% of individuals infected with *M.tb* will develop active disease during their life time (5). When the immune response cannot eliminate or control the infection it can lead to the death of an infected individual. Symptoms of TB include but are not limited to coughing, fever, night sweats and weight loss.

1.1.3 Innate immune response

After bacteria enter the alveoli they bind to receptors on antigen presenting cells (APCs), and are phagocytosed by APCs such as alveolar macrophages and dendritic cells (DCs) (4). Receptors on macrophages that can recognise and bind to *M.tb* are called pattern recognition receptors (PRRs). They include Toll-like receptors (TLRs) (6), the C-type lectin receptors; more specifically the mannose receptor, and nucleotide-binding oligomerization domain (NOD) -like receptors (7),(8). PPRs recognise highly conserved structures on the surface of pathogens called pathogen associated molecular patterns (PAMPs) (6). Binding of PAMPs to PRRs is needed for macrophage activation and subseqeun phagocytosis of *M.tb*. TLR ligands such as 19-kDa lipoprotein (LpqH), LprA (Rv1270) and LprG (Rv1411c) interact with TLR2 to induce tumour necrosis factor (TNF)-α and nitric oxide production in macrophages (9-11). LpqH is also a major inducer of interleukin (IL)-12 production in human monocytes (9). Mice lacking the TLR adaptor molecule MyD88 were found to be highly susceptible to TB that in many cases were lethal (12). This might be due to defective TLR signalling or defective signalling in response to IL-1α and IL-1β since signalling depends on MyD88 (12). Phosphatidyl-*myo*-inositol mannoside (PIM) can also activate macrophages by interacting with TLR2 by activating a signal cascade through nuclear factor kappa B NFκB (13). PIM can also induce (NFκB) activation via interaction with TLR4 (14). Fortune *et al.* have shown that *M.tb* ligand-induced activation of TLR2 with can lead to the inhibition of macrophage antigen presentation through major histocompatibility complex (MHC) class II and block responsiveness to interferon (IFN) -γ (15). It is hypothesised by Ernst that IL-10 induction by TLR2 in response to *M.tb* forms part of a negative feedback mechanism that inhibits the inflammatory response to limit damage to host tissues (12). NOD-like receptor 2 (NOD-2) recognises peptidoglycan from the cell walls of bacteria and has been shown to be required for the optimal production of pro-inflammatory cytokines in response to *M.tb* infection (7). The mannose receptor recognises glycolipids, such as lipoarabinomannan (LAM) and

mannosylated LAM (ManLAM), in the cell wall of *M.tb* (7). It has been suggested that the mannose receptor might be targeted by *M.tb* to enhance its survival, as the binding of ManLAM to the mannose receptor limits phagosome lysosome fusion (7).

DCs play an important role as APCs and activators of T cells (4). Following the phagocytosis of *M.tb* and activation of TLR signalling the APCs migrate from the lung to draining lymph nodes, here mature DCs present antigens to naïve T cells to initiate the adaptive immune response (16). T cells that are specific for *M.tb* antigens then migrate to the site of disease where they initiate the formation of granulomas.

1.1.4 Adaptive immune response

Different tuberculus granulomas can occur among infected individuals or even within the same individual (17). A caseous granuloma contains epithelial macrophages and neutrophils surrounded by lymphocytes, these cells can also be surrounded by fibroblasts (17). This type of granuloma can be found in patients with active TB and latent infection. A non-necrotizing granuloma is mainly seen in patients with active TB and is made up of macrophages and some lymphocytes (17). The granuloma seen in patients with latent infection is the fibrotic lesion, which is composed mainly of fibroblasts and a low number of macrophages (17). Another granuloma, the nectrotic granuloma can also be formed. This granuloma is formed when T helper (Th) cells reach the site of disease where they interact with *M.tb* antigen presenting macrophages.. Upon binding these T cells produce chemokines that attract more macrophages to the site of disease (18). The recruited macrophages are activated and fuse to form multinucleated giant cells or differentiate into epithelioid cells (18). These cells limit the growth of the TB bacilli, but many undergo necrosis leading to the formation of the necrotic centre of the granuloma. The necrotic centre is surrounded by giant cells and epithelioid cells, which in turn are surrounded by T cells (19). When the bacilli are contained within these granulomas, they do not show signs of multiplying or spreading within the infected individual and the individual does not display any of the symptoms of active disease, a state called latent infection is reached.

1.1.5 Cytokine response to TB.

The interaction of *M.tb* with TLRs and subsequent activation of T cells leads to the secretion of an abundance of cytokines and chemokines such as TNF-α, IL-12 and IFN-γ. These molecules regulate granuloma formation and are in part responsible for the initiation of the adaptive immune response. The induction of a Th1 cellular immune response is essential for protection against *M.tb* (18),(20). There are three well-defined cytokines that steer the naïve T cells towards a Th1 response namely IL-12, IL-23 and IL-27. IL-12 is the dominant cytokine and affects the induction and maintenance of the Th1 immune response (4). IL-23, which shares the p40 component with IL-12, functions in the

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activation of memory T cells (4). IL-27 is secreted prior to IL-12 by APCs and is involved in the initiation of the Th1 response (4). Early studies showed that IL-12 is needed to maintain a Th1 response and together with IFN-γ is necessary for protection against *M.tb* infection. Studies done in murine models where the p40 component of IL-12 was absent and IFN-γ was knocked out found these animals had a higher susceptibility to *M.tb* infection. However in the IFN-γ knockout mice addition of IL-12 did not restore protection from infection, suggesting that IFN-γ is a downstream effector molecule that is required for protection (21). In contrast mice that did not express IL-12p40 had a higher level of protection against infection after the addition of exogenous p40 (22). Another important cytokine during the Th1 response is TNF-α. TNF-α together with IFN-γ mediate the activation of macrophages, which are needed to control the growth of *M.tb* and for the formation of granulomas. It also induces the killing of *M.tb* together with IFN-γ by the generation of reactive nitrogen intermediates (23). TNF-α can also induce apoptosis in alveolar macrophages, which indirectly leads to the reduction of bacterial burden (24). The importance of TNF-α during *M.tb* infection was discovered when patients treated with TNF-α blocking agents, for the treatment of inflammatory diseases such as rheumatoid arthritis, experienced reactivation of TB (24).

DCs and macrophages can produce the immunosuppressive and anti-inflammatory cytokine IL-10 (4). It has been shown that monocytes secreting IL-10 can lead to lower levels of IFN-γ responses by T cells in pulmonary tuberculosis (25). *In vitro* IL-10 down regulates the expression of IL-12 in macrophages infected with *M.tb* (26). IL-10 also represses the activity of CD4 and CD8 T cells by down-regulation of co-stimulatory molecules on macrophages and inhibits the proliferation of IFN-γ producing T cells (27).

There are many other cytokines that are important during infection with *M.tb* and TB such as IL-2 and IL-3 that have not been discussed.

1.2 MicroRNAs

MicroRNAs (miRNAs) are small, non-coding double-stranded RNA molecules that are 19-22 nucleotides (nt) in length (28). These miRNAs have key roles in the regulation of a wide variety of biological processes including development, cancer, neuronal cell fate and both innate and adaptive immune function in many disease states (29).

The expression of the majority of miRNAs is thought to be controlled by transcription factors that are activated or repressed during the immune response by TLR ligands (28), antigens or cytokines (30). When the expression of a miRNA is induced, a cascade of reactions is initiated (Figure 1.2), the first being the transcription of the DNA sequence where the miRNA is encoded by RNA-polymerase II. This results in the formation of a pri-miRNA (28)(31). The pri-miRNA folds over on to itself to form

a hairpin structure. This structure is cleaved by the enzyme Drosha to form a pre-miRNA that consists of a hairpin structure of approximately 65 nucleotides in length (31). The pre-miRNA structure is transported across the nuclear membrane by Exportin 5 into the cytoplasm, where the hairpin is cleaved by the enzyme Dicer to form a mature miRNA of roughly 21 nucleotides (31). The miRNA duplex is unwound and the single stranded RNA is bound to the protein complex miRNA-induced silencing complex (miRISC). This miRISC complex is then transported by Importin 8 to its messenger RNA (mRNA) target (30). The miRNA-miRISC complex mediates its effects blocking mRNA translation, reducing mRNA stability by accelerated uncapping and deadenylation (30, 32) or by inducing mRNA cleavage after imperfect binding to the miRNA recognition elements within the 3' and 5' untranslated regions (UTRs) of target mRNAs (28).
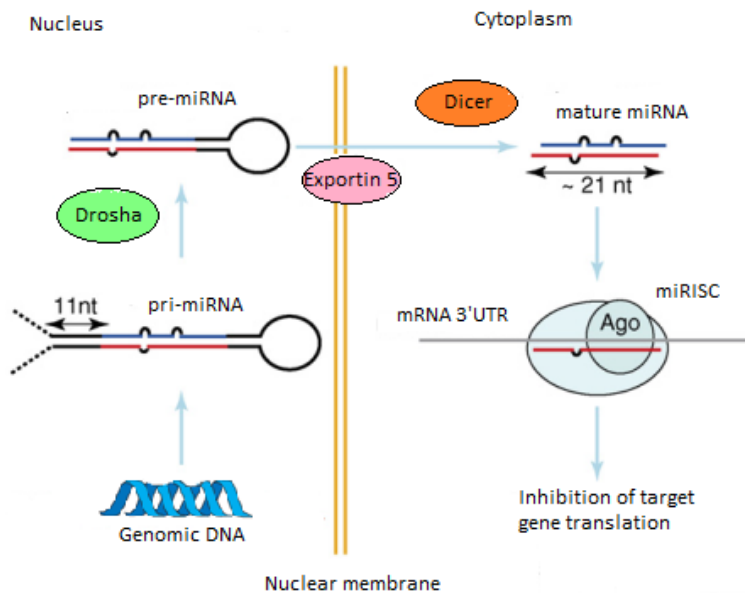
Figure 1.2 Pathway of miRNA biogenesis adapted from Lindsay 2008 (28).
DNA is transcribed to form a pri-miRNA which is cleaved by Drosha to form a pre-miRNA structure of approximately 65 nt. This structure is transported from the nucleus into the cytoplasm by Exportin 5. The enzyme Dicer then cleaves the hairpin structure to form a mature miRNA structure of between 19 and 23 nt. The lead strand binds to the protein complex miRISC, which subsequently binds to the complementary sequence of the target mRNA and inhibits its translation.

The regulation of miRNA expression occurs at three different levels namely: transcription, processing and sub-cellular localisation (30). Expression is induced due to different environmental stimuli or during the development of cells from progenitors. Examples of the expression of miRNA that are controlled by transcription factors in immune cells are miR-155 and miR-146a; the expression of both

are up-regulated in response to inflammatory stimuli like TLR ligands or the expression of pro-inflammatory cytokines (30,33).

1.2.1 MiRNAs and innate immunity

The first line of defence against infection consists of cells of the innate immune system; this includes granulocytes, monocytes (that differentiate upon stimulation into DCs or macrophages) and natural killer (NK) cells. There is an increasing amount of evidence suggesting that miRNAs play an integral role in the development and functioning of these cells. For the purpose of this study the function of miRNAs in the development and function of monocytes and the cells that are derived from monocytes will be focused on.

1.2.1.1 MiRNAs and NK cells and granulocytes

Granulocytes are derived from granulocyte-monocyte progenitor cells that are differentiated when in contact with certain stimuli, most commonly that of transcription factor growth factor independent 1 (GFI1). GFI1 can bind to the promoter regions of several miRNAs including miR-21 and miR-196b and can repress their expression (34). Sustained expression of miR-155 can increase the number of immature granulocytes *in vivo* and it is likely that several of the targets of miR-155s such as SH2-domain-containing inositol-5-phosphatase 1 (SHIP1) may be involved in this process (33).

MiRNAs have also been shown to regulate the function of granulocytes. The transcription factor PU.1 induces the expression of miRNA-223; this miRNA in turn plays a role in negatively regulating the proliferation and activation of neutrophils (35).

NK cells play an important role in host defence against pathogens and the initiation of the adaptive immune response (36). The deletion of miRNA-processing enzyme Dicer leads to defective NK cell survival, activation and function during mouse cytomegalovirus infection (36), showing that miRNAs are important for the regulation of these processes. How individual miRNAs affects these processes is still largely unknown, however it is known that miR-150 is expressed in NK cells and is up-regulated during the cellular maturation of these cells (36). The transcription factor c-Myb is a direct and functional target of miR-150 (37). It is therefore possible that miR-150 exerts its effect through the signalling of this transcription factor.

1.2.1.2 MiRNAs in monocytes, macrophages and DCs

Macrophages and DCs play vital roles during the initial immune response to pathogens. Both are derived from a common myeloid progenitor cell (28) and miRNAs have been shown to play a role in the regulation of the specific transcription factors that are involved in the differentiation of these cells

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from their common progenitor (38). The expression of miR-17p, miR-20a and miR-106a, all members of the miR-17-92 and miR-106a-92 clusters, has been shown to be decreased during the differentiation of progenitor cells into monocytes. This leads to the transcription factor runt-related transcription factor 1 (RUNX1) being under less stringent control, which in turn leads to increased expression of this transcription factor (39). It is believed that RUNX1 is part of a negative feedback loop as it can bind to the promoter regions of the miR-17-92 and miR-106a-92 clusters decreasing their expression (39). An increase in RUNX1 expression results in increased monocyte differentiation, presumably by up-regulation of colony-stimulating factor receptor (CSFR) expression.

The inflammatory response to an infection by macrophages involves the up-regulation of several miRNAs, including miR-155, miR146, miR-147, miR-21 and miR-9 (30),(40),(41). MiR-155 expression is induced through the activation of numerous TLR ligands (42). In mouse bone marrow-derived macrophages the expression of miR-155 is increased due to pro-inflammatory cytokines TNF-α and IFN-β (30); the interferon does not induce miR-155 expression directly but rather through TNF autocrine and paracrine signalling (30). The coding region of miR-155 is known to be transcriptionally activated by the transcription factor activator protein 1 (AP1) and NFκB. MiR-146 expression is also activated in an NFκB dependent manner (43). In macrophages derived from peripheral blood it was found that miR-9 is up-regulated in a similar way by pro-inflammatory signals by a MyD88 and NFκB dependent manner (44).

Most of the miRNAs mentioned negatively regulate the inflammatory response in a direct or indirect manner. MiR-146, for example, targets and thus down-regulates several molecules involved in TLR signalling, including IL-1R-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), both of which promote inflammation (43),(45). MiR-9 in turn represses the expression of NFκB subunit 1 (NFκB1) during TLR 4 mediated activation of monocytes (44). When miR-155 is knocked down in human myeloid-derived DCs, the expression of IL-1β, a pro-inflammatory cytokine, increases significantly (30). Furthermore, when a miR-155 mimic is added to cells the expression of NFκB decreases (30).

MiRNAs play a role in the regulation of various pathways that regulate the differentiation, activation and function of cells in the innate immune system. These functions play a role in the direct or indirect initiation of the antigen-specific response of the adaptive immune response.

1.2.2 MiRNAs and adaptive immunity

1.2.2.1 T cells and miRNAs

The development of T cells in the thymus and their activation in the periphery are known to be controlled by complex interactions of various signalling networks, there are many components in

these pathways that are proven or putative targets of miRNAs. For this reason it is believed that the development and activation of T cells are regulated by miRNAs to a greater extent than has been shown up to now. Until now, very few miRNAs have been directly implicated in T cell development.

The miR-17-92 cluster has been implicated in the impairing mRNA expression of the proteins encoding pro-apoptoptic proteins, including BCL-2 interacting mediator of cell death (BIM) and phosphatase and tensin homologue (PTEN) (46). It is thought that this cluster of miRNAs increases the survival of T cells during development as it is expressed during thymopoiesis.

Recent studies have also shown that miRNAs have a role in the differentiation of T cells into their different effector T helper subsets. For example, mice that were deficient in miR-155 had a bias towards Th2 cell differentiation, possibly indicating that miR-155 promotes differentiation into a Th1 immune response (47). The inhibition of miR-106a seems to act as a regulator of IL-10, alleviates inflammation and leads to increased Th2 responses (48). MiR-21 directly targets IL-12p35 and dysregulates the IL-12/IFN-γ pathway leading to increased levels of IFN-γ and polarising the immune system towards a Th1 response (48).

T cell receptor (TCR) signalling determines whether cells are positively or negatively selected during development in the thymus. The expression of miR-181 is increased in early T cell development and is thought to increase TCR signalling strength by directly targeting a group of protein phosphatases, including SH2-domain-containing protein tyrosine phosphatase 2 (SHP2) (49).

1.2.2.2 B cells and miRNAs

The expression and function of miRNAs during B cell development has not been as extensively studied as other aspects of the immune system, but some miRNAs have been shown to be involved in different stages of B cell generation.

Over-expression of miR-181 causes an increase in the development of B cells, leading to a two to three fold increase in the number of B cells with no corresponding increase in T cells (50).

A miRNA that is readily expressed during early B cell development, miR-150, causes a block in the development of B cells, preventing the transition of pro-B cells to pre-B cells (51). Mice that are deficient in miRNA-150 also have increased levels of secreted antibody, both in naïve cells and following T cell dependent antigenic stimulation (46). MiRNA-34a targets the transcription factor FOXP1 which regulates the expression of recombinating-activating genes. This leads to a perturbation of B cell development causing an increase in the number of cells at the pro-B to pre-B cell transition (52). MiR-155 is up-regulated following B cell activation in the germinal centre. B cells deficient in miR-155 have defective antibody class switching and differentiation into plasma cells, which leads to an impaired humoral response to T cell dependent antigenic stimulation (53). MiR-155 regulates

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many targets which are important for T cell dependent antibody responses namely: PU.1 and activation-induced cytidine deaminase (AID), the expression of both is repressed by miR-155 (54). The effects on B cell function mentioned above are likely effects of this direct repression.

1.2.3 MiRNAs and the immune response to TB

It is only recently that the role of miRNAs in the immune response to TB has been investigated. Studies have also looked at the possibility of measuring expression of certain miRNAs or miRNA signatures as biomarkers to differentiate between diseased, latently infected and healthy individuals. Table 1.1 summarises the functions of the miRNAs discussed in this section.

Table 1.1 MiRNAs shown to be involved in the immune response to TB.

MiRNA	Function	Reference
MiR-29	Down regulated IFN-γ by directly targeting 3'UTR of the IFN-γ mRNA.	Ma *et al.* 2011 (55)
	Promotes association with Ago2 to form a Dicer complex and suppress IFN-γ posttranscriptionally,	Harapan *et al.* 2013 (56)
MiR-29 and let-7	Inhibits apoptosis by caspase 3 and caspase 7	Sharbati *et al.* 2011 (57)
MiR-147	Attenuates expression of TNF-α and IL-6	Liu *et al.* 2009 (41)
MiR-21	Inhibits IL-12 by possibly targeting the 3'UTR of the mRNA	Lu et al. 2011 (58) Wu et al. 2012 (59)
MiR-99b	Negatively regulates expression of TNF-α, IL-6, IL-12 and IL-1β, blocking this miRNA leads to reduced growth of *M.tb*	Singh *et al.* 2013 (60)
MiR-125b	Targets 3'UTR TNF mRNA, enhances stability kB-Ras2 an inhibitor NFkB, decreasing inflammatory response	Rajaram *et al.* 2011 (61)

MiR-155	Targets 3'UTR SHIP1 mRNA a negative regulator TNF, leading to increased expression. Inhibits IL-6 and Cox-2	O'connell *et al.* 2009 (33) Kumar *et al.* 2012 (29)
MiR-144*	Targets MAPK, TLR signalling and inhibits TNF-α and IFN-γ production	Liu *et al*. 2011 (62)(29)(29)(31)

Some miRNAs, namely miR-187 and miR-365, have not been directly implicated during *M.tb* infection, but affects or are affected by cytokines that are important during the immune response to TB. MiR-187, when induced by IL-10, negatively regulates TNF-α, IL-6 and IL-12p40 production in TLR-4 stimulated monocytes (48). MiR-365 is up-regulated by Sp1 and NFκB and directly targets IL-6 (63).

MiR-29 suppresses IFN-γ production directly by targeting T-bet, a transcription factor known to induce IFN-γ in T cells (64). MiR-29 also targets the 3'UTR of the IFN-γ mRNA and promotes association of the IFN-γ mRNA with Ago2 to form a RISC complex and suppress the expression of IFN-γ posttranscriptionally (56). Ma *et al*. found that naive T cells have higher levels of miR-29 than effector T cells and miR-29a and miR-29b were down-regulated in T cells secreting IFN-γ in a murine model after BCG infection (55). The higher level of miR-29 present in naive T cells and the miRNAs ability to bind to the 3'UTR of the IFN-γ mRNA leads to the lower production of IFN-γ in these cells. A study investigating levels of circulating miRNAs in patients with active pulmonary TB Fu *et al.* found 92 miRNAs that were differentially expressed in serum between TB patients and healthy controls. Only two of the miRNAs (miR-938 and miR-29a) that were over expressed in serum were also over expressed in the sputum of these patients (65). Of these, miR-29a expression levels could differentiate TB patients from healthy controls with a sensitivity of 83% and a specificity of 80% (65).

MiR-147 is thought to have potent anti-inflammatory functions and has been shown to be induced through the TLR/NFκB pathway and attenuate the expression of pro-inflammatory cytokines such as TNF-α and IL-6(41). When the expression of miR-99b was blocked in the DCs of MyD88 deficient mice it resulted in reduced *M.tb* growth and the up regulation of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and IL-1β (60). Indicating that miR-99b has an important role in the survival of *M.tb* in the host and its ability to evade the host's immune system (60). MiR-125b directly targets the 3'UTR of the TNF mRNA transcript and contributes to the inhibition of translation and accelerates the degradation of TNF-α (61).

MiR-144* was found to be over-expressed in T cells of patients with active TB compared to healthy controls (62). When T cells were transfected with a precursor of miR-144* it was found that this miRNA could inhibit the production of TNF-α and IFN-γ and also inhibit the proliferation of T cells. Therefore it is hypothesised that miR-144* plays an important role in anti-tuberculosis immunity by modifying the cytokine production of cells and also by inhibiting the proliferation of T cells during infection (62).

As stated previously T cells that do not express miR-155 are biased towards Th2 differentiation. Banerjee *et al.* showed that miR-155 is induced during T cell activation and promotes Th1 differentiation (66). When cells are transfected with a miR-155 antagonist the IFN-γ receptor α-chain (IFN-γRα) is expressed, showing that IFN-γRα might be a target of miR-155 and that this miRNA contributes to Th1 differentiation of T cells by inhibiting IFN-γ signalling (66). Recently is has also been shown that virulence associated secreted protein early secretory antigenic target (ESAT)-6 plays a role in the induction of miR-155 expression during *M.tb* infection and that the up-regulation of miR-155 is linked to the inhibition of Bach1 and SHIP1 (29). Bach1 represses the expression of haem oxygenase-1 (HO-1), which is a documented activator of the *M.tb* dormancy regulon; whereas SHIP1 inhibits the activation of the serine/threonine kinase AKT which is needed for *M.tb* survival (29). MiR-155 also inhibits Cox-2 and IL-6 that are both modulators of the innate immune response. When Kumar *et al* inhibited the expression of miR-155 in RAW264.7 and murine BMDM cells the *in vivo* survival of *M.tb* was decreased. Taken together, these results suggest that the ESAT-6-induced expression of miR-155 might benefit the pathogen.

Spinelli *et al.*2012 recently showed that the miRNA signature differs between mononuclear cells collected from peripheral blood (PBMCs) and pleural fluids (PFMCs) of the same patients. MiR-146a was down-regulated in both PFMCs and PBMCs, a down-regulation of miR-144*, miR-421 and miR-223 was seen in PFMCs of TB patients (67). MiR-424 expression was increased in PBMCs but not PFMCs (67). They also showed that the expression profiles of patients changed during treatment and that these changes were associated with levels of IL-6 (67), a cytokine that plays an important role in the immunopathology of TB.

MiR-21 targets the 3'UTR of the IL-12 mRNA, suppressing the expression of this cytokine and the Th1 responses of the host (59). MiR-21 also targets the mRNAs of TNF-α and IL-6, but a corresponding decrease in the levels of the cytokines was not seen (56).

1.2.4 MiRNA nomenclature

MiRNAs have been discovered in many different species, in this study the miRNA profile of humans and mice were examined. When referring to human miRNAs they will be referred to as hsa-miR-xxx and murine miRNAs as mmu-miR-xxx. Many miRNAs are evolutionarily conserved between mammalian species, more specifically humans and mice (68). Examples are miR-29(55), miR-329, miR322 and miR-346 (69) in humans and mice. The sequences of all miRNAs that were found to be differentially expressed in this study were conserved between humans and mice. Hsa-miR-454 did not have a homolog in mice and mmu-miR-292-3p, mmu-miR-467c and mmu-miR-467e had no human homologs (http://www.mirbase.org).

1.3 Glucocorticoids

Steroid hormones are a group of hormones that are derived from the precursor cholesterol. Cholesterol is converted to pregnenolone from where the synthesis pathway diverges into different steroid hormone sub-families namely glucocorticoids (eg. cortisol), mineralocorticoids (eg. aldosterone), progestagens (eg. progesterone), androgens (eg. DHEA and dihydro testosterone) and estrogens (eg. estradiol).

Glucocorticoids (GCs), of which cortisol is the most important in humans, are steroid hormones that are secreted when the hypothalamic-pituitary-adrenal (HPA) axis is activated by stimuli such as inflammation, pain, infection or mental stress (70). In this state the hypothalamus is excited and secretes corticotropin-releasing hormone (CRH) (70). CRH activates the anterior pituitary to induce the release of adrenocorticotropic hormone (ACTH), which in turn stimulates the synthesis and release of cortisol by the adrenal cortex (70). GCs have a wide range of effects in the body, they have an anti-inflammatory effect, are anti-proliferative and pro-apoptotic (71). They affect many cells of the immune system and their function. GCs suppress DC maturation, cell migration and apoptosis (72). GCs stimulate the innate immune response by inducing TLR2 together with TNF-α in macrophages (73). GCs can suppress the migration of neutrophils to the site of infection by repressing the expression of adhesion molecules such as integrins, selectins intracellular adhesion molecules (73)(74). GCs suppress adhesion of neutrophils by reducing the expression of L-selectin on neutrophils and E-selectin and P-selectin on endophelial cells (73). GCs also play a role in the shifting of the immune response from a Th1 (a pro-inflammatory response characterised by the secretion of IFN-γ, TNF-α and IL-12) to a Th2 (an anti-inflammatory response characterised by the secretion of

IL-4, IL-10 and IL-13) response to limit inflammation during infection (75). It is due to this anti-inflammatory property that synthetic GCs are often used as therapies during chronic inflammatory conditions such as autoimmune diseases and asthma. GCs can mediate their actions by classical genomic glucocorticoid receptor (GR) signalling and by non-genomic mechanisms (76).

The GR molecule has three functional domains, 1) a ligand binding domain, 2) a DNA binding domain and 3) a N-terminal domain (77). The GR, when ligand is absent, is found mainly in the cytoplasm of a cell as part of a multi-protein complex (70). This complex consists of amongst others two heat shock protein (Hsp) 90 molecules, Hsp 70, Hsp 23, the immunophilins p59 and calreticulinin and Src (78). GCs are lipophilic molecules and can diffuse across the cell membrane. Once they have entered the cell they bind to the GR at the site of the ligand binding domain; this leads to a conformational change in the receptor which causes the dissociation of the Hsp-complex and other proteins from the GC-GR complex. The dissociation of these proteins allows the GC-GR complex to translocate to the nucleus of the cell where it acts as a transcription factor (79). Once the GC-GR complex is in the nucleus it can up- or down-regulate the transcription of genes by binding of the DNA-binding domain to specific DNA sequences called glucocorticoid response elements (GREs). When it down-regulates transcription of a specific gene it is due to the GR binding to a negative GRE (nGRE) or by a tethering mechanism whereby the GC-GR complex binds to a transcription factor and prevents it from binding and activating the gene of interest (80). This is known as transrepression. When the GC-GR complex binds to a GRE and up-regulation occurs it is known as transactivation (71)(81). GREs are regions within the promoters of genes that allow the expression of the genes to be regulated by GCs (70). The receptor complex binds to the GRE sites as homodimers (78). When binding to a GRE, the GR complex functions by increasing the transcription of inhibitory and anti-inflammatory proteins such as IκB, which in turn inhibits transcription factors that promote the inflammatory response. In the case of IκB it is the transcription factor NFκB (82). When bound to nGREs the GR complex directly inhibits the expression of genes that have an inflammatory function such as IL-1 (70). The expression of these proteins is usually only seen after a few hours, which suggests that they affect the long term anti-inflammatory effects of glucocorticoids. However, recently more rapid effects of GCs have been observed. These are thought to not be caused by the classical genomic GR signalling as the effects are seen much too rapidly to be caused by altered gene expression. It is hypothesised that GCs can bind to the GR in the cytoplasm and cause the activation of signalling pathways such as the mitogen activated protein kinase (MAPK) pathway (71), which form part of the GR-hsp complex and could mediate some of the rapid effects of GCs known as non-genomic GR signalling (70).

GCs are believed to influence cell survival during thymocyte differentiation. Thymocytes that express TCR that bind to MHC too strongly undergo apoptosis due to the strong TCR signal; this cannot be overruled by GC signalling (73). Thymocytes that have suboptimal TCR signalling would have GC

signalling through the GR that will result in GC-induced apoptosis (73). Thymocytes with moderate TCR activity for MHC will have TCR and GC signalling. These signals neutralise each other leading to the survival of the cell and further differentiation (73). Short term treatment with GCs caused a disruption of the TCR protein complex in peripheral T cells; this impaired the TCR signalling (83). The GR makes up part of the TCR protein complex and GC treatment induces the dissociation of this protein complex and leads to reduced TCR signalling (83).

1.3.1 Progesterone

Progesterone can influence the immune response by shifting a Th1 response to a Th2 response by inhibiting Th1 responses and signalling of peripheral T lymphocytes (75). It was shown that stimulated cells treated with progesterone produced more anti-inflammatory cytokines such as IL-4 and IL-10 and less IFN-γ than untreated controls (75). Progesterone significantly increases the expression of IL-10, IL-1β and TNF-α (84). Progesterone suppresses the generation of effector T helper cells by steering T helper cell differentiation towards T regulatory (Treg) cells (85). Progesterone mediates its effects by binding to cytosolic progesterone receptors (cPRs) as well as membrane-associated progesterone receptors (mPRs) (86).

1.3.2 Medroxyprogesterone acetate

Previous findings in our group showed that PBMCs of women using Medroxyprogesterone acetate (MPA) produced lower levels of IL-1α, IL-12p40, IL-10, IL-13 and G-CSF in response to BCG stimulation (87). These women also had lower levels of circulating monocytes (87). The lower levels of circulating monocytes and the reduced levels of IL-1 and IL-12 in response to BCG stimulation could possibly lead to a greater susceptibility to TB and increased risk of progression from latent infection to active disease (87). In a murine model of *M.tb* infection MPA treated animals produced lower levels of TNF-α 3 weeks after infection (88). Taken together this evidence suggests that MPA can modulate the immune response to *M.tb* in mice as well as in humans. The consequences thereof should be studied in large clinical trials in future.

 MPA is a contraceptive that is administered once every three months. MPA is administered intramuscularly at a dose of 150mg/ml by injection into the gluteus or deltoid muscle (89). MPA is highly effective as a contraceptive and accomplishes its function by inhibiting the hormone cycle of users and so preventing ovulation (90). Women in countries such as South Africa use MPA because of the convenience of only having to go to the clinic once every three months and also because it is free of charge at health care clinics. MPA is the contraceptive most frequently used in South Africa, which is also a high endemic TB region (91). Since the formulation of MPA in the 1950s newer contraceptives have been developed that have fewer side effects.

MPA is a synthetic progestin which binds with a strong affinity to the PR. It can also bind to the GR whereas the synthetic progestin, norethisterone enanthate (NET), which is a two monthly injectable contraceptive, binds to the PR with high binding affinity, but to the GR only with a very low binding affinity **(92)**. It has been reported that MPA binds to the GR with a higher affinity than cortisol does **(93)** and it can mediate a suppressive effect on the IL-2 promoter via the GR and PR **(94)**.

1.3.3 MiRNAs and glucocorticoids

It is known that steroid hormones are anti-inflammatory and that they exert their effect through the GR. Transcription factors that are inhibited by signalling through the GR such as NFκB and activator protein (AP)-1 (95) are also targets of specific miRNAs such as miR-155. Due to this it would be interesting to look at the effect GCs might have on the expression of miRNAs in the context of mycobacterial infection. Studies have looked at GCs and miRNA expression in the context of cancers such as breast and ovarian cancer. Some of these findings might be relevant to the expression of miRNAs and the effect that GCs have on their expression in the context of infectious diseases. The miRNAs that influence the expression of the GR or are influenced by the GR are summarised in table 1.2.

When the re-expression of miR-128b was induced it was shown to restore the sensitivity to glucocorticoid treatment in acute lymphoblastic leukemia cells (RS4;11 and SEM cell lines) in which miR-128b expression is down-regulated (96). Over expression of miR-128b positively correlates to GC-induced apoptosis (96). However the direct target of this miRNA is still unknown. MiR-130b has been shown to bind directly to the 3' UTR of the GRα and down regulates the expression of this receptor (97). In contrast to miR-128, miR-130b inhibits GC induced apoptosis in multiple myeloma cells (97). MiR-124a is predicted to target the GR directly by binding to the 3'UTR of the mRNA transcript *in silico* and regulate the expression of the receptor (98). GC treatment also induces the expression of miR-124, which leads to the down-regulation of GR-α expression limiting the anti-inflammatory effects of GCs (99). However, how expression of this miRNA is regulated is unknown.

MiR-98 is up-regulated by human T cells when exposed to the GC methylprednisolone, this up-regulation leads to the suppressed expression of the predicted targets FAS, FASL and IL-13(100). GCs appear to mediate a T cell specific anti-inflammatory function through the induction of miR-98 (100). GCs are also thought to target miR-155 and suppress the expression of this miRNA to induce their anti-inflammatory effect (101).

MiR-182 has been shown to be increased in human and mouse GC-resistant cell lines when compared to GC-sensitive cell lines. Concordant with higher expression of miR-182 GC-resistant cell lines had lower levels of FOXO3A, a molecule that can translocate to the nucleus and can induce apoptosis in

lymphocytes (102). This suggests that miR-182 plays a role in GC resistance and GC-induced apoptosis by targeting FOXO3A.

TLR-activated plasmacytoid DCs are resistant to GC-induced apoptosis, lessening the anti-inflammatory effect of GC treatment in type-I interferon-related autoimmune diseases (103). MiR-29b and miR-29c were shown to be involved in TLR-inhibited GC-induced DC apoptosis and promoted apoptosis directly by targeting Bcl-2 (103).

Table 1.1 MiRNAs that expression has been shown to be altered by GC treatment or that targets GCs or the GR directly.

MiRNA	Target	Reference
MiR-128b	Restores sensitivity to GCs	Kotani *et al.* 2009 (96)
MiR-130b	Binds to 3'UTR GRα and regulates its expression	Tessel *et al.* 2011 (97)
MiR-124a and miR-124	Regulates GR activity and can bind to the 3'UTR of the mRNA. GC treatment induces miR-124, which down regulates GR-α, limiting anti-inflammatory effects of GCs	Vreugdenhil *et al.* 2009 (98) Ledderose *et al.* 2012 (99)
MiR-98	Up regulated by GCs and has anti-inflammatory function by suppressing IL-13	Davis *et al.* 2013 (100)
MiR-182	Targets FOXO3A and is involved in GC resistance	Yang *et al.* 2012 (102)
MiR-155	Inhibited by GCs in a GC-receptor, NFκB dependent manner.	Zheng *et al.*2012 (104)
MiR-29b and miR-29c	Targets Bcl-2 in human DCs during TLR-inhibited GC-induced apoptosis	Hong *et al.*2013 (103)

1.4 Hypotheses and aims

Human study: Hypothesis

It is known that miRNAs influence the immune response to pathogens and that the expression of some miRNAs is altered in cell lines when treated with GCs. In a setting where many women use the contraceptive MPA, which has been shown to have GC activity, we hypothesised that the use of MPA, but not NET, can suppress the immune response to a pathogen such as *M.tb* by altering the miRNA expression levels.

Human study: Aims

1. The aim of this study is to determine whether high concentrations of MPA, NET, cortisol and progesterone alter the expression of cytokines in PBMCs stimulated with BCG.
2. To determine if MPA and NET act like cortisol or progesterone by altering cytokine expression.
3. To determine the miRNA expression profile in PBMCs stimulated with BCG and treated with MPA, NET, cortisol and progesterone and compare these data to cytokine data obtained from the culture supernatant of these cells.

Mouse study: Hypothesis

We hypothesise that MPA, but not NET,has a negative effect on the immune response to Mycobacteria in mice and inhibits clearance of BCG from their lungs and spleens.

Mouse study: Aims

1. We aim to correlate the colony forming unit (CFU) data to the miRNA expression data in the blood and lungs of mice.
2. To determine the miRNA expression profile in the whole blood and lungs of mice and compare these profiles to each other as well as the profile obtained in human PBMCs stimulated with BCG.

Chapter 2: Materials and Methods

2.1 Human study

2.1.1 PBMCs

2.1.1.1 Steroids

Medroxyprogesterone 17-acetate (MPA) (M1629-1G), 19-norethindrone (NET) (N1200000-100MG), hydrocortisone (cortisol) (98%) (H4001-1G) and progesterone (4-pregnene-3,20-dione) (P-8783) were purchased from Sigma-Aldrich, South Africa. The hormones were dissolved in 96.5% ethanol (Merck. Johannesburg, South Africa) to a concentration of 10^{-2} M and stored at -20°C.

2.1.1.2 *M. bovis* BCG

Mycobaterium bovis Bacille Calmette-Guérin (*M. bovis* BCG) is classified as a bio-safety category two organism and all work was therefore conducted in a bio-safety level (BSL)-2 laminar flow hood. The initial stock of *M. bovis* BCG Pasteur was a kind gift from Dr Léanie Kleynhans. Further stocks were made as follows: *M. bovis* BCG Pasteur was grown in liquid cultures of Difco Middelbrook 7H9 broth (BD), supplemented with 0.2% (v/v) glycerol (Sigma), 0.05% (v/v) Tween 80 (Sigma) and 10% Middlebrook oleic acid albumin dextrose catalase (OADC) enrichment media (BD (Becton, Dickinson and Company. New Jersey, USA)) in 50 cm^{3} tissue culture flasks at 37°C.

The liquid BCG cultures were declumped using a 29 gauge syringe and frozen at -80°C in 10% (v/v) glycerol while in the logarithmic growth phase (optical density (OD) between 0.6 and 0.8). The number of viable bacteria were determined by thawing an aliquot and plating serial dilutions of the liquid cultures on Difco Middlebrook 7H11 (BD) agar plates, supplemented with 0.2% glycerol (v/v) and 10% Middlebrook OADC (BD). The plates were incubated inverted for 21 days at 37°C, in semi-sealed in plastic bags, at which point the CFUs were enumerated.

Using these frozen cultures ensures that the bacteria used for each experiment were in the same growth phase and that the inoculum size between experiments were the same.

2.1.1.3 Ziehl-Neelsen (ZN) stain

Acid-fast ZN stains were done on all cultures to ensure that no contaminants were present in the cultures. Briefly, a drop of the culture was put on a microscope slide, spread out to the size of a thumb print and fixed by incubation of the slide at 88°C for two hours. The slide was flooded with carbol fuchsin (National Health Laboratory Service, Sandringham, RSA), heated and incubated for five

minutes. The slide was rinsed with distilled water (dH$_2$O) and flooded with 5% acid-alcohol (National Health Laboratory Service, Sandringham, RSA) and incubated for two minutes. It was rinsed with dH$_2$O and flooded with methylene blue (National Health Laboratory Service, Sandringham, RSA) and incubated for one to two minutes. The slide was then rinsed with dH$_2$O and allowed to air dry before being examined using the 100x oil immersion objective of the microscope. The *M. bovis* BCG bacilli was stained a bright pink and if contaminants were present they were stained blue.

2.1.1.4 Blood collection

Blood was collected from healthy tuberculin skin test (TST) positive Caucasian females, not using any contraceptives, into 9ml Sodium Heparin vacutainer blood collection tubes (Becton Dickinson (BD)). All blood samples were processed within two hours of collection.

2.1.1.5 PBMC isolation

Blood was diluted in a 1:1 ratio with sterile phosphate buffered saline (PBS) (Lonza. Basel, Switzerland). 10ml of diluted blood was carefully layered onto 3 ml Histopaque (Sigma-Aldrich. Missouri, USA) at room temperature (RT), in a 15 ml Greiner tube (Greiner Bio-one. United Kingdom). The tubes were centrifuged at 400 x g for 25 minutes at RT (with the acceleration and the brakes turned off). The cells were separated by density gradient (D>1.077g/ml) and the PBMCs formed a layer between the plasma and Histopaque, while the red blood cells formed a pellet at the bottom of the tube. The plasma was removed and the layer of cells collected in a separate 50ml tube for each patient. The PBMCs were washed once with 50ml PBS and twice with 10ml PBS at 400 x g for 10 minutes at RT. After the last wash the supernatant was discarded and the pellet resuspended in 1 ml pre-warmed AIM-V (Sigma-Aldrich) media. A 1:5 dilution of cells was prepared with tryptophan blue (Sigma-Aldrich). In order to count the cells and check their viability, 10 microlitres (μl) of the solution was loaded onto a haemocytometer. The total number of cells was calculated using the following formula:

Cell count x dilution factor x 10000 = number of cells per ml

2.1.2 Cell culture

1x10^6 cells per well were cultured in 24-well tissue culture plates (Greiner Bio-one) in a total volume of 2 ml AIM-V media. The following conditions were included: unstimulated and BCG stimulated. For the BCG stimulated, 5 x 10^5 CFUs at a multiplicity of infection (MOI) of 5:1 was used, assuming that macrophages make up 10% of PMBCs (105). Cells were stimulated with BCG in the presence and absence of MPA, NET, cortisol and progesterone. As a control cells were also cultured in

presence of each of the hormones without BCG. Two microlitres each of 10^{-2} mM contraceptive or hormone was added to the total cell culture (final concentration of 10^{-5} mM) and CD3 (eBioscience. San Diego, USA) (final concentration of 1μg/ml). The plates were incubated at 37°C and 5% CO_2 for 6h, 24h and 3 days respectively and thereafter supernatants were collected and stored at -80°C and RNA was extracted from the cells as described below.

2.1.3 Interferon (IFN)-γ Enzyme-linked Immunosorbent Assay (ELISA)

An IFN-γ ELISA was performed using the culture supernatants from the PBMC stimulations to confirm PBMC stimulation assays worked. Briefly, ELISA plates (Greiner Bio-one) were coated with 50 μl of 2 μg/ml capture mouse anti-human IFN-γ monoclonal antibody (BD) and incubated at 4°C overnight. The next day plates were washed four times with wash buffer (PBS with 0.05% Tween 20) and blocked for two hours with blocking buffer (PBS with 10% heat-inactivated foetal calf serum (FCS)). IFN-γ standards diluted in AIM-V media (BD), samples and blanks (AIM-V media) were added in duplicate and the plates incubated at 4°C overnight. The standard concentrations ranged from 31 pg/ml to 4000 pg/ml. On day three the plates were washed and incubated for 45 minutes at room temperature with 100 μl of biotinylated anti-human IFN-γ detection antibody (BD) at a final concentration of 1 μg/ml. The plates were washed and incubated with 100 μl of 2.5 μg/ml avidin peroxidase (Sigma) for 30 minutes at RT. The detection solution, o-Phenylenediamine dihydrochloride (OPD) Fast solution (Sigma) was prepared by dissolving one tablet each of OPD and urea hydrogen peroxide in 20 ml distilled water to obtain final concentrations of 0.4 mg/ml for both OPD and urea hydrogen peroxide and 0.05 M phosphate-citrate. The plates were washed with wash buffer as described above and 100 μl OPD fast solution added to each well followed by incubation in the dark for 40 minutes. The colour reaction was stopped by adding 50 μl 1M H_2SO_4 to each well. The plates were then read at 490 nm and analysed using a linear-linear curve fit on a Biorad ELISA plate reader. The ELISA data were managed using Microplate Manager Software (Bio-Rad) and the results were interpreted with Graph Pad Prism software.

2.1.4 RNA extraction and supernatant harvest

RNA extraction was performed using the mirVana miRNA Isolation Kit (Ambion. California, USA) according to the manufacturer's instructions. The contents of each PBMC stimulation well was transferred into a 2 ml RNase-free tube by scraping the bottom of each well with a cell scraper (Greiner Bio-one) to ensure all adhering cells were loosened. The well was washed with 1 ml media to ensure the cells were collected and transferred to the labelled tube. The tubes were centrifuged for five minutes at 15990 g. The supernatants were taken off and stored at -80°C until cytokine expression analysis was done. The pellet was washed and resuspended in 500 μl sterile PBS and again

centrifuged. Three-hundred microlitres of Lysis Solution was added and the sample vortexed to dissolve the cell pellet and complete cell lysis.

One tenth of the final volume of miRNA Homogenate Additive was added and mixed well by vortexing and the tube incubated on ice for ten minutes. A volume equal to the total volume of the mixture before addition of the miRNA Homogenate additive (~300 μl) of Acid-Phenol:Chloroform was added to each tube. The tubes were vortexed for roughly 20-30 seconds and centrifuged for five minutes at maximum speed (~15990 g). The aqueous phase was removed and 1.25 volumes (~375μl) of RT absolute ethanol (Sigma) were added and the mixture inverted several times to mix. For each sample a filter was placed in a new collection tube and 700μl of the mixture added to the filter and centrifuged for 15 seconds at 522 g. This process was repeated for the entire volume of the sample and the flow-through discarded. Seven-hundred microletres miRNA Wash Solution 1 was applied to each filter, centrifuged for 10 seconds and the flow-through discarded. 500μl of Wash Solution 2/3 was applied and the tubes centrifuged for 15 seconds and the flow through again discarded. This step was repeated. The tube with the filter was centrifuged for one minute at 5220 g to remove any residual fluid from the filter. The filter was then placed on a new collection tube and 100μl pre-heated (95°C) nuclease-free water applied to the centre of the filter. The tube was centrifuged for one minute at the maximum speed (15990g) to recover the RNA. The filter was removed and the RNA was stored at -80°C until later analysis.

2.1.5 DNA contamination and DNase treatment

2.1.5.1 DNase treatment

RNA samples were treated for genomic (g)DNA contamination using the TURBO DNA-free kit (Ambion) according to the manufacturer's guidelines. In brief, all samples and the reagents were allowed to thaw on ice. A volume amounting to 10% (10 μl) of the total volume of the RNA sample of 10X TURBO DNase Buffer and 1μl TURBO DNase was added to the RNA samples and gently mixed. Samples were incubated at 37°C for 30 minutes. DNase Inactivation Reagent was resuspended by briefly vortexing the tube. A volume equal to 10% (10 μl) of the total volume of the RNA sample volume of DNase Inactivation Reagent was added to each sample. Samples were incubated at room temperature for five minutes during which time tubes were agitated three times to resuspend the DNase inactivation reagent. Samples were centrifuged at 10 000 g for 90 seconds. Supernatant containing RNA was transferred into fresh collection tubes.

2.1.5.2 Primer design

PCR Primers were designed to be specific for the human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (gene sequence as found on www.ensembl.org). Primers where designed to cross an intron-exon boundary to amplify the sequence only if gDNA was present in the sample. Primer Premier 5 was used to design the primers and they were obtained from Whitehead Scientific (WhiteSci). Primers sequences were as follows:

Forward (F) primer: 5' TTCCCCAACTTTCCCGCCTCT 3'

Reverse (R) primer: 5' TTTCTCTCCGCCCGTCTTCAC 3'

These primers have a T_m of 61.2 and 59.3 ^{o}C respectively.

Stocks were made up to a concentration of 1000 pmol/μl and working stocks were made up to 50 pmol/μl and stored at –20°C.

2.1.5.3 Genomic DNA contamination Polymerase chain reaction (PCR)

To determine if RNA samples had residual genomic DNA (gDNA) contamination the HotStarTaq DNA polymerase HotStarTaq Master mix kit (Qiagen. Limburg, Netherlands) was used. The master mix for each sample was made as shown in the table 2.1 below:

Table 2.1 PCR composition.

		1 sample (μl)
1	H$_2$O	7.2
2	QB	5
3	10X	2.5
4	MgCl$_2$	2
5	dNTPs (2.5 mM)	2
6	F primer (GAPDH)	1
7	R primer (GAPDH)	1
8	Hotstar Taq	0.3
9	RNA	4
	TOTAL	25

The reaction was then run for 45 cycles as described in table 2.2 below:

Table 2.2 PCR conditions

Cycles	Step	Temp	Time (min)
1	Activation Hotstar Taq	95	15
45	Denaturation	94	1
	Annealing	62	1
	Elongation (1min/kb)	72	1
1	Final elongation	72	10
1	Final hold	4	

PCR products were resolved on a 1% agarose gel with 1X Sodium Borate (SB) buffer containing Ethidium Bromide (Sigma). Five microlitres 6x DNA loading dye (Fermentas, Burlington Canada) was added to each tube containing PCR product. Eight microlitres from each tube was then loaded into individual wells on the gel and 5μl 100bp ladder (GeneDireX) was loaded to determine whether the 551bp product was present. On each gel a positive control (human genomic DNA kindly donated by Dr Craig Kinnear) and a negative control (H$_2$O containing all PCR reagents added) were also loaded onto the gel. The gel was then run at 100V for 45 minutes and visualised under UV light using the GeneSnap software.

2.1.5.4 RNA integrity and concentration

The concentration of RNA was determined using the Nanodrop photospectometry system (Inqaba Biotech. Pretoria, RSA). 1.5 μl of each sample was loaded and a photospectric analysis generated. The software compared each sample profile to the blank (nuclease-free H$_2$O) to calculate the concentration of RNA that was present in each sample.

The integrity and of the RNA samples were then determined using the Experion RNA Std Sens Analysis Kit (Bio-Rad, Hercules, California, USA). This was done according to the manufacturer's instructions. Samples and ladder were thawed on ice while the loading buffer, gel and RNA stain are equilibrated at RT. Two μl of each sample and 2μl of ladder were transferred into a fresh RNase-free tube and incubated at 70°C for two minutes. The gel-stain solution was prepared and covered in foil to prevent exposure to light. The ladder and samples were incubated on ice for five minutes, briefly centrifuged and kept on ice until analysed. The electrodes of the experion chip reader was cleaned according to the manufacturers guidelines, by loading 800μl of electrode cleaner into a cleaning chip

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and incubating it in the machine for two minutes. This was repeated using a fresh cleaning chip containing 800μl DEPC-treated water and incubating for five minutes. The electrode was allowed to dry for one minute after which the DEPC-treated water wash step was repeated. The chip was primed by adding 9μl of gel stain to the appropriate well, placing the chip in the priming station and selecting B1. The loading buffer, gel, sample and ladder was added to the appropriate wells. The chip was vortexed for one minute, placed in the machine and run as 'Eukaryotic total RNA'. Electrodes were cleaned by rinsing with DEPC-treated water for one minute between each chip.

2.1.5.5 cDNA

Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems. Carlsbad, California, USA) and Megaplex reverse transcription primers (Applied Biosystems) was used to synthesize single stranded cDNA from total RNA samples according to the manufacturer's instructions. The reagents and RNA samples were thawed on ice and added to a 1.5ml tubes as detailed in table 2.3:

Table 2.3 Reagents and volumes used during the reverse transcription reaction.

RT Reaction Mix Components	Volume for one sample (μl)
Megaplex RT Primers (10x)	0.8
dNTPs with dTTP (100mM)	0.2
MultiScribe Reverse Transciptase (50 U/μl)	1.5
10X RT Buffer	0.9
$MgCl_2$ (25 mM)	0.9
RNase Inhibitor (20 U/μl)	0.1
Nuclease-free water	0.2
Total	4.5

The reaction mix was mixed by inverting the tubes several times, and the tube centrifuged. For each reverse transcription reaction 4.5 μl of the reaction mix was added to a well of a 96-well MicroAmp Optical Reactions Plate (Applied Biosystems), 3 μl of total RNA was added to each well and the plate was sealed. The content was mixed by inverting the plate several times and then centrifuging it briefly after which it was incubated on ice for five minutes.

The 7900HT Real-time machine was set up for each run by setting the ramp speed to 'standard', the reaction volume to 7.5 μl and the thermal-cycling conditions as shown in table 2.4. The cDNA was stored at -20°C until required.

Table 2.4 Thermal cycling conditions for reverse transcription reaction.

Stage	Temperature	Time
Cycle (40 Cycles)	16°C	2 minutes
	42°C	1 minute
	50°C	1 second
Hold	85°C	5 minutes
Hold	4°C	Hold

2.1.5.6 MicroRNA array

cDNA was thawed on ice and the concentration and integrity determined using the Nanodrop. Five-hundred ng cDNA was used per reservoir and the volume adjusted with nuclease free water to 50 µl. 50 µl Taqman Universal PCR Master Mix without AmpErase UNG (Applied Biosystems) was added and the tube was briefly vortexed and pluse centrifuged to collect the contents at the bottom of the tube and eliminate any air bubbles.

The Taqman Human MicroRNA Array A card (Applied Biosystems) was allowed to reach room temperature and 100 µl of the cDNA PCR reaction mix is added to the fill port of each reservoir. The array was centrifuged at 350 x g for 2x1 minute intervals to move the cDNA PCR reaction mix from the fill port into the array. The card was carefully sealed using a Taqman Array Micro Fluidic Card sealer. After sealing the fill port was trimmed off.

A 7900HT Fast Real-Time PCR System was used to run the cards. The SDS Software was imported and the SDS 7900HT Document and the settings set according to the specifications of the manufacturers of the array cards. RQ manager (Applied Biosystems) was used to obtain a threshold cycle (Ct) value for each expressed miRNA. The delta (Δ) Ct value was calculated using the ΔCt method. The formula $2^{(Ct(MammU6)-Ct(MiRNA))}$ was used to obtain the relative expression of the specific miRNAs compared to the expression of the endogenous control MammU6. The fold difference of miRNA expression in stimulated PBMCs can be calculated by dividing the relative expression of the miRNA by the relative expression of the corresponding miRNA in unstimulated PBMCs. However, during this study we have opted to present the data as relative expression to an endogenous control rather than fold-changes. This allowed us to better visualise basal expression of miRNAs in untreated and uninfected cells. For future publications fold-changes can be used. The results were analyzed using Graph Pad Prism software.

2.1.6 Luminex

A custom human 13-plex Luminex assay (MPXHCYTO-60K-13, Merck-Millipore) was used to simultaneously quantify the levels of the following cytokines: Granulocyte colony-stimulating factor (G-CSF), IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-13 and Tumour Necrosis Factor (TNF) –α from the 6 hour, 24 hour and 72 hour culture supernatants of the PBMC stimulations. The assay was performed according to the manufacturer's instructions and all samples were run in duplicate.

A standard curve was prepared using a serial dilution of the reconstituted standard (3.2 pg/ml to 10 000 pg/ml). The plate was incubated with assay buffer for 10 minutes, which was then removed by a vacuum. Assay buffer was added to each of the sample wells and AIM-V (BD) media was added to each standard, quality control (QC) and blank well. The standards, QCs and samples were added in duplicate after which the beads, which are coated with antibodies specific for each cytokine, were added. The plate was incubated at room temperature on the orbital shaker (55 rpm) for one hour. The fluid was removed by vacuum and the plate washed with Wash Buffer. Detection antibody (a biotinylated secondary antibody) was added and the plate incubated on the shaker for 30 minutes at room temperature. Steptavidin-Phycoerythrin was added and the plate incubated for another 30 minutes. The plate was washed twice, sheath fluid added to each well and the plate read immediately on a Bio-plex platform (Bio Plex, Bio Rad Laboratories) using a 5PL regression curve. The levels of all analytes in the QC samples were checked to ensure that they were within the expected range as indicated in the package insert.

2.1.7 Statistical analysis

Cytokine levels measured above or below the highest or lowest concentration of the standard curve were extrapolated using the Bio-Plex Manager software. Luminex data were analysed using a mixed model repeated measures analysis of variance (ANOVA) with a Fisher LSD Post-Hoc test. Results are given as least squares (LS) means and 95% confidence intervals (CI). Luminex data analysis was done using Statistica 10, Statsoft (Ohio, USA). Unbiased clustering of miRNA expression of PBMCs stimulated with BCG and treated with MPA, NET or cortisol was done using Qlucore Omincs Explorer (http://www.qlucore.com) (Lund, Sweden). A heat map was generated using the same software. The expression of individual miRNAs was analysed using the mixed model repeated measures ANOVA using Statistica. Ingenuity Systems Pathway Analysis (Redwood City, California, USA) was used to generate a network indicating signalling molecules affected by the expression of miRNAs expressed under each condition. For all analysis a p-value of < 0.05 was considered as

significant. Statistical analysis was done under consultation of Prof Martin Kidd at the Department of Statistical Services at the University of Stellenbosch.

2.1.8 Ethical clearance

Ethical clearance for the sample processing and PBMC isolation was granted to the Stellenbosch University Immunology Research group (SUN-IRG) under the ethics number: N05/11/187 with the project title 'Biomarkers of protective immunity and surrogate markers of TB disease in Africa – Gates Grand Challenge project 6-74'.

2.2 Murine study

2.2.1 Mice

Specified pathogen-free (SPF) wild-type female C57BL/6 mice were purchased from the University of Cape Town (UCT) Animal Unit. All mice were housed at the Stellenbosch University (SU), Faculty of Medicine and Health Sciences Biosafety-level 2 Animal Unit. The animal unit has been accredited by the association for assessment and accreditation of laboratory animal care. The mice were allowed to acclimatize to the animal unit for at least six days prior to the start of experiments. All mice were aged between six and eight weeks at the start of experiments. Mice were aneathetised by 200 μl intraperitoneal (i.p.) injection with a mixture of 5mg/kg Xylazine and 80mg/kg Ketamine.

2.2.2 BCG infection of mice

BCG infections were performed intranasally with $2x10^6$ CFUs in 20μl.

2.2.3 Contraceptive injections

Clinical grade MPA 150 mg/ml (Depo Provera/Petogen-Fresenius) and Nur-Isterate (NET) (Bayer Limited) were obtained from the pharmacy (Tygerberg Hospital, Parow, Cape Town). Mice receiving contraceptives were injected weekly with 50μl of either 20mg/ml MPA or 26mg/ml NET intramuscularly (i.m.) into the right thigh. MPA was diluted in sterile filtered PBS (Lonza) and NET in sterile filtered olive oil (Sigma). The control groups were injected with either sterile filtered 1X PBS or olive oil i.m.

2.2.4 Tissue harvest

Mice were killed at days 21 or 56 post infection with a lethal dose of Xylazine/Ketamine mixture. Blood was collected by cardiac puncture, no more than 500µl blood, using a 26G hypodermic needle (Kimix, RSA) and a 1ml insulin syringe (Kimix). Blood was collected in EDTA minicollect blood collection tube (Greiner Bio-one). Blood was later removed and placed into RNAlater® (Ambion) for RNA extraction the next day.

Following euthanasia the lungs were aseptically removed. The right upper lobe was immediately placed into a cryovial and snap frozen by immersion in liquid nitrogen. RNA was therefore preserved until RNA extraction.

The remaining part of the lung was used to determine the bacterial loads in the mice. The remaining lobes and spleen were placed in 2ml of PBS (Lonza) containing 0.04% Tween 20 (Sigma). The tissue was homogenized using an Omni-prep homogenizer (Omni International) in a bio-safety level 2 laminar flow hood. Serial dilutions (10^{-1} to 10^{-4}) were plated out on Middlebrook 7H11 (BD) agar plates supplemented with 0.2% glycerol and 10% OADC. The plates were inverted, semi-sealed in plastic bags and left for 3 to 4 weeks in a 37°C incubator to grow.

2.2.5 RNA extraction from blood samples

Extraction of RNA from the blood was performed using the Mouse RiboPure[TM]-Blood RNA Isolation Kit (Ambion) according to the manufacturer's protocol. All steps were performed using certified RNase-free equipment and techniques to minimise enzymatic degradation of RNA. Tubes containing blood and RNA later solution were centrifuged at maximum speed (15990 g) for three minutes and all residual fluid removed. Lysis solution was added and the samples vortexed until the pellet was resuspended. Two-hundred microlitres 3M Sodium acetate was added. 1.5ml of Acid-Phenol:Chloroform was added, the samples were mixed well for 30 seconds and incubated for five minutes at room temperature. Samples were then centrifuged at 2000 x g for 10 min. The aqueous phase was recovered into a fresh 15ml tube and 0.6 volumes of nuclease free water was added and the mixture vortexed for five seconds. 1.2 volumes 100% ethanol was added and vortexed until the mixture cleared. Each sample was passed through a fresh filter by vacuum filtration using a manifold. The filter was put into a collection tube, 750µl of wash solution 1 was added to the filter and the assembly was centrifuged for 10 seconds at maximum speed (15 990 g) to allow the solution to pass through the filter. The flow through was discarded and 750µl of wash solution 2/3 is added and

centrifuged for 10 seconds and the flow through again discarded. This step was repeated. The assembly was centrifuged for one minute at maximum speed (15 990 g) to remove any residual fluid. The filter was placed in a new labelled collection tube and 150μl of nuclease free water (pre-heated to 80°C) applied to the filter and left to stand for one minute at room temperature. The assembly was centrifuged at maximum speed (15 990 g) for one minute to recover the RNA. RNA was then stored at -80°C.

2.2.6 RNA extractions from lung samples

The extraction of RNA from the lung samples was done using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. Lung samples were ground into small pieces while frozen before being added to 300μl Lysis solution. One tenth of the final volume of miRNA Homogenate Additive was added and mixed well by vortexing and the tube incubated on ice for ten minutes. A volume of Acid-Phenol:Chloroform equal to the total volume of the mixture (before addition of the miRNA Homogenate additive) (~300μl) was added to each tube. The tubes were vortexed for 20-30 seconds and centrifuged for five minutes at maximum speed (15990 x g). The aqueous phase was removed and 1.25 volumes (~375μl) of room temperature absolute ethanol (Sigma) was added and the mixture inverted several times to mix. For each sample a filter was placed in a new collection tube and 700μl of the mixture added to the filter and centrifuged for 15 seconds at 5220 x g. This process was repeated for the entire volume of the sample and the flow through discarded. 700μl miRNA Wash Solution 1 was applied to each filter, which was centrifuged for 10 seconds and the flow through discarded. 500μl of Wash Solution 2/3 was applied and the tubes centrifuged for 15 seconds and the flow through again discarded. This step was repeated. The tube with the filter was centrifuged for one minute at 5220 x g to remove any residual fluid from the filter. The filter was then placed on a new collection tube and 100μl pre-heated (95°C) nuclease-free water was applied to the centre of each filter. The tube was centrifuged for one minute at the maximum speed to recover the RNA. The filter was removed and the RNA was stored at -80°C until later analysis.

2.2.7 DNase treatment

Refer to section 2.1.5.1

2.2.8 cDNA

The concentration of RNA was determined using the Nanodrop photospectometry system (Inqaba Biotech). As described in section 2.1.5.5.

RNA samples from five mice from each of the BCG, MPA BCG, NET BCG and PBS groups were pooled adding the same amount of RNA from each mouse and resulting in the same RNA concentration of RNA between each of the groups before cDNA was made. This was done for two individual experiments.

Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) and Megaplex reverse transcription primers (Applied Biosystems) was used to synthesize single stranded cDNA from total RNA samples according to the manufacturer's instructions. As described in section 2.1.5.5.

2.2.9 MicroRNA array

As described in section 2.1.5.6.

2.2.10 Statistical analysis

CFU data were analysed using Statsitica 10 using mixed model repeated measures ANOVA and data presented as standard error of the mean (SEM). Please refer to section 2.1.7 for miRNA analysis. Statistical analysis was done under consultation of Prof Martin Kidd at the Department of Statistical Services at the University of Stellenbosch.

2.2.11 Ethical clearance

The animals were housed in accordance to the SU guidelines for the care of animals and all the experiments performed were approved by the Animal Ethics Committee of US (Project number 11LG_THI01) prior to comencing of experiments.

Chapter 3: Medroxyprogesterone acetate alters the immune response to mycobacteria by modulating expression of cytokines and miRNAs in PBMCs

3.1 Introduction

MPA is a three monthly injectable progestin only contraceptive. MPA is the contraceptive of choice in South Africa and many other areas with a high TB prevalence. It is known that MPA has selective glucocorticoid activity (93). MPA alters the cytokine expression in response to BCG stimulation in the PBMCs of TST positive household contacts of TB patients in a similar way to cortisol (87). MiRNAs such as miR-99b and miR-155 are known to influence the immune response to *M.tb* (29)(60). While miRNAs such as miR-124 and miR-130b are known to target the GR and suppress expression of this receptor (97), (98), it has not been investigated before if the synthetic progestin NET changes the BCG-induced cytokine expression in the same way MPA does or whether it acts more like progesterone which shifts the immune response from a Th1 to a Th2 response (75). The effect synthetic progestins, MPA and NET, and the endogenous glucoccorticoid cortisol have on the expression of miRNAs in PBMCs stimulated with BCG has also not previously been investigated. This study therefore investigates the effect NET has on cytokine expression compared to MPA, cortisol and progesterone. The effect that MPA, NET and cortisol have on the expression of miRNAs in PBMCs during BCG stimulation is also investigated.

3.2 Experimental design

Blood was collected from healthy, skin test positive, non-contraceptive using females. The PBMCs isolated from the blood were stimulated with live BCG at a MOI of 5:1. BCG is classified as a biosafety level 2 pathogen and was chosen to avoid working in a biosafety level 3 facility. The effect high concentrations (10^{-5}M) of MPA, NET and endogenous hormones cortisol and progesterone have on the immune response to BCG was determined. This was done by measuring the expression of G-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-13 and TNF-α in the culture supernatant 6, 24 and 72 hours after stimulation with BCG and addition of hormones using a Luminex assay. Due to high costs the level of progesterone was only measured at the six hour time point and only the PBMCs harvested six hours after stimulation were used for miRNA expression analysis using a Taqman miRNA array.

3.3 Results

3.3.1 Altered cytokine expression in peripheral blood mononuclear cells infected with BCG and treated with MPA, NET, cortisol and progesterone.

3.3.1.1 G-CSF

At six hours post stimulation with BCG the level of G-CSF was significantly higher in BCG stimulated compared to unstimulated (US) cells (p=0.000001; >1.0 $\log_{10}$ increase) (Figure 3.1.a). 24 hours post infection the level of G-CSF was significantly higher in all BCG stimulated cells compared to the US cells (>1.5 $\log_{10}$) and hormone only treated groups (p<0.000001; >1.0 $\log_{10}$) (Figure 3.1.b). The level of G-CSF at 72 hours post infection seen in the BCG (p=0.000001), BCG and MPA (p=0.000001), BCG and cortisol (p=0.000001), BCG and NET (p=0.000001) and BCG and Progesterone (p=0.000001) stimulated cells were higher than that of the US cells (>1.5 $\log_{10}$). No difference was seen between the BCG stimulated cells and the BCG stimulated and hormone treated cells (Figure 3.1.c). The expression of G-CSF was induced by the presence of BCG at all time points. At all three time points the hormones had no effect on the BCG-mediated expression of G-CSF. The hormones alone, without BCG had no significant effect (p>0.05) on G-CSF expression.

3.3.1.2 IFN-γ

The expression of IFN-γ was induced at all time points by BCG. Six hours post-infection the expression was inhibited by cortisol and progesterone. 24 hours after infection the expression was inhibited by MPA and cortisol but not by NET and progesterone. 72 hours after infection the expression was inhibited by NET and progesterone, but it was inhibited to a greater extent by the presence of MPA and cortisol.

The expression of IFN-γ six hours after infection was significantly higher in the BCG stimulated cells (p=0.004; > 0.5 $\log_{10}$) compared to the US cells (Figure 3.2a). The BCG-induced expression if IFN-γ was inhibited by cortisol (p=0.007; 0.8 $\log_{10}$). Cortisol had a greater suppressive effect on the BCG-induced expression of IFN-γ than MPA (p=0.007; 0.7 $\log_{10}$) and NET (p=0.003; 1.2 $\log_{10}$) (Figure 3.2a). The expression of IFN-γ in BCG stimulated and progesterone treated cells was lower than the level in the BCG stimulated NET treated cells (p=0.004). Twenty four and 72 hours post infection, IFN-γ was induced by BCG (p=0.000001; > 2 $\log_{10}$) (Figure 3.2b). MPA (p=0.0002; 1 $\log_{10}$) and cortisol (p=0.000001; > 1.5 $\log_{10}$) significantly inhibited the BCG-mediated secretion of IFN-γ. NET and progesterone had no effect on the BCG-induced expression of IFN-γ (Figure 3.2b). The expression of IFN-γ in the BCG stimulated cells 72 hours after BCG stimulation was suppressed by MPA (p=0.000015; 0.9 $\log_{10}$), NET (p=0.02; 0.3 $\log_{10}$), cortisol (p=0.000001, 1.4 $\log_{10}$) and progesterone (p=0.004; 0.4 $\log_{10}$) (Figure 3.2c). Cortisol suppressed the BCG-mediated expression of

IFN-γ to a greater extent than MPA (p=0.001; 0.5 $\log_{10}$), NET (p=0.000001; 1.1 $\log_{10}$) and progesterone (p=0.000006; 1 $\log_{10}$). The suppression by MPA was greater than that of NET (p=0.003; 0.5 $\log_{10}$) and progesterone (p=0.019; 0.4 $\log_{10}$).

3.3.1.3 IL-1α

24 and 72 hours after stimulation with BCG, MPA and cortisol had a suppressive effect on the BCG-induced expression of IL-1α. This effect was not seen in the presence of NET and progesterone.

The expression of IL-1α was induced, after six hours, by BCG stimulated (p=0.00005; 1.4 $\log_{10}$) (Fig.3.8a). The hormones had no effect on the expression of this cytokine at this time point. 24 hours after infection BCG induced the expression of IL-1α (p=0.000001; 1.3 $\log_{10}$) (Figure 3.3a). MPA (p=0.001; 0.6 $\log_{10}$) and cortisol (p=0.000003; 1 $\log_{10}$) inhibited the BCG-mediated expression of IL-1α. NET and progesterone did not alter the BCG-induced secretion of IL-1α. At 72 hours post infection, the IL-1α expression was induced by BCG (p=0.000001; 1.4 $\log_{10}$) compared to the unstimulated control (Figure 3.3c). The BCG-mediated expression of IL-1α was suppressed by MPA (p=0.00003; 0.8 $\log_{10}$) and cortisol (p=0.000001; 1.3 $\log_{10}$). NET and progesterone did not alter the BCG stimulated expression of IL-1α at any of the three time points.

3.3.1.4 IL-1β

Six hours after the stimulation of PBMCs, BCG induced IL-1β secretion (p=0.000001; 1.9 $\log_{10}$). At this early time point the hormones had no effect on the BCG-mediated expression of IL-1β (Figure 3.4a). Similarly 24 hours post BCG stimulation the expression of IL-1β was up-regulated (p=0.000001; 1.4 $\log_{10}$) (Figure 3.4a). Cortisol inhibited this up-regulation (p=0.003). The inhibitory effect was not observed with any of the synthetic progestins or with progesterone. At 72 hours post BCG infection, cortisol (p=0.000000; 1.2 $\log_{10}$) and MPA (0.000001; 0.9 $\log_{10}$) suppressed IL-1β production strongly, however NET (p=0.03) and progesterone (0.03) also suppressed IL-1β production at this time point although not to the same degree as cortisol and MPA.

BCG induced the expression of IL-1β, but at the six hour time point the hormones had no effect on the expression of this cytokine. 24 hours after the addition of BCG, only cortisol suppressed the level of IL-1β significantly. 72 hours after infection all the hormones suppressed the level of expression, but MPA and cortisol to a greater extent than NET and progesterone.
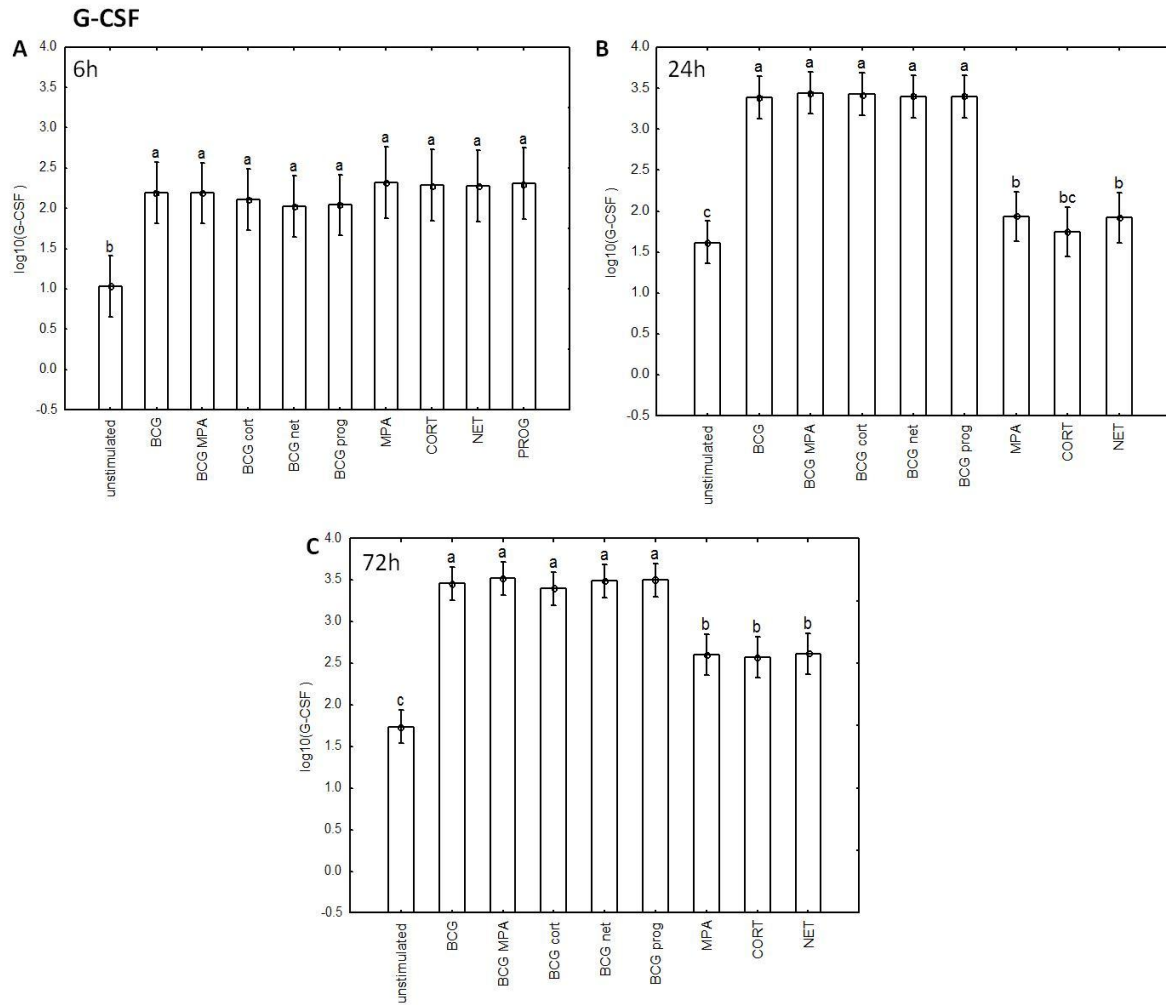
Figure 3.1 G-CSF expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.
The expression of G-CSF was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b and c indicate statistical significance, values with the same letter are not statistically different from each other.

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Figure 3.2 IFN-γ expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.
The expression of IFN-γ was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c, d, e and f indicate statistical significance, values with the same letter are not statistically different from each other.

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IL-1α

Figure 3.3 IL-1α expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone. The expression of IL-1α was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c, d, e and f indicate statistical significance, values with the same letter are not statistically different from each other.

IL-1β

Figure 3.4 IL-1β expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone. The expression of IL-1β was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c, d, e and f indicate statistical significance, values with the same letter are not statistically different from each other.

3.3.1.5 IL-2

Six hours after BCG stimulation PBMCs treated with MPA (p=0.001; 1.1 $\log_{10}$) and NET (p=0.002; 1 $\log_{10}$) showed an increase in the expression level of IL-2 compared to US cells (Figure 3.5a). This up-regulation could be due the androgenic effect of the synthetic progestins. BCG alone did not induce expression of IL-2.

24 hours post BCG infection, BCG induced the production of IL-2 (p=0.00001; 0.6 $\log_{10}$) (Figure 3.5a). The BCG-induced expression if IL-2 was suppressed in by MPA (p=0.012) and cortisol (p=0.004). NET and progesterone did not significantly alter the BCG-induced expression of IL-2. 72 hours after stimulation with BCG the secretion of IL-2 was not induced (Figure 3.5c).

The expression of IL-2 was only induced by BCG after 24 hours. At this time point MPA and cortisol suppressed the BCG-mediated expression of IL-2. NET and progesterone did not have the same effect on IL-2 production. 72 hours after stimulation the induction of IL-2 by BCG has disappeared and all cells stimulated with BCG showed expression levels of IL-2 that are comparable to that seen in the US cells.

3.3.1.6 IL-4

Six hours after BCG stimulation the expression of IL-4 was not induced (Figure 3.6a). The level of IL-4 was higher in the BCG infected MPA treated (p=0.006; 0.4 $\log_{10}$) and BCG infected NET treated (p=0.04) cells compared to the US control. 24 hours after stimulation the expression of IL-4 was not significantly induced by BCG (Figure 3.6b). 72 hours post stimulation the expression of IL-4 was induced by BCG compared to the unstimulated control (p=0.002; 0.6 $\log_{10}$) (Figure 3.6c). The BCG-induced expression of IL-4 was inhibited by cortisol (p=0.02) and progesterone (p=0.05). MPA (p=0.061) and NET (p=0.051) showed a trend of suppressing the BCG-induced expression of IL-4.

The expression of IL-4 was only induced by BCG after 72 hours and significantly inhibited by cortisol and progesterone. PBMCs infected with MPA and NET did have higher levels of IL-4 present after six hours.
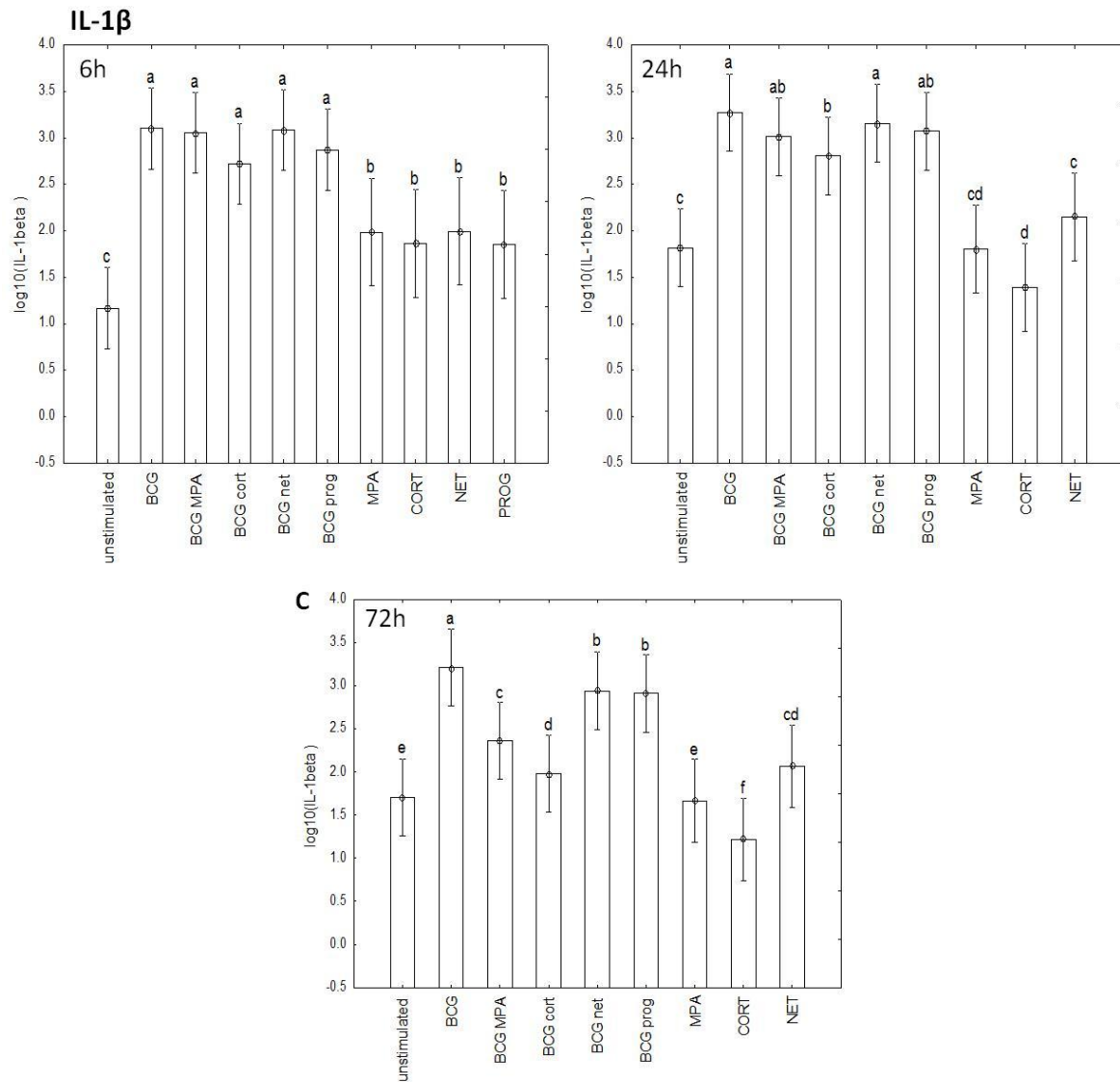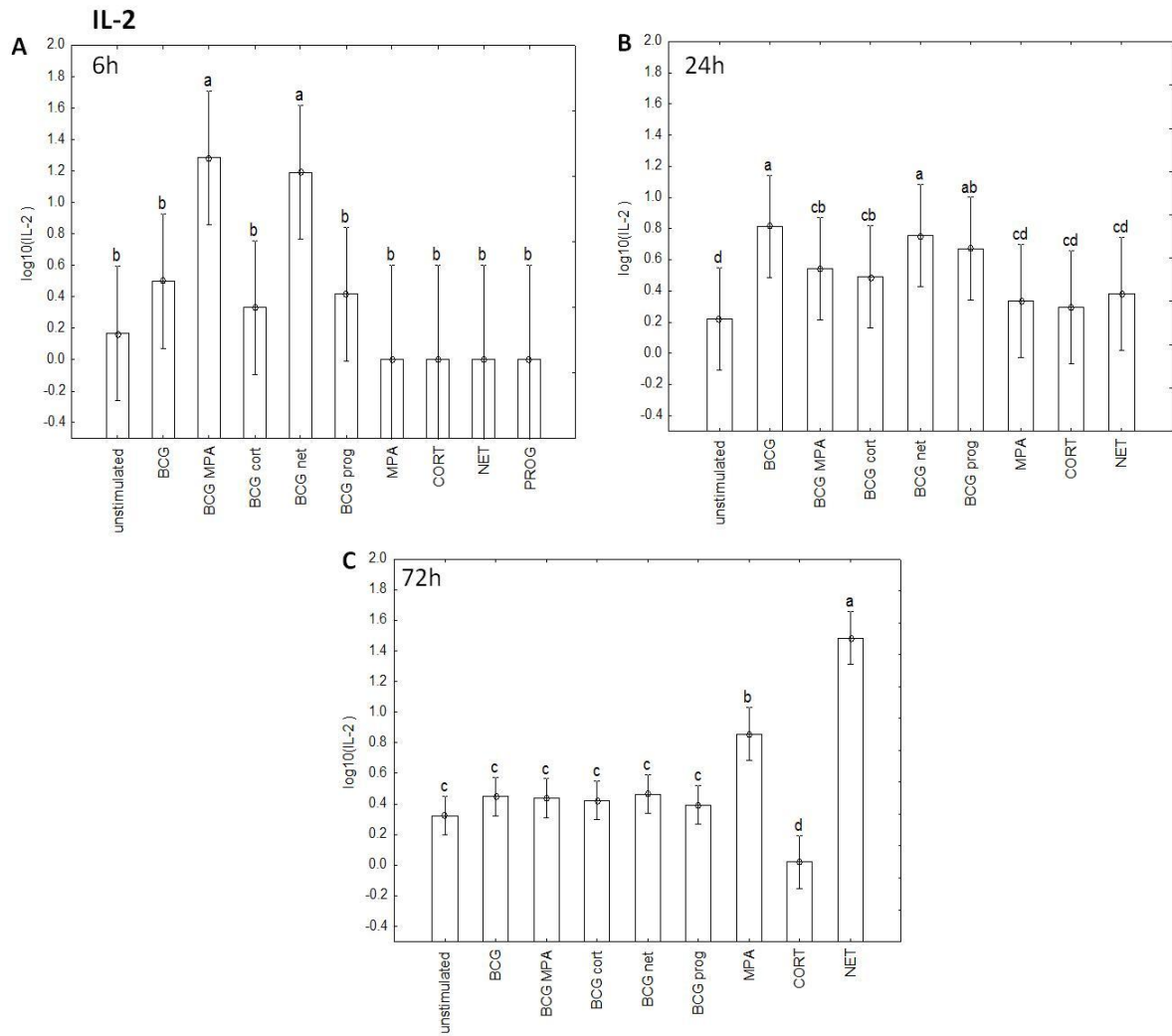
Figure 3.5 IL-2 expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.
The expression of IL-2 was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c and d indicate statistical significance, values with the same letter are not statistically different from each other.

IL-4
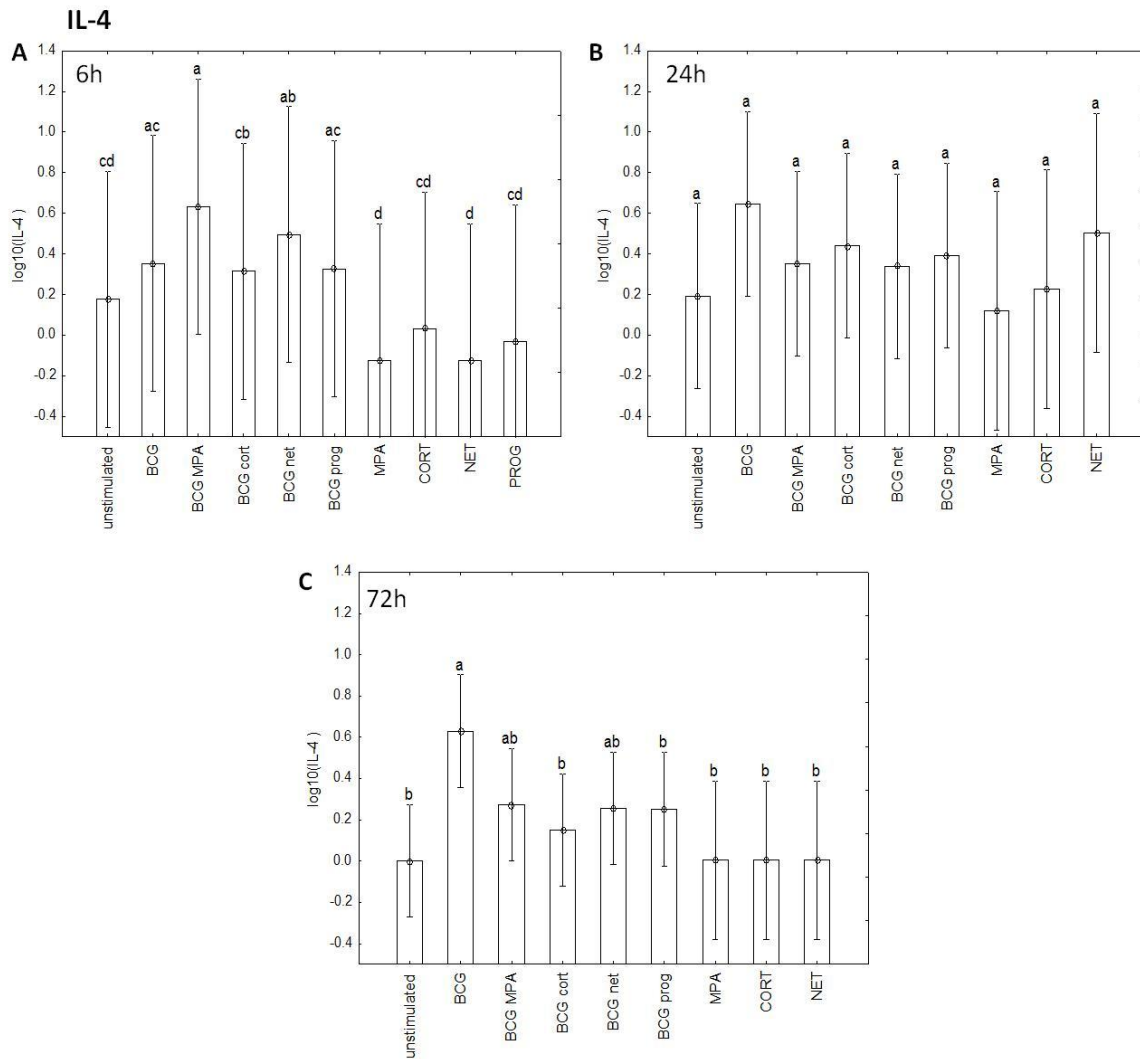
Figure 3.6 IL-4 expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.
The expression of IL-4 was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c and d indicate statistical significance, values with the same letter are not statistically different from each other.

3.3.1.7 IL-6

Six hours post-stimulation BCG induced expression of IL-6 in the BCG stimulated cells (p=0.00004; 1.5 $\log_{10}$) (Figure 3.7a). The presence of the hormones had no effect on the expression of IL-6. 24 hours after BCG stimulation the expression of IL-6 remained elevated (p=0.00004; 0.9 $\log_{10}$) (Figure 3.7b). The hormones still had no effect on the BCG-mediated expression of IL-6 at this time point. Similarly, 72 hours post stimulation the BCG-induced expression was higher than in the US (p=0.00009; 0.7 $\log_{10}$) cells and the hormones had no effect on the expression of this cytokine.

BCG-induced expression of IL-6 however the hormones had no effect on the BCG-induced expression of IL-6 at any time point measured.

3.3.1.8 IL-8

The expression of IL-8 was induced by BCG (p=0.0009; 0.7 $\log_{10}$) and the hormone only after six hours (Figure 3.8a). 24 hours after stimulation BCG did not induce the expression of IL-8. Cortisol did however suppress the level of IL-8 compared to BCG infected and uninfected cells (p=0.02) (Figure 3.8b). 72 hours after stimulation BCG did not induce the expression of IL-8. However NET together with BCG up-regulated IL-8 secretion compared to BCG only stimulated cells (p=0.058) (Figure 3.8c).

IL-8 expression was only induced by the presence of BCG six hours after the stimulation and the hormones had no effect on this BCG-induced expression of IL-8. 72 hours after BCG infection NET induced the expression of IL-8. IL-8 secretion was also induced on cells treated with MPA and NET only, but this did not reach significance.

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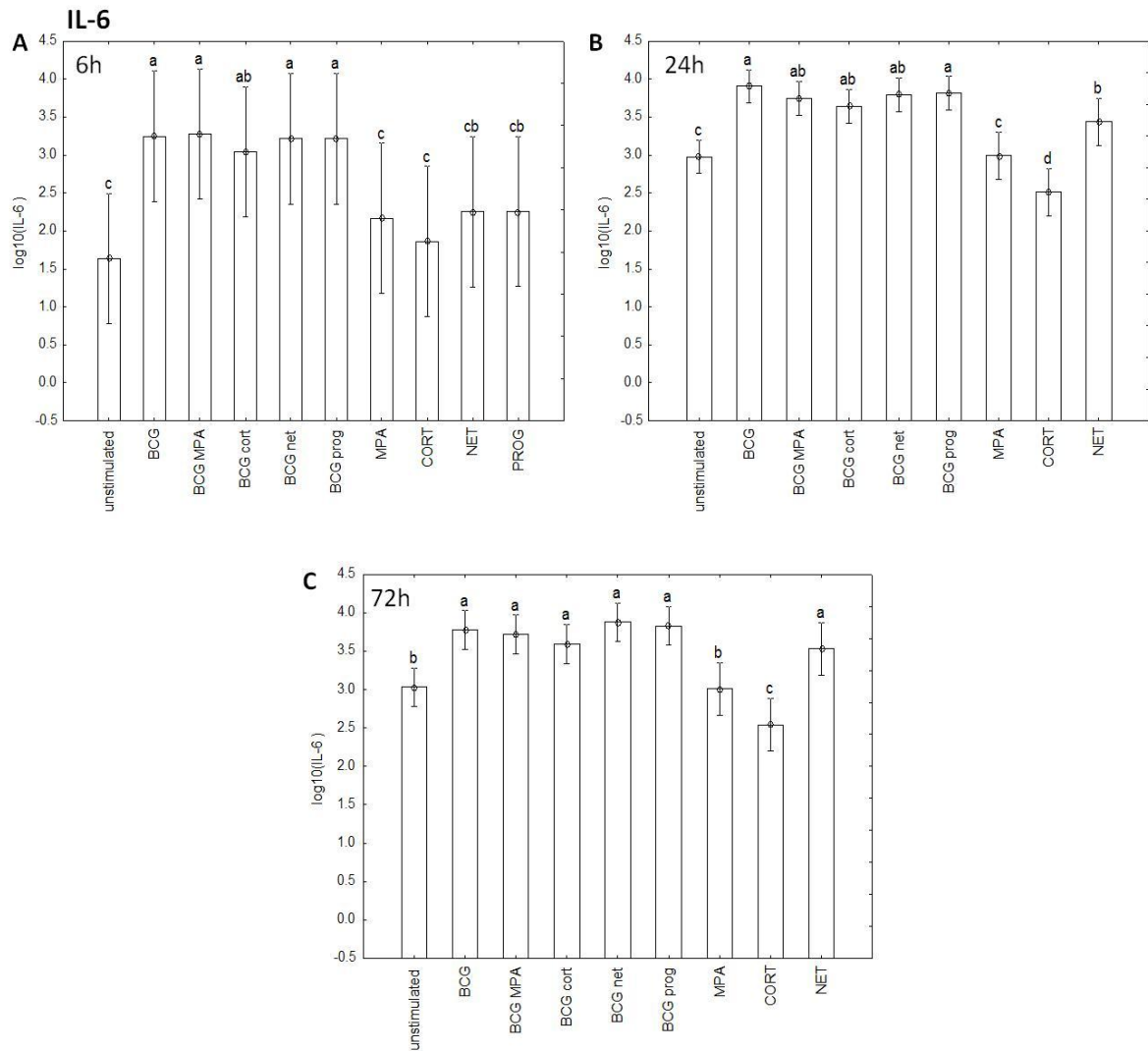
Figure 3.7 IL-6 expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.
The expression of IL-6was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c and d indicate statistical significance, values with the same letter are not statistically different from each other.

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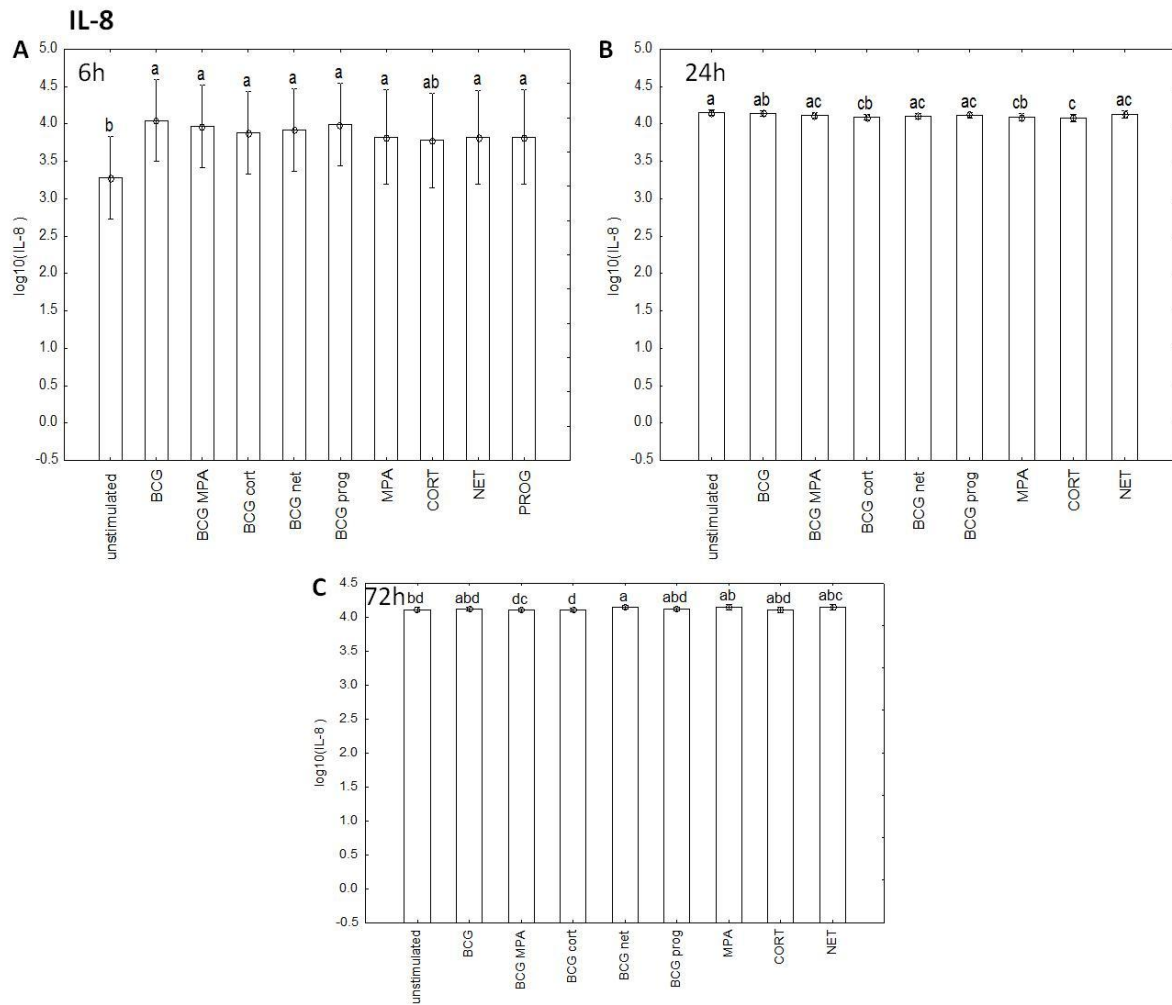
Figure 3.8 IL-8 expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.
The expression of IL-8 was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c and d indicate statistical significance, values with the same letter are not statistically different from each other.

3.3.1.9 IL-10

Six hours after BCG stimulation the expression if IL-10 was induced (p=0.000001; 1.2 $\log_{10}$) (Figure 3.9a). The hormones had no effect on the BCG-induced expression of IL-10. The cells treated with only MPA, NET, cortisol or progesterone also showed an up-regulation in the production of IL-10. 24 hours post BCG stimulation the expression of IL-10 was induced (p=0.00001; 1.2 $\log_{10}$) (Figure 3.9b). The hormones had no effect on the BCG-induced expression of IL-10. Suppression in IL-10 secretion was observed in cells treated with the hormone only. 72 hours post BCG stimulation the expression of IL-10 was still induced (p= 0.000001; 2.1 $\log_{10}$) (Figure 3.9c). The BCG-induced expression of IL-8 was suppressed by MPA (p=0.04) and cortisol (p=0.003). The cells treated with hormone only had an up-regulation in IL-10 expression at this time point, although this up-regulation was not to the same extent as than seen in BCG-infected cells.

The expression of IL-10 was induced by BCG at all time points, MPA and cortisol suppressed the expression of this cytokine 72 hours after BCG stimulation.

3.3.1.10 IL-12p40

Six hours post BCG stimulation IL-12p40 expression was not induced by BCG (Figure 3.10.a). 24 hours after stimulation BCG induced the expression of IL-12p40 (p=0.000001; 1.5 $\log_{10}$) (Figure 3.10b). The BCG-mediated expression of IL-12p40 was suppressed by progesterone (p=0.04), MPA (p=0.001) and cortisol (p=0.00002). 72 hours after BCG stimulation IL-12p40 expression was induced by BCG (p=0.000003; 1.5 $\log_{10}$) (Figure 3.10c). Again this induction was suppressed by progesterone (p=0.05), MPA (p=0.0007) and cortisol (p=0.000001).

The expression of IL-12p40 was induced by BCG only 24 and 72 hours after stimulation. This induction was suppressed by progesterone, MPA and cortisol at both time points. The suppression seen by MPA mimicked the level of suppression of cortisol rather than that of progesterone.
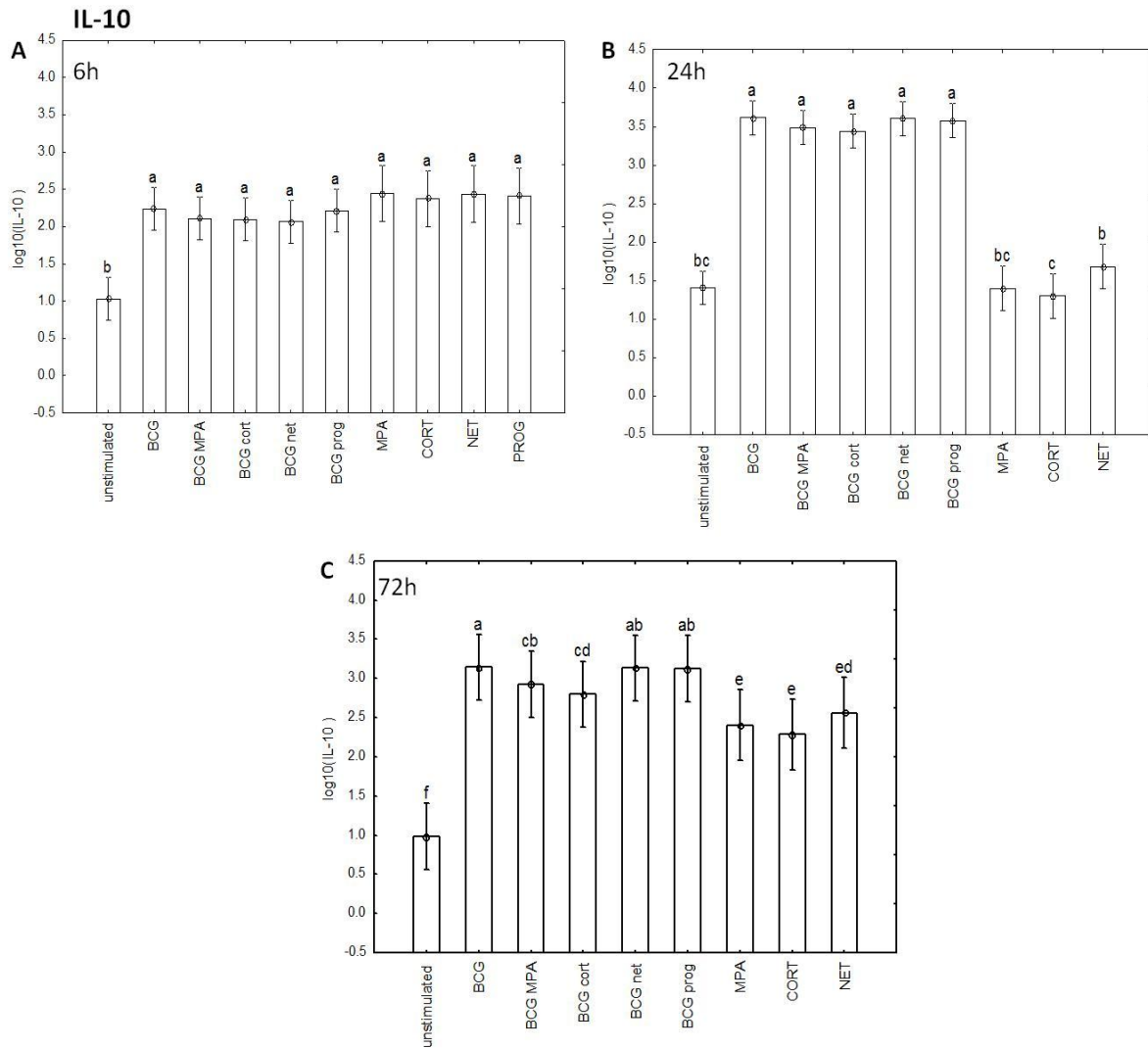
Figure 3.9 IL-10 expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.
The expression of IL-10 was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c, d, e and f indicate statistical significance, values with the same letter are not statistically different from each other.
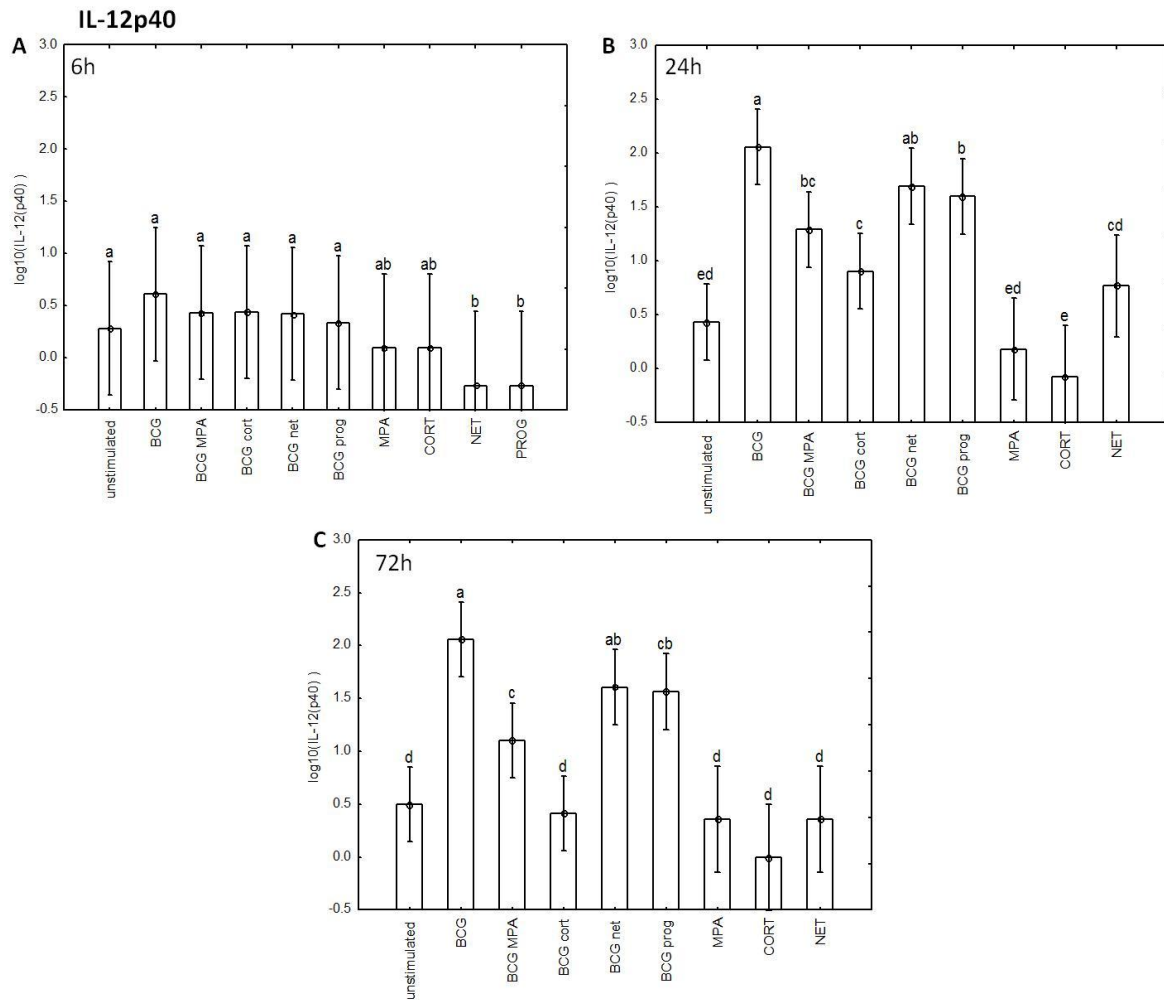
Figure 3.10 IL-12p40 expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.

The expression of IL-12p40 was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c, d and e indicate statistical significance, values with the same letter are not statistically different from each other.

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3.3.1.11 IL-12p70

Six hours after BCG stimulation the expression of IL-12p70 was not induced by BCG (Figure 3.11a). MPA caused an up-regulation of IL-12p70 secretion compared to the unstimulated control (p=0.03; 0.1 $\log_{10}$) at this time point. 24 hours after stimulation BCG induced the expression of IL-12p70 (p=0.003; 0.4 $\log_{10}$). This induction was suppressed by MPA (p=0.013) and cortisol (p=0.014) (Figure 3.11b). 72 hours after infection BCG mediated the expression of IL-12p70 (p=0.00002; 0.4 $\log_{10}$) (Figure 3.11c). The BCG-induced production of IL-12p70 was suppressed by progesterone (p=0.0005), NET (p=0.013), MPA (p=0.0003) and cortisol (p=0.0001).

The BCG-induced expression of IL-12p70 was suppressed by MPA and cortisol after 24. 72 hours after stimulation the BCG-induced expression IL-12p70 was suppressed by MPA, cortisol, progesterone and NET. The suppression by MPA was comparable to that of cortisol and progesterone.

3.3.1.12 IL-13

Six hours after BCG stimulation, BCG only induced the production of IL-13 in PBMCs that were treated with the synthetic hormones MPA (p=0.003; 0.3 $\log_{10}$) and NET (p=0.016; 0.28 $\log_{10}$) (Figure 3.12a). After 24 hours of BCG stimulation the expression of IL-13 was induced (p=0.000001; 1.3 $\log_{10}$) (Figure 3.12b). The BCG-mediate expression of IL-13 was suppressed by progesterone (p=0.05), MPA (p=0.000001) and cortisol (p=0.000003). 72 hours after stimulation the BCG-induced the expression of IL-13 (p=0.000001; > 1.5 $\log_{10}$) (Figure 3.12c). MPA (p=0.00001) and cortisol (p=0.000001) suppressed the BCG-induced expression of IL-13.

BCG induced the expression of IL-13 after 24 hours of stimulation. At this time point the induction by BCG was suppressed by Prog, MPA and Cort. The suppression seen by MPA was greater than the suppression by Prog and mimicked the suppression of Cort. After 72 hours the suppression by Prog disappeared and only MPA and Cort showed suppression of IL-13 secretion.

3.3.1.13 TNF-α

Six hours after BCG stimulation BCG induced the expression of TNF-α (p=0.00003; 1.4 $\log_{10}$). The hormones had no effect on the BCG-induced expression of TNF-α (Figure 3.13a). 24 hours after BCG stimulation BCG still induced the expression of TNF-α (p=0.000001; 1.5 $\log_{10}$) (Figure 3.13b). MPA (p=0.000001), cortisol (p=0.000003) and progesterone (p=0.049) inhibited the BCG-induced expression of TNF-α at this time point. Similarly 72 hours after BCG stimulation the secretion of

TNF-α was induced by BCG (p=0.000003; 1.9 log$_{10}$) (Figure 3.13c). The BCG-mediated expression of TNF-α was inhibited by MPA (p=0.000003), cortisol (p=0.000001), NET (p=0.02) and progesterone (p=0.02).

The expression of TNF-α was induced by BCG as soon as six hours after stimulation. Six hours after stimulation the hormones had no effect on the expression of this cytokine. 24 hours after stimulation MPA, cortisol and progesterone suppressed the BCG-induced expression of TNF-α, MPA and cortisol to a greater extent than progesterone. 72 hours after stimulation MPA, cortisol, progesterone and NET suppressed the expression of TNF-α. MPA again mimiced cortisol in the level of suppression and NET progesterone.
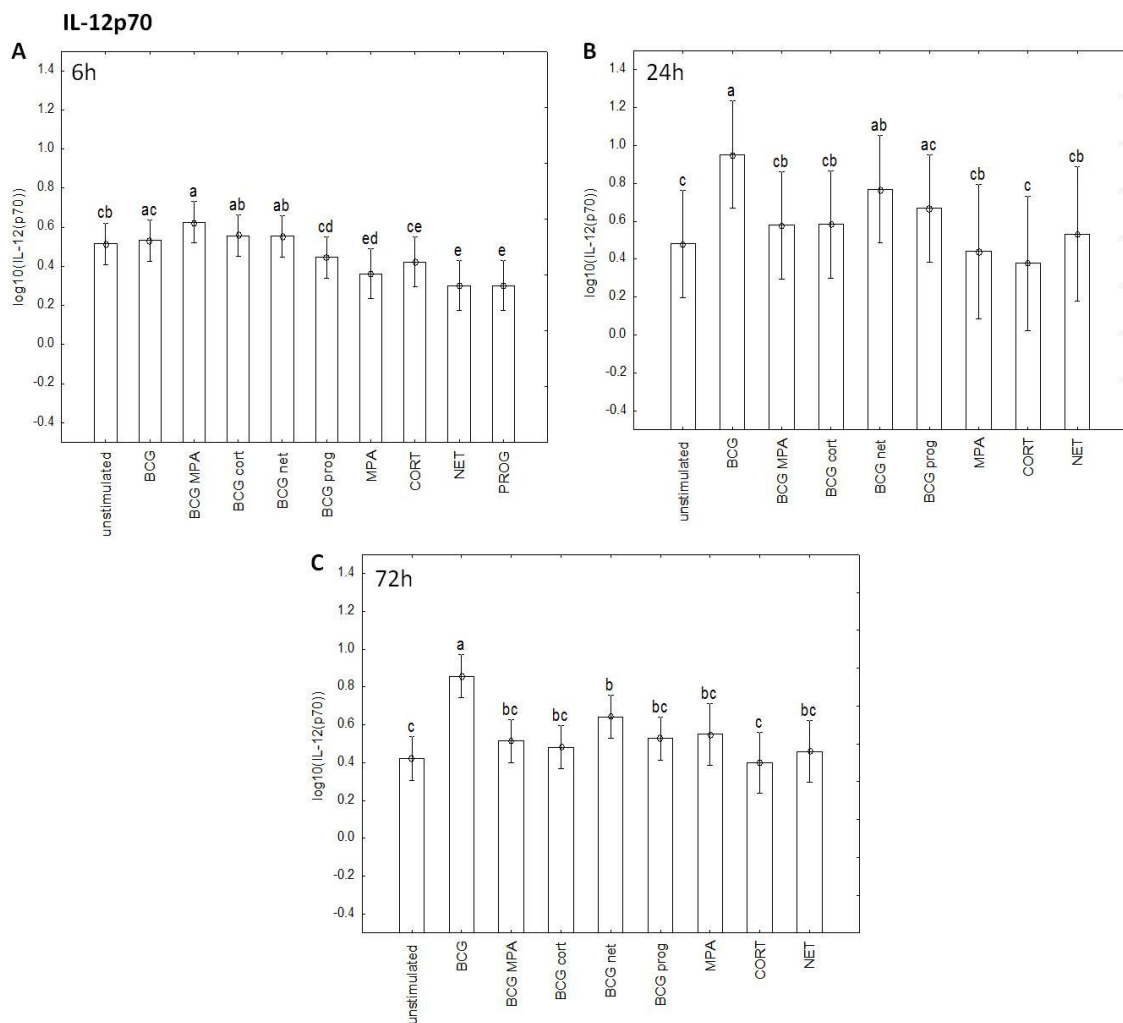
Figure 3.11 IL-12p70 expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.
The expression of IL-12p70 was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c, d and e indicate statistical significance, values with the same letter are not statistically different from each other.
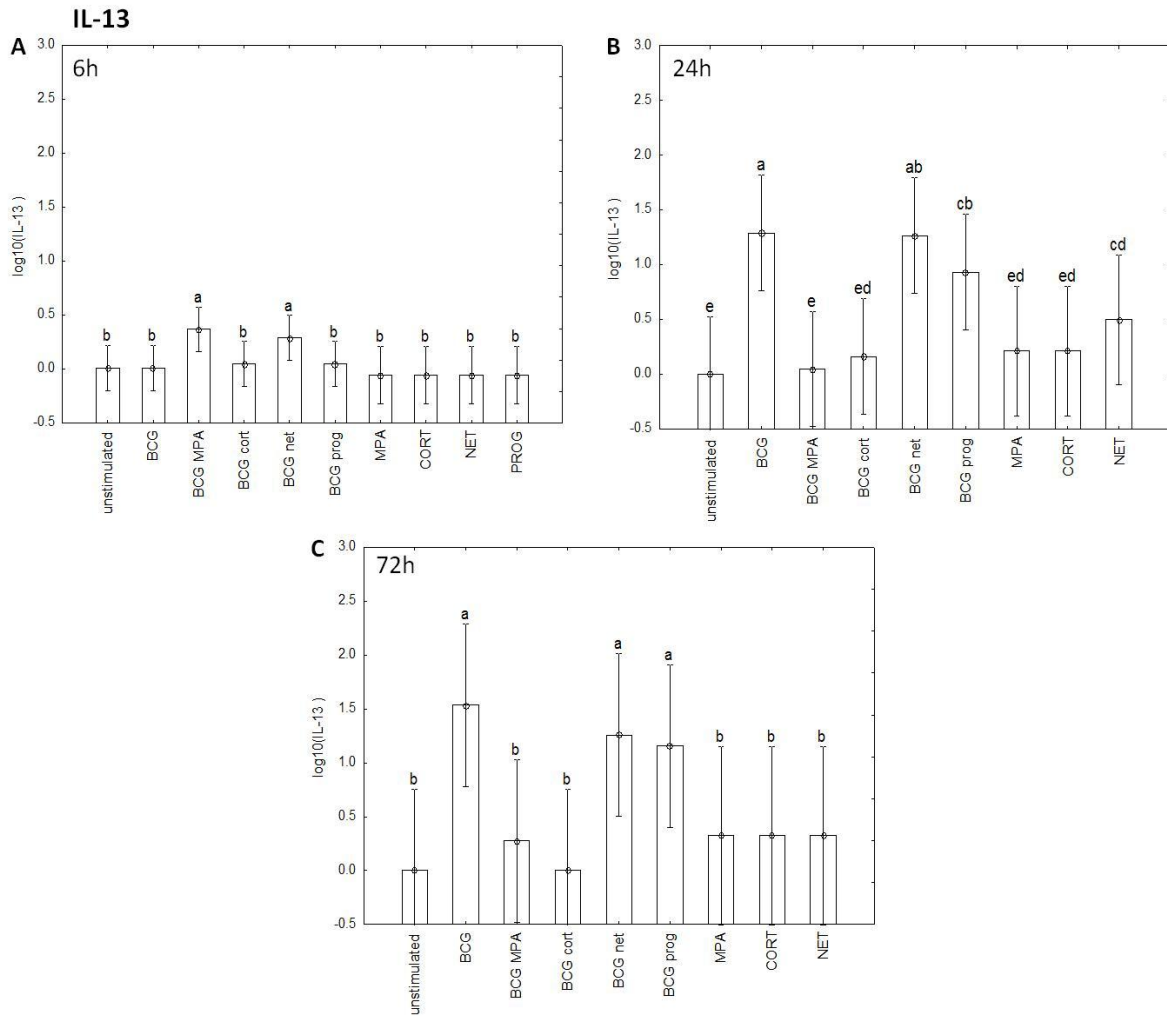
Figure 3.12 IL-13 expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.
The expression of IL-13 was measured at six hours (a), 24 hours (b) and 72 hours (c) post infection with BCG. Each condition was done in duplicate and the pooled results from two separate experiments shown. Data were analysed by variance estimation, precision and comparison ANOVA (LS means, 95% CI). Error bars indicate the 95% CI. A p-value of < 0.05 was considered significant. The letters a, b, c, d and e indicate statistical significance, values with the same letter are not statistically different from each other.

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Figure 3.13 TNF-α expression in PBMCs infected with BCG and treated with MPA, NET, cortisol and progesterone.

3.3.2 MiRNA expression profiles in PBMCs differ between unstimulated, BCG infected and BGC infected and MPA, NET or cortisol treated groups.

MiRNAs play an important role in fine tuning the immune response to pathogens (42), their expression can be quickly induced or suppressed giving them a fast-acting effect. The expression of some miRNAs can be altered not only by the host, but also by the infecting pathogen (60). The expression of certain miRNAs is affected by the presence of steroid hormones such as cortisol. It is known that some miRNAs (eg. hsa-miR-130b and hsa-miR-124) directly target the mRNA of the GR (55,97).

The miRNA expression pattern differed between the treatment with the various hormones, showing little similarities between the conditions. The expression of individual miRNAs was then investigated.

For this reason the expression profile of the 22 miRNAs found to be differentially expressed in BCG infected PBMCs treated with cortisol, MPA or NET was determined by an unbiased analysis approach using the Qlucore Omics Explorer software. It was investigated whether the expression profiles could distinguish between the different stimulation conditions and if the profile of the MPA treated cells showed similarities to that of the cortisol treated cells as it is known that MPA binds and signals through the GR. Each miRNA was normalised to the mean of the expression in all stimulations. The up- or down-regulation of the expression of miRNAs were determined using the variance of the mean expression of a specific miRNA between the stimulations. The colour red corresponds to a variance of 2+ of the mean expression across the stimulations and the colour green to a variance of -2 to the mean expression of the miRNA across the stimulations. The colour black indicates the mean. Hierarchical clustering of miRNAs by the software was unbiased and based on correlation.

In the unstimulated cells the miRNA in cluster C was up-regulated (hsa-miR-30c) (Figure 3.14). In the BCG infected cells some miRNAs of cluster A (consisting of has-miR-146a, hsa-miR-126, hsa-miR-122, hsa-miR-150, hsa-miR-422a, hsa-miR-335, hsa-miR-331-3p and hsa-miR-342-5p), cluster D (hsa-miR-484, hsa-miR-186 and hsa-miR-29a) and cluster E (consisting of hsa-miR-454 and hsa-miR-222) were up-regulated compared to the unstimulated cells. The BCG infected and MPA treated cells showed an up-regulation in the expression of the miRNAs in cluster B (hsa-miR-19b, hsa-miR-16, hsa-miR-30b, hsa-miR-191, hsa-miR146b-5p,hsa- miR-342-3p, hsa-miR-142-3p, and hsa-miR-223) and cluster C was up-regulated compared to BCG infected cells. PBMCs infected with BCG and treated with NET had an up-regulation of the miRNAs in cluster A, cluster B and cluster C compared to the BCG infected only cells. The up-regulation of cluster D was less than that of BCG infected and MPA treated cells. BCG infected and cortisol treated cells had an up-regulation of cluster A, cluster B and cluster C compared to the BCG infected cells. The up-regulation of cluster B in these cells was less than the up-regulation seen in BCG infected and NET treated cells.

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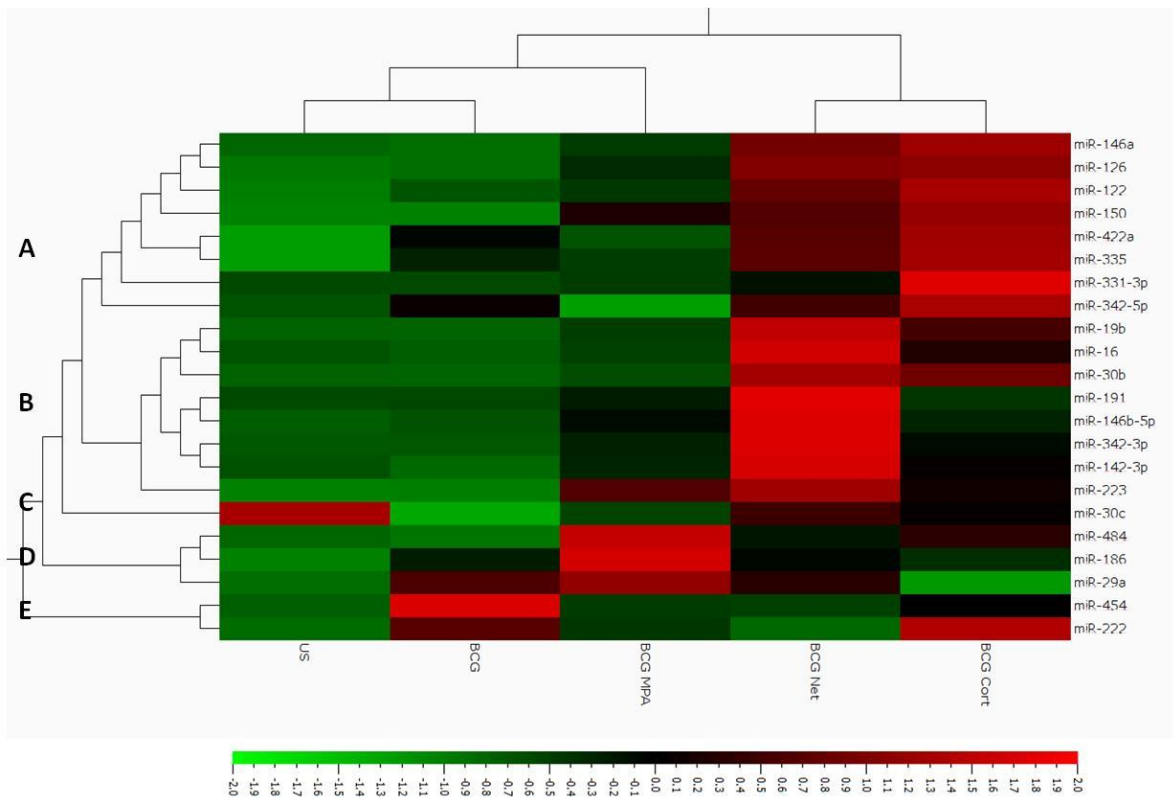
Figure 3.14 Differential expression of miRNAs in the RNA of PBMCs stimulated with BCG and MPA, NET or cortisol for 6 hours.
Over-expressed miRNAs are shown in red and under-expressed miRNAs in green. LS means were used to for the unbiased clustering of the conditions. A heatmap was generated using the Qlucore omics explorer software. The letters A, B, C, D and E represent miRNA clusters that the software created based on the correlation between the miRNAs. Variations in miRNA expression was determined using a variance to the mean approach. The colour red indicates a variance of 2+ from the mean and the colour green a variance of 2- from the mean.

3.3.3 Expression of individual miRNAs in PBMCs differs between unstimulated, BCG infected and BGC infected and hormone treated groups.

The expression pattern of the 22 miRNAs found to be differentially expressed between the stimulations varied between each condition. Therefore statistical analysis was done on the expression of each individual miRNA to assess the BCG-induced response and the effect of the hormones MPA, cortisol and NET on this response.

Hsa-miR-222 was up-regulated by BCG, but did not reach statistical significance. Cortisol caused a further up-regulation in the expression of hsa-miR-222 and it was significantly higher than the expression in unstimulated (p=0.043), BCG infected NET treated (p=0.03) and BCG infected MPA treated (p=0.049) cells (Figure 3.15a). NET treatment caused a suppression of BCG-induced

production of hsa-miR-222 (p=0.048). The expression of hsa-miR-222 in the BCG infected MPA treated and BCG infected NET treated cells was comparable to the unstimulated cells.

The level of hsa-miR-30c was suppressed in all conditions when compared to the expression in unstimulated cells. The level of down-regulation in the BCG infected (p=0.012) and the BCG infected MPA treated (p=0.032) cells when compared to the unstimulated cells was significant (Figure 3.15b). The BCG-induced suppression of the expression of hsa-miR-30c was partially reversed by NET (p=0.02) but did not restore the level of this miRNA to basal level.

The BCG infected cortisol treated cells showed significant up-regulation in expression of hsa-miR-331-3p compared to the unstimulated (p=0.021), BCG infected (p=0.015), BCG infected MPA treated (p=0.017) and BCG infected NET treated (p=0.026) cells (Figure 3.14c).

The expression of hsa-miR-454 was significantly up-regulated in the BCG stimulated cells compared to unstimulated cells (p=0.003) (Figure 3.15d). Cortisol (p=0.008), MPA (p=0.002) and NET (p=0.002) suppressed this BCG-mediated up-regulation and resulted in hsa-miR-454 expression comparable to basal levels.

Other miRNAs that were expressed did not show significant differences in the expression level between the different conditions. Although the differences were not significant some do show patterns that are interesting and could possibly still have a physiological effect as only small up- or down-regulations in the expression of miRNAs can still lead to an alteration in the expression of their targets.

The miRNAs found to be expressed but not altered by BCG stimulation or the presence of hormones are: hsa-miR-122, hsa-miR-126, hsa-miR-142-3p , hsa-miR-146a, hsa-miR-146b-5p, hsa-miR-150 , hsa-miR-16, hsa-miR-186, hsa-miR-191, hsa-miR-19b, hsa-miR-223, hsa-miR-29a , hsa-miR-30b, hsa-miR-335, hsa-miR-342-3p, hsa-miR-342-5p, hsa-miR-422a and hsa-miR-484 . Graphs can be found in appendix A.
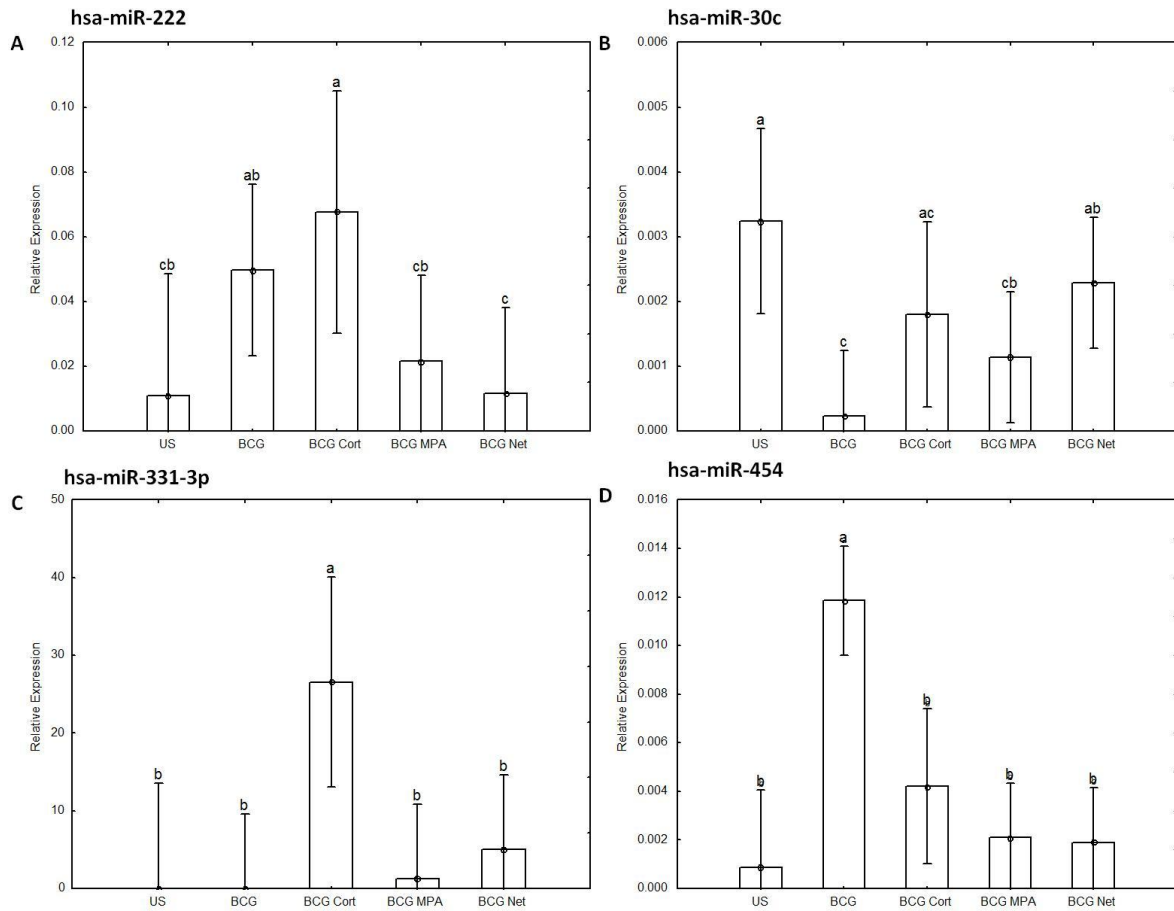
Figure 3.15The relative expression of miRNAs is not different between uninfected, BGC infected, BGC infected and cortisol, MPA or NET treated PBMCs.

Relative expression levels of miR-122 (a), miR-126 (b), miR-142-3p (c), miR-146a (d), miR-146b-5p (e) and miR-150 (f) in PBMCs stimulated with BCG in the presence of cortisol, MPA and NET for 6 hours. Experiments were done in duplicate and whiskers indicate 95% confidence interval. Each condition was done in duplicate. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a, b and c indicate statistical significance, values with the same letter are not statistically different from each other.

3.3.4 Ingenuity Pathway Analysis (IPA) of miRNAs in PBMCs

The Ingenuity Software was used to analyse the relationship between the miRNAs found to be expressed in PBMCs under different stimulation conditions. The software uses the expression pattern of the miRNAs during a certain stimulation and determines the and networks they are most likely involved in. The IPA software puts the data into the biological context and identifies any other miRNAs that are either influenced by the miRNAs tested in our panel or influence those miRNAs. In other words the software shows how the miRNAs are connected and regulated each other.

It is not surprising that IPA analysis showed that the miRNAs up-regulated in BCG infected cells play important roles in immunological, inflammatory and infectious diseases. miRNAs involved in cellular developmment, function, maintenance, growth and profileration were also up-regulated (Fig.3.19). The relative abundance of miRNAs expressed in the BCG infected MPA treated cells was lower compared to the other stimulations for the predicted cellular functions; cell death and survival, cellular growth and proliferation, cell-to-cell signalling and interaction and cellular assembly and organization.
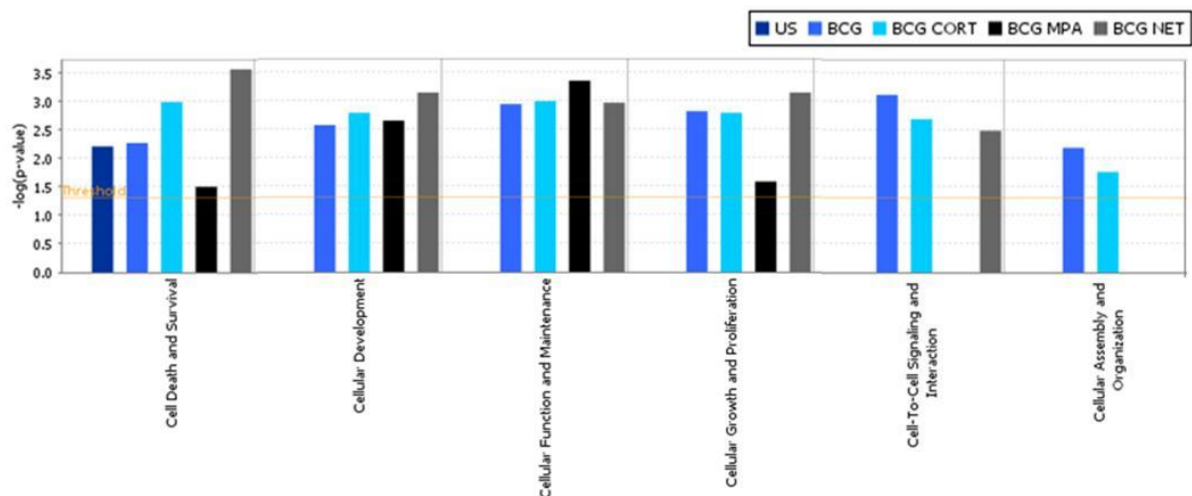
Figure 3.16 Relative abundance of miRNAs linked to the immune system in the different stimulation conditions.
The Ingenuity Software was used to depict the relative abundance of miRNAs expressed in unstimulated, BCG infected and BCG infected and MPA, cortisol and NET treated PBMCs that have predicted functions in cellular procesess.

In unstimulated cells not many netwotks were hypthesised to be activated and only hsa-miR-30c was found to be expressed (Figure 3.20). The B-cell lymphoma 6 (BCL6) protein was predicted to be directly regulated by hsa-miR-30c. This protein modulates the STAT-dependant IL-4 responses. Compared to unstimulated cells the cells stimulated with BCG were predicted to have many networks activated and an extensive miRNA-target molecule network exist (Figure 3.21). The expression of the transcription factor PTEN was predicted to be directly and indirectly influenced and this could

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influence the survival of T cells. MiRNAs could be regulating their own function and expression as Ago2 and DICER are part of the network. Ago2 forms an integral part of the network and regulates many miRNAs found to be expressed. The MAPK signalling pathway is also predicted to be directly and indirectly influenced by the expression of miRNAs in these cells. The Ras signalling pathway was also implicated. This pathway can activate the transcription factor NFκB. It is know that NFκB is important during the immune response to *M.tb*. The network generated for the BCG and MPA stimulated cells is much less complex than the one generated for the BCG stimulated cells and could be indicative of the immune suppressive effect of MPA (Figure 3.22). Ago2 forms an integral part of this network and had interactions with all the miRNAs expressed in these cells. The cells stimulated with BCG and cortisol had a more extensive network than the BCG and MPA stimulated cells (Figure 3.23). Insulin receptor substrate 1 (IRS1), a molecule that transmits signals from insulin like growth factor to the MAPK signalling pathways is found in the network. Consistent with this MAPK2, which itself is regualted by miRNA, forms part of this network. The network generated for cells stimulated with BCG and NET Ago2 and MAPK2 was shown to be regulated by miRNAs expressed in these cells (Figure 3.24).
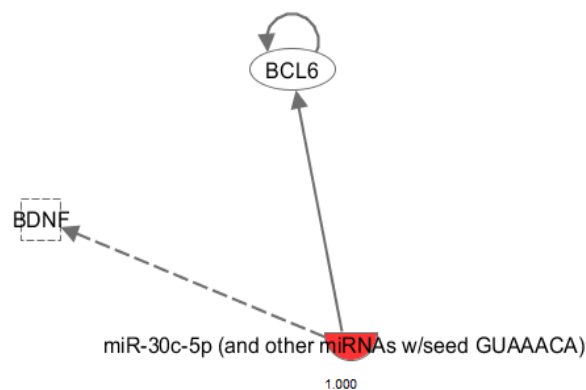
Figure 3.17 Network of interactions predicted using the IPA Software for miRNAs up-regulated in unstimulated PBMCs.
Network of miRNAs predicted to be involved in a biological network after six hours in unstimulated PBMCs from healthy TST positive non-contraceptive using females. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.
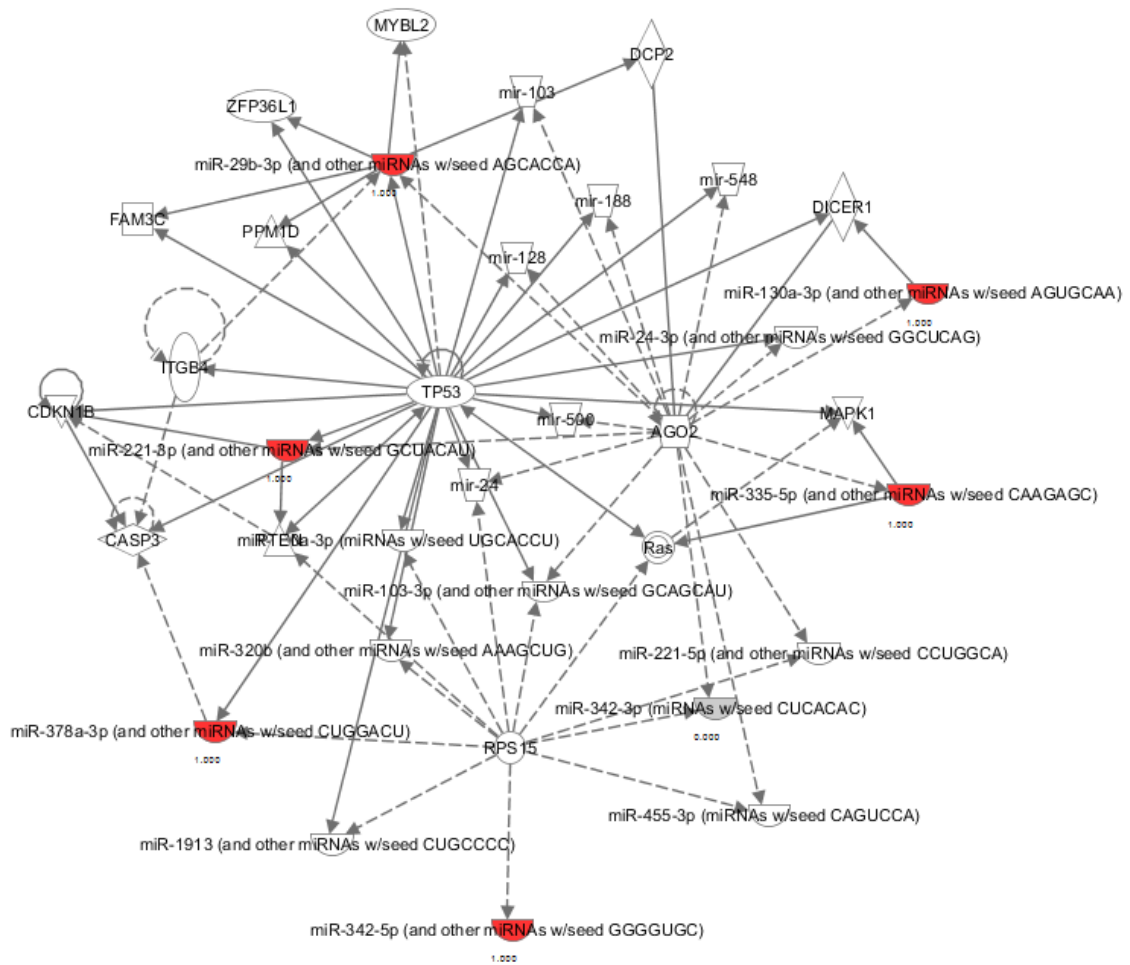
Figure 3.18 Network of interactions predicted using the IPA Software for miRNAs up-regulated in BCG stimulated PBMCs.
Network of miRNAs predicted to be involved in a biological network after six hours in unstimulated PBMCs from healthy TST positive non-contraceptive using females. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.

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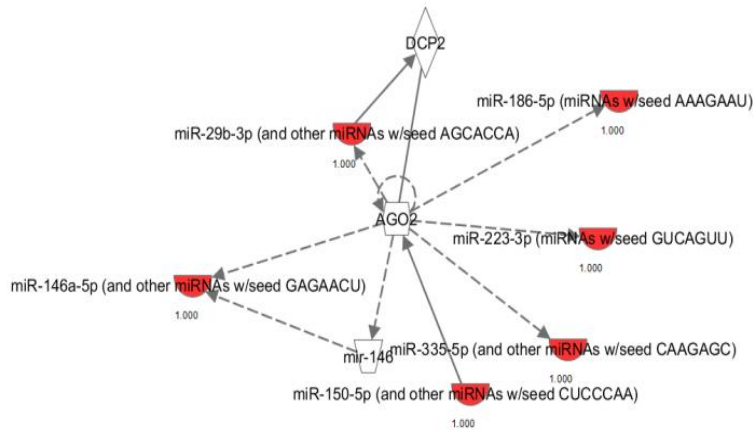
Figure 3.19 Network of interactions predicted using the IPA Software for miRNAs up-regulated in BCG and MPA stimulated PBMCs.
Network of miRNAs predicted to be involved in a biological network after six hours in unstimulated PBMCs from healthy TST positive non-contraceptive using females. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.
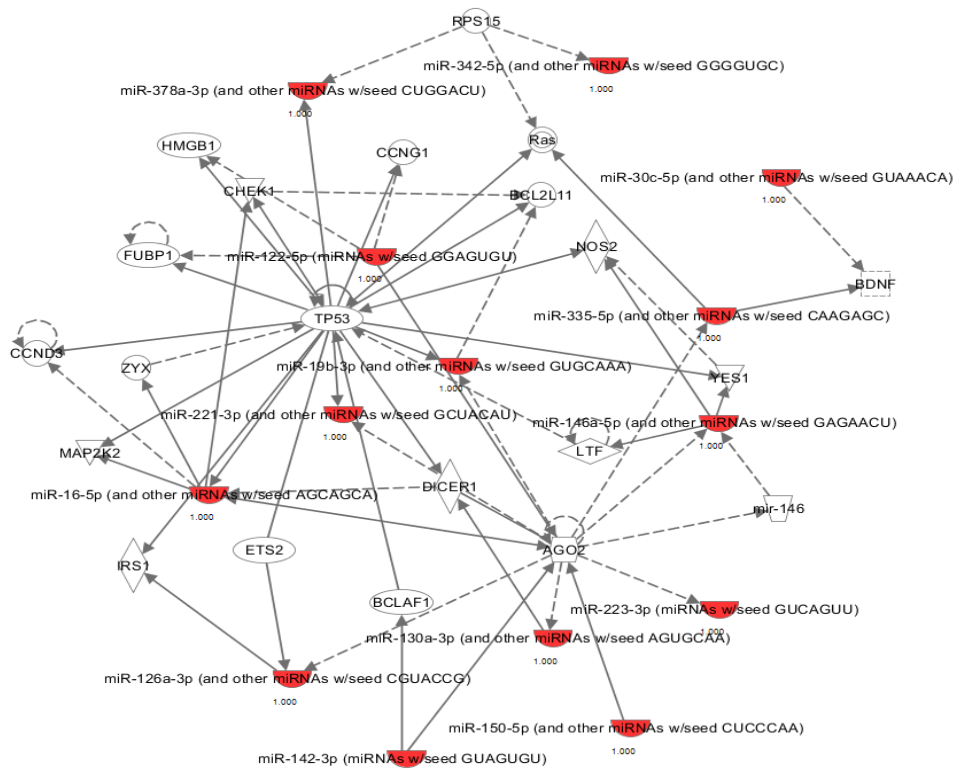
Figure 3.20 Network of interactions predicted using the IPA Software for miRNAs up-regulated in BCG and cortisol stimulated PBMCs.
Network of miRNAs predicted to be involved in a biological network after six hours in unstimulated PBMCs from healthy TST positive non-contraceptive using females. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.
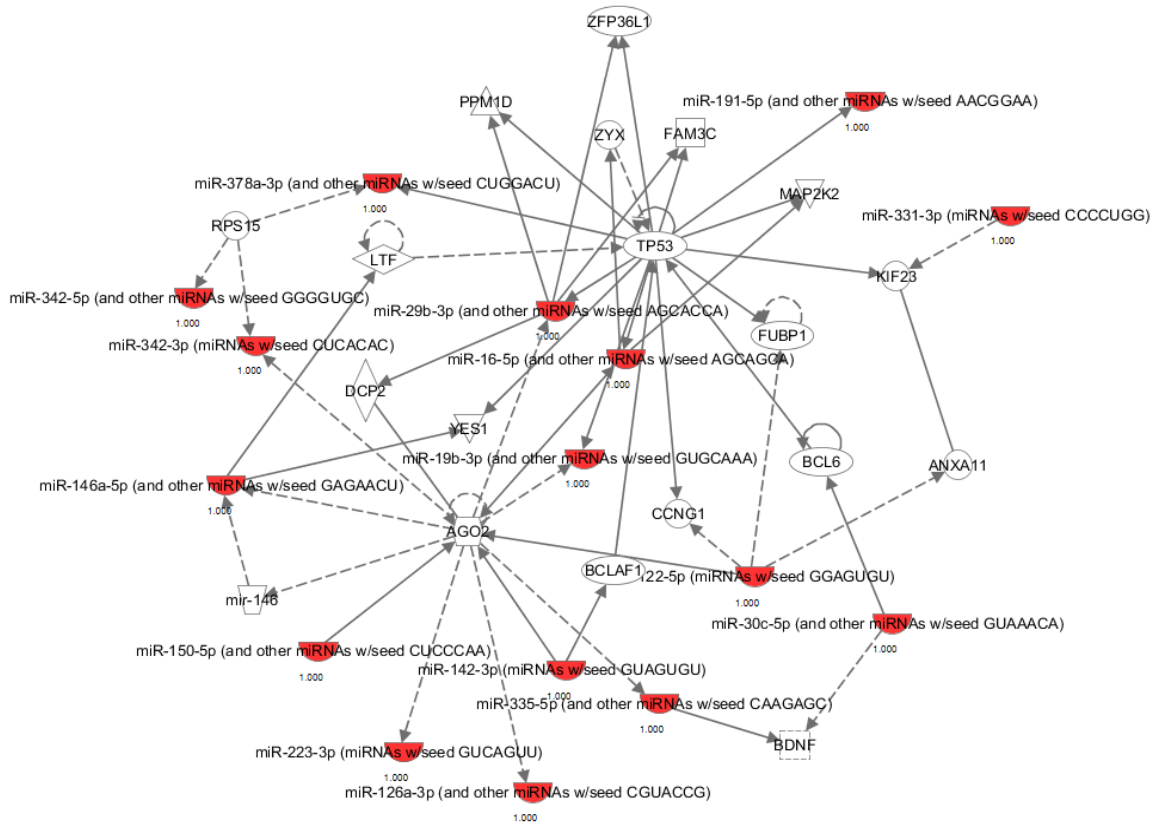
Figure 3.21 Network of interactions predicted using the IPA Software for miRNAs up-regulated in BCG and NET stimulated PBMCs.
Network of miRNAs predicted to be involved in a biological network after six hours in unstimulated PBMCs from healthy TST positive non-contraceptive using females. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.

3.4 Summary

MPA is a synthetic progestin that binds to the PR with high affinity but also has selective GC activity and can bind to the GR and alter the expression of GC-sensitive genes (93). This study investigated the effect of high concentrations of MPA, cortisol, NET and progesterone on the BCG-induced cytokine and miRNA expression in PBMCs of healthy, TST positive women that are not using any hormonal contraceptives. The results of this study indicate that MPA alters the BCG-induced expression of many cytokines (IFN-γ, IL-1α, IL-1β, IL-2, IL-12p40 and TNF-α) in a way that mimics cortisol rather than progesterone, while NET alters the cytokine expression in a way that mimics progesterone.

The miRNA expression patterns in BCG stimulated cells as well as BCG stimulated and MPA, NET and cortisol treated cells are distinctly different form each other. This highlights the unique pharmacological characteristics of each steroid hormone and demonstrates that the similarities between MPA and cortisol are not reflected on miRNA level.

Nevertheless, the drastic effect of each of the hormones on miRNA expression in these cells suggests that they are modulating the immune response to BCG at transcriptional level.

Chapter 4: Medroxyprogesterone acetate and norethisterone enanthate differentially alter miRNA expression in a mouse model

4.1 Introduction

MPA is known to have selective glucocorticoid activity and it is possible that pharmacological doses of MPA could modulate the immune response to BCG. MPA has previously been found to alter the immune response to *M.tb* in mice (88). Mice treated with MPA had a higher bacterial burden in their lungs and altered cytokine expression (88). The effect of NET during mycobaterial infection has not been investigated; similarly the effect of MPA and NET on miRNA expression during mycobacterial infection has not yet been described. This part of the study aimed to determine whether a relationship exists between miRNA expression and bacterial burden in mice infected with BCG and treated with MPA or NET.

4.2 Experimental design

C57BL/6 mice were injected weekly with 20 mg/ml and 26 mg/ml MPA and NET respectively. One week after the first injection they were intranasally infected with $2x10^6$ CFUs BCG in 20 µl saline. Twenty one and 56 days after infection the mice were killed and their lungs, spleens and blood harvested. The right upper lobe of the lung from each mouse as well as the blood was analysed for miRNA expression experiments. The remainder of the lung and spleen were homogenized to determine the bacterial burden. Due to financial constraints only the RNA from the day 21 time point could be used for miRNA expression analysis. Two independent experiments were done and the data pooled.

4.3 Results

4.3.1 BCG infected mice treated with MPA have a higher bacterial load in the spleen.

The CFU counts at day one after infection did not significantly differ between experiments and results of the two experiments were comparable (Figure 4.1). At 21 and 56 days post infection five mice per group were killed and their lungs and spleens harvested to determine the bacterial burden. The lungs were used because it is the site of infection and the spleens because it is indicative of disease dissemination. The CFUs observed in the lung of BCG infected mice 21 and 56 days post infection

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did not differ from the CFUs counted in the BCG infected MPA treated or BCG infected NET treated mice (Figure 4.2a-b). The CFUs counted in all groups at day 21 was higher than that counted at day 56 (Figure 4.2c).

The bacterial burden observed in the spleens of mice infected with BCG and treated with MPA was higher than BCG infected mice 21 days after infection (p=0.02) (Figure 4.3a). The CFUs counted in the BCG infected NET treated mice did not differ from the BCG infected or the BCG infected MPA treated mice. This difference observed in bacterial burden between the BCG infected mice and BCG infected and MPA or NET treated mice disappeared by day 56 (Figure 4.3b). The CFUs of mice infected with BCG and treated with MPA or NET did not increase from day 21 to 56, BCG infected mice had a significant increase (p=0.006; 1 $\log_{10}$) from day 21 to 56 which appeared to account for the disappearance of a difference by day 56 (Figure 4.3c).
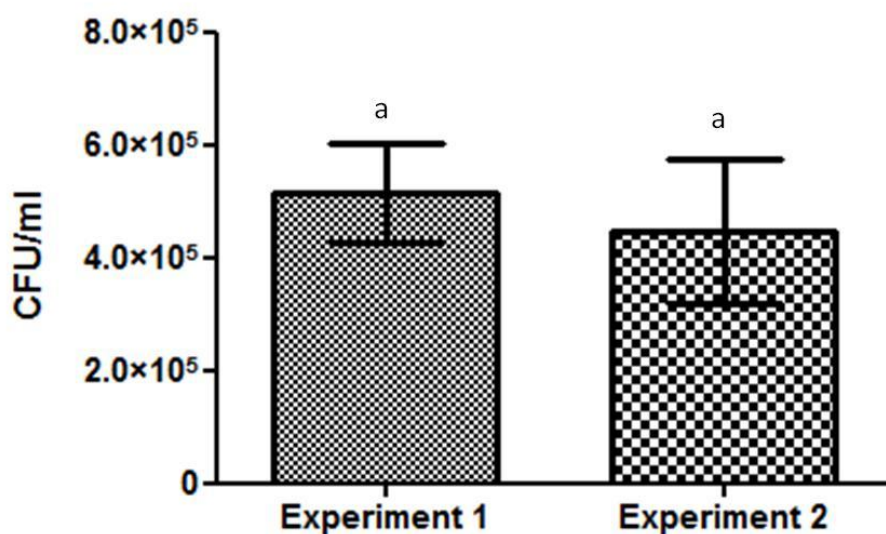
Figure 4.1 The bacterial burden in C57BL/6 mice one day after infection does not differ between experiments.
C57BL/6 mice were intranasally infected with 2×10^6 CFUs of BCG in 20 µl of PBS to confirm up take of bacilli in the lungs one day post-infection. Each experiment represents the CFU counts of 5 individual mice. No difference was seen between the two experiments. Differences were determined by the unpaired t-test and represented as the mean and standard error of the mean (SEM). The letter a indicates statistical significance, values with the same letter are not statistically different from each other.
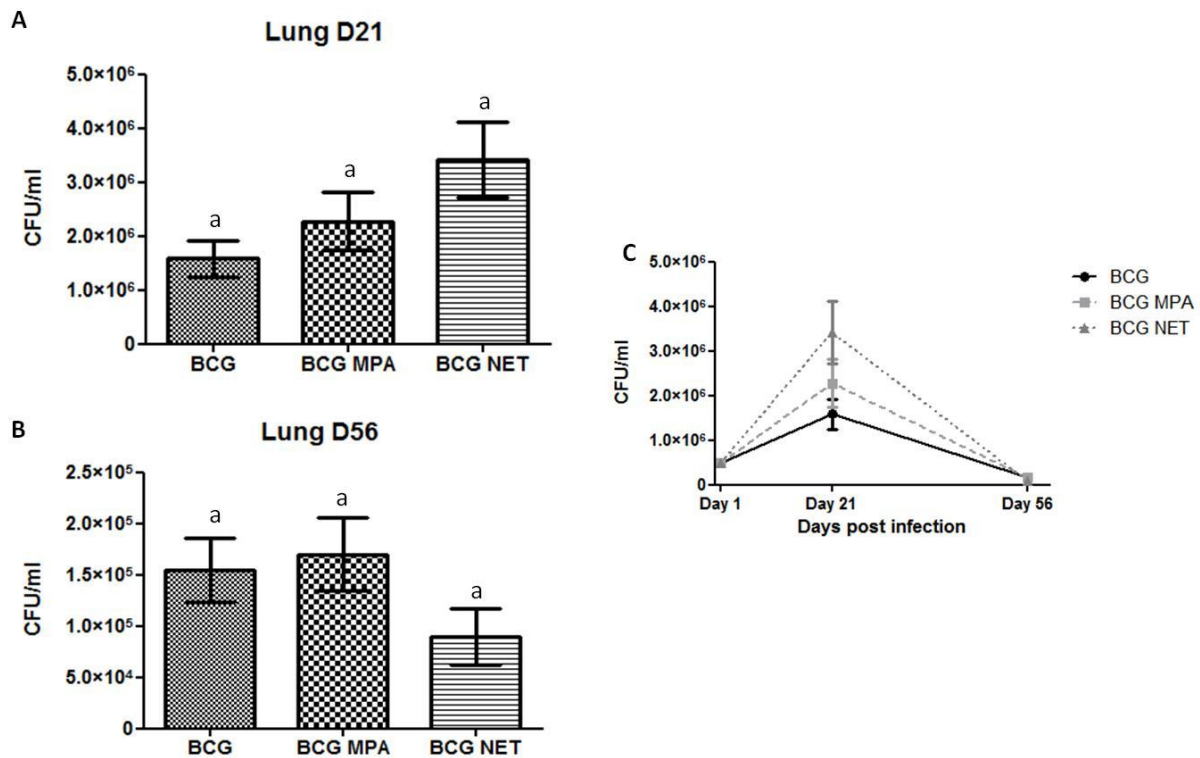
Figure 4.2 MPA and NET does not alter the bacterial burden in the lungs of BCG infected C57BL/6 mice.
C57BL/6 mice were intranasally infected with $2x10^6$ CFUs of BCG one week after commencing with intra-muscular injections into the right thigh with MPA, NET or saline. Mice were treated weekly with 50µl containing 1mg MPA or 1.3 mg NET while controls were injected with 50 µl saline. Five mice of the BCG, BGC MPA and BCG NET groups were killed 21 and 56 days post infection. The right upper lobe of lung from each mouse was removed for RNA extraction, while the rest was homogenized and plated in serial dilutions on 7H11 agar for CFU determination. No statistical difference was observed between the BCG, BCG MPA and BCG NET groups (a-c). In all groups the CFUs decreased from day 21 to 56 (c). Data were analysed by ANOVA (means and SEM). A p-value of ≤ 0.05 was considered significant. The letter a indicates statistical significance, values with the same letter are not statistically different from each other. The results represent 2 pooled experiments of which each experiment had 5 mice per group.
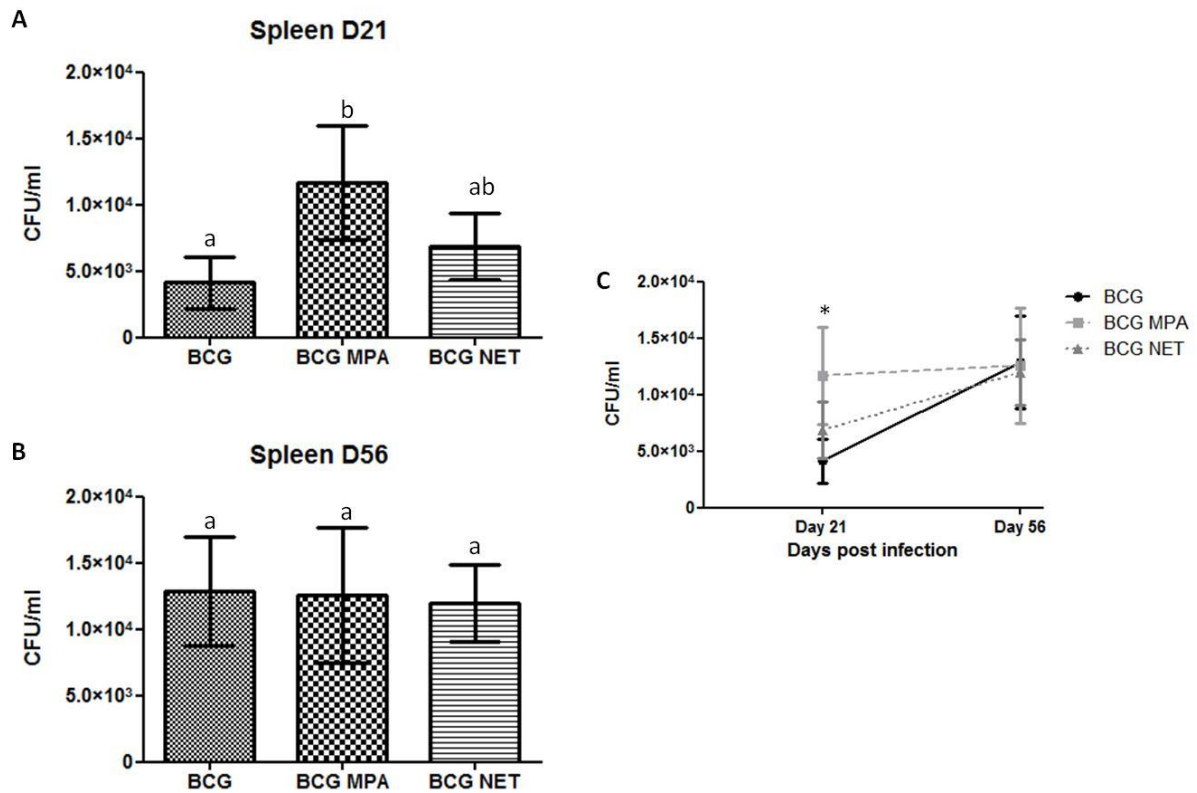
Figure 4.3 MPA and NET does alter the bacterial burden in the spleens of BCG infected C57BL/6 mice 21 days after infection.

C57BL/6 mice were infected with $2x10^6$ CFUs of BCG one week after commencing with intra-muscular injections into the right thigh with MPA, NET or saline. Mice were treated weekly with 50μl containing 1 mg MPA or 1.3 mg NET while controls were injected with 50 μl saline. Five mice of the BCG, BGC MPA and BCG NET groups were killed 21 and 56 days post infection. The spleen was homogenized and plated in serial dilutions on 7H11 agar for CFU determination. The BCG infected MPA treated mice had a higher bacterial burden 21 days post-infection (a). The difference disappeared by day 56 post-infection (b). In all groups the CFUs increased from day 21 to 56 (c). Data were analysed by ANOVA (means and SEM). A p-value of ≤ 0.05 was considered significant. The letter a and b indicates statistical significance, values with the same letter are not statistically different from each other. The results represent 2 pooled experiments of which each experiment had 5 mice per group.

4.4.1 MiRNA expression profiles in mouse whole blood differs between BCG infected and BCG infected and MPA or NET treated mice.

The mice infected with BCG showed an up-regulation in the expression in a group of miRNAs compared to the uninfected (PBS) mice (Figure 4.4). The expression profile of mice infected with BCG and treated with MPA differed from the BCG infected mice, with a different set of miRNAs being up-regulated. The miRNAs found to be up-regulated in the BCG infected mice were down-regulated in BCG infected MPA treated mice. The expression profile in mice infected with BCG and treated with NET differed to some degree from the profile of mice infected with BCG and also from the BCG infected MPA treated mice. These results demonstrate that both NET and MPA directly alter BCG-induced miRNA expression.

The expression of individual miRNAs was investigated next to determine how the synthetic progestins MPA and NET affect BCG-induced miRNA expression.
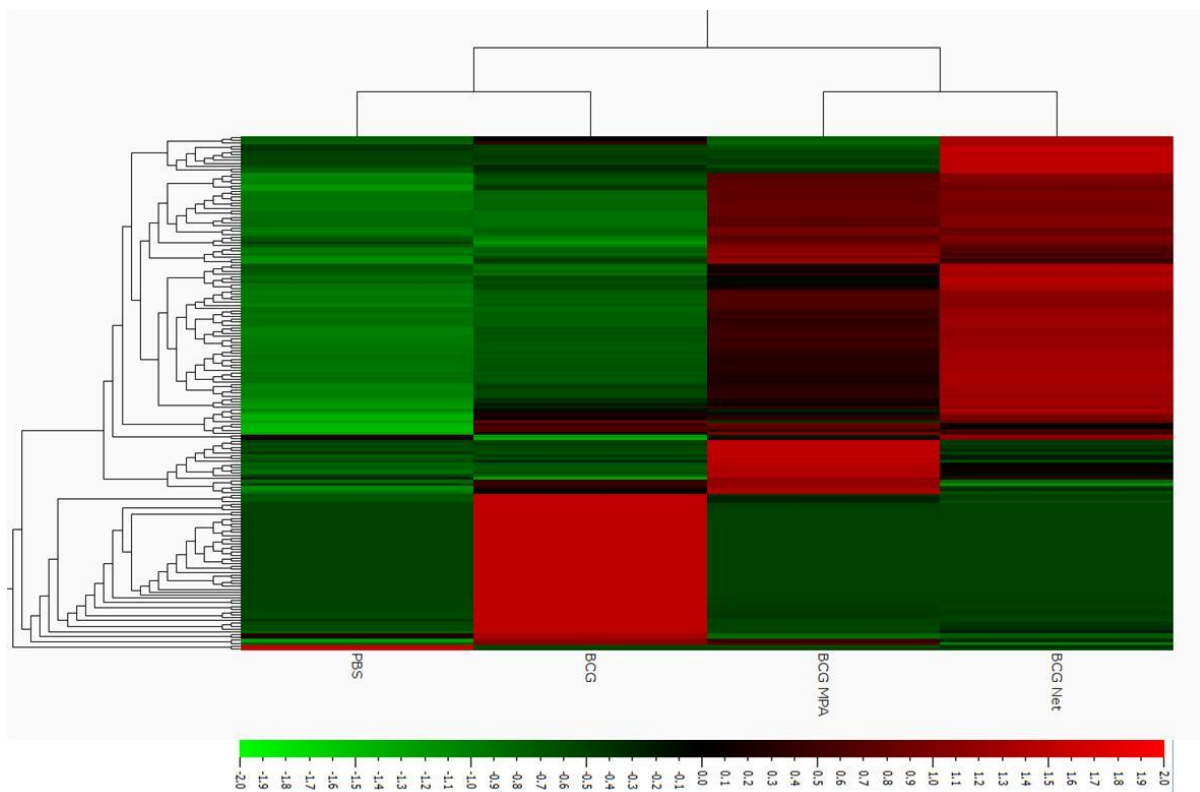
Figure 4.4 Differential expression of miRNAs in the RNA of the whole blood of mice infected with BCG and treated with MPA or NET 21 days after BCG infection.
Over-expressed miRNAs are shown in red and under-expressed miRNAs in green. LS means were used to for the unbiased clustering analysis. A heatmap was generated using the Qlucore omics explorer software. Experiments were done in duplicate and each group for each experiment consisted of the pooled total RNA of 5 mice Variations in miRNA expression was determined using a variance to the mean approach. The colour red indicates a variance of 2+ from the mean and the colour green a variance of 2- from the mean.

4.4.2 Expression of individual miRNAs in whole blood differs between PBS treated, BCG infected, BCG infected MPA treated and BCG infected NET treated mice.

The expression level of mmu-miR-100 was significantly increased in the BCG infected mice compared to the uninfected (PBS treated) mice (p=0.019) (Figure 4.5a). MPA (p=0.019) and NET (p=0.02) suppressed the BCG-induced expression of mmu-miR-100 to basal levels.

Mmu-miR-509-3p had a significant increase in expression in the BCG infected mice compared to the uninfected (p=0.008) mice (Figure 3.5b). MPA (p=0.01) and NET (p=0.01) inhibited the BCG-induced mmu-miR-509-3p expression.

The expression of mmu-miR-99a was not different between the BCG infected and uninfected mice (Figure 3.5c). This miRNA had an increased expression level in the BCG infected MPA treated mice compared to the BCG infected (p=0.0002) mice. The NET treated mice did not have an increase in the expression of mmu-miR-99a, therefore MPA itself up-regulates this miRNA.

Because whole blood was used for the extraction the variation in the expression level of the miRNAs between groups were large and not many individual miRNA differed significantly.
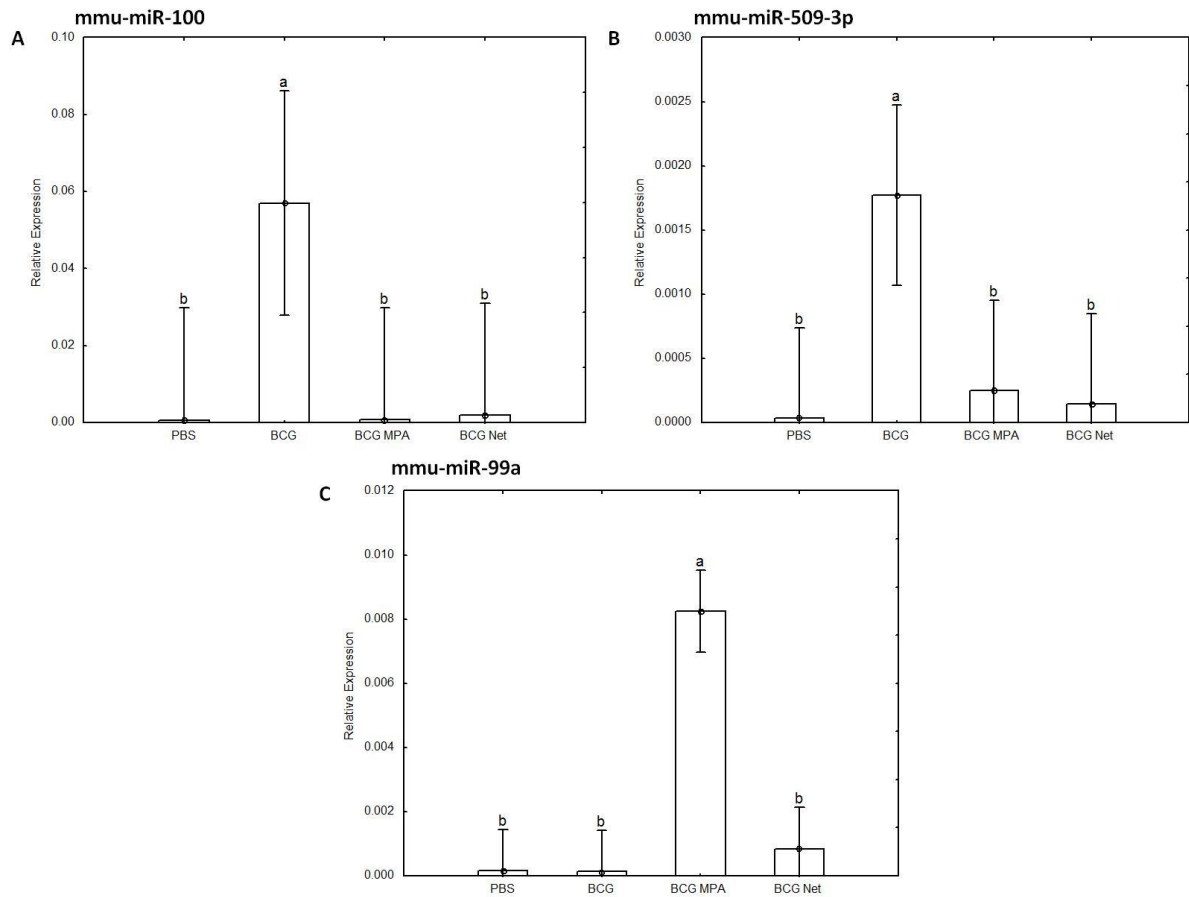
Figure 4.5 The relative expression of miRNAs in the whole blood of C57BL/6 mice differs between BCG infected, BCG infected MPA treated and BCG infected NET treated mice.

Relative expression levels of mmu-miR-100 (a), mmu-miR-509-3p (b) and mmu-miR-99a (c) in the whole blood RNA of C57BL/6 mice infected intranasally with 2×10^6 CFUs in 20μl sterile PBS and injected weekly with 50μl of either 20mg/ml MPA or 26mg/ml NET. Samples were collected 21 days post infection with BCG. Experiments were done in duplicate using the pooled RNA from 5 mice. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a, b and c indicate statistical significance, values with the same letter are not statistically different from each other.

4.4.3 Ingenuity Pathway Analysis of miRNAs in whole blood

The relative abundance of miRNAs expressed in the whole blood of mice was predicted by the IPA software (Figure 4.6). The software indicated that the miRNAs expressed could be invovled in the cell cycle, cell death and survival, cellular growth and development.
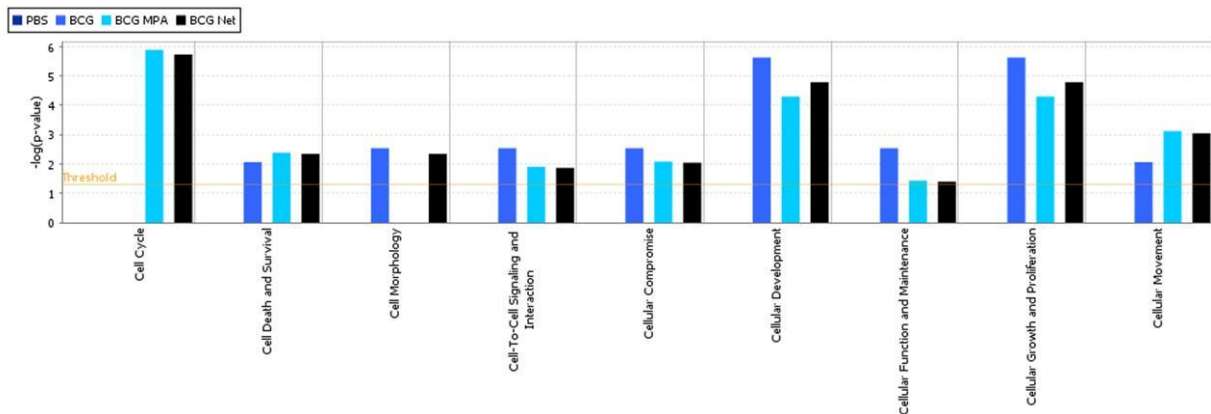
Figure 4.6 Relative abundance of miRNAs linked to the immune system in the different stimulation conditions.
The Ingenuity Software was used to depict the relative abundance of miRNAs expressed in the whole blood of uninfected, BCG infected and BCG infected and MPA or NET treated mice that have predicted functions in cellular procesess.

Not many miRNAs were found to be expressed in the uninfected mice and therefore the network generated contained few interacting molecules (Figure 4.7). The network generated for BCG infected mice was more extensive and signalling molecules such as the transcription factors RUNX2 and nuclear factor activated T cells 1 (NFATC1) were predicted to be regulated by the BCG-indiced miRNAs (Figure 4.7). The signalling molecules Smad 2 and Smad 3 which form part of the transforming growth factor β (TGF-β) signalling pathway were also implicated in this network. The network of the BCG infected and MPA treated mice predicted the regulation of IL-6 and C-reactive protein by the miRNAs expressed under these stimulation conditions (Figure 4.8). The kinase cyclin-dependant kinase 7 (CDK7) was hypothesised to be implicated. This kinase regulates the progression of the cell cycle and could be involved in the clonal expansion of T cells during the immune response to *M.tb*. Myc a transcription factor important during differentiation, proliferation and apoptosis is also predicted to be regulated in these mice. DICER and DROSHA, molecules involved in miRNA processing and function are also thought to be regulated. This could indicate that MPA affects the expression of these molecules by altering the expression of the reguatory miRNAs. The BCG infected and NET treated mice had an extensive network with many signalling molecules predicted to be regulated (Figure 4.9). Cyclin E1 (CCNE1) and CDK7 expression is hypothesised to be regulated by miRNAs expressed in NET treated mice. The expression of hsp20 is predicted to be regulated by miRNAs expressed in NET treated mice.
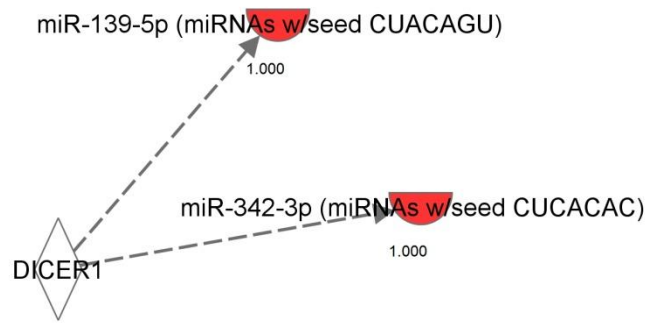
Figure 4.7 The network of interactions of up-regualted miRNAs predicted using the IPA Software in the whole blood of uninfected mice.

Network of miRNAs predicted to be involved in a biological network 21 days after infection in uninfected C57BL/6 mice. Data is representative of 2 independant experiments and the pooled RNA of five mice per experiment. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.
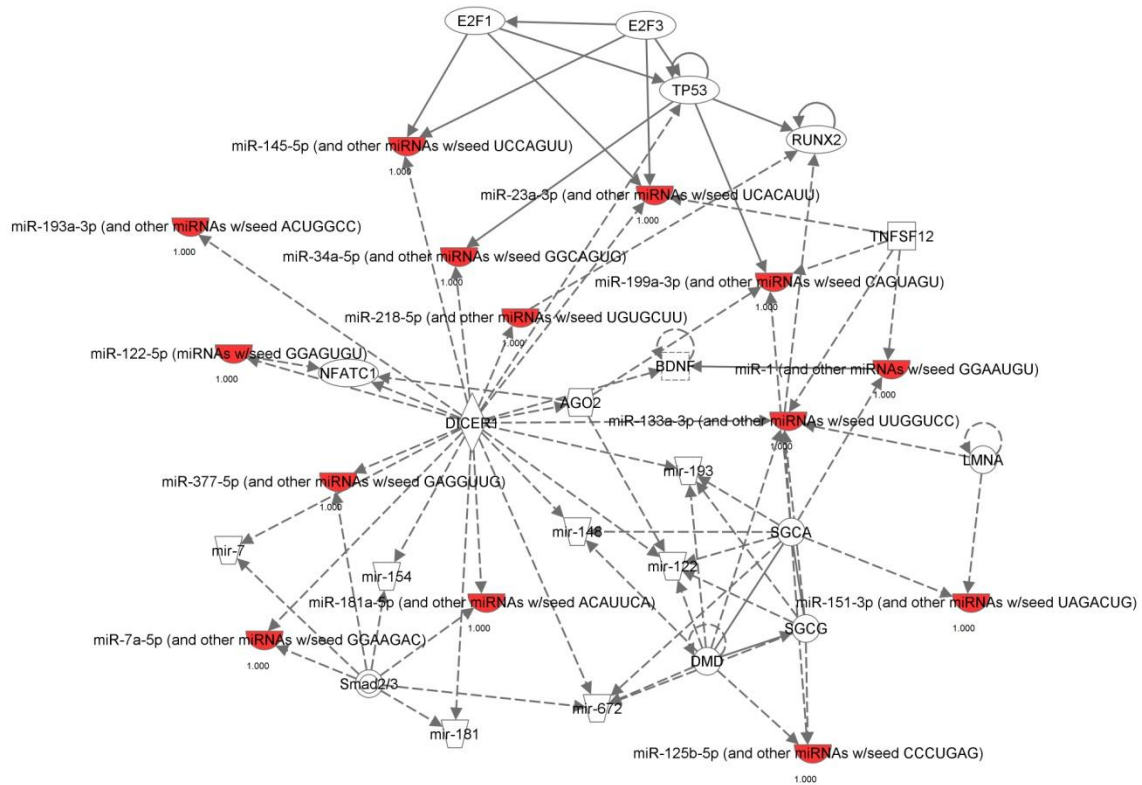
 Figure 4.8 The network of interactions of up-regualted miRNAs predicted using the IPA Software in the whole blood of BCG infected mice.

Network of miRNAs predicted to be involved in a biological network 21 days after infection in uninfected C57BL/6 mice. Data is representative of 2 independant experiments and the pooled RNA of five mice per experiment. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.
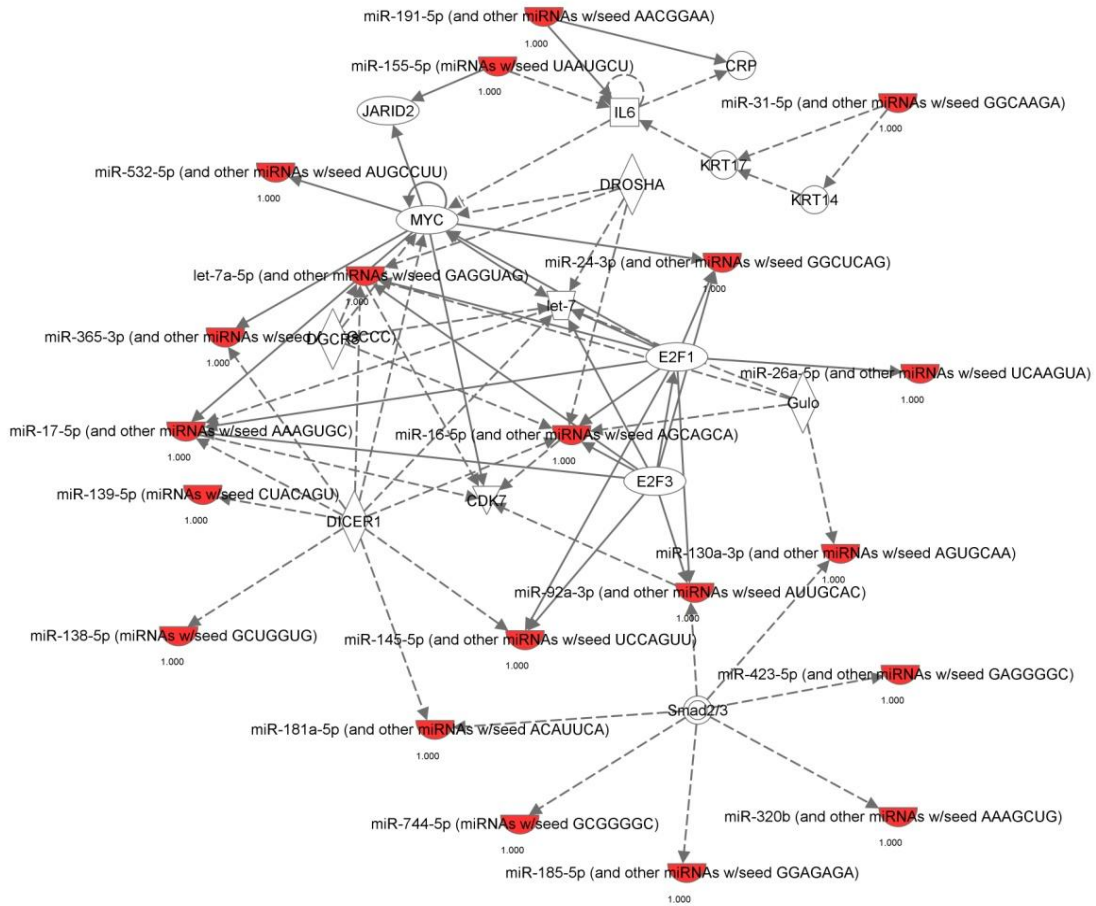
Figure 4.9 The network of interactions of up-regualted miRNAs predicted using the IPA Software in the whole blood of BCG infected MPA treated mice.
Network of miRNAs predicted to be involved in a biological network 21 days after infection in uninfected C57BL/6 mice. Data is representative of 2 independant experiments and the pooled RNA of five mice per experiment. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.
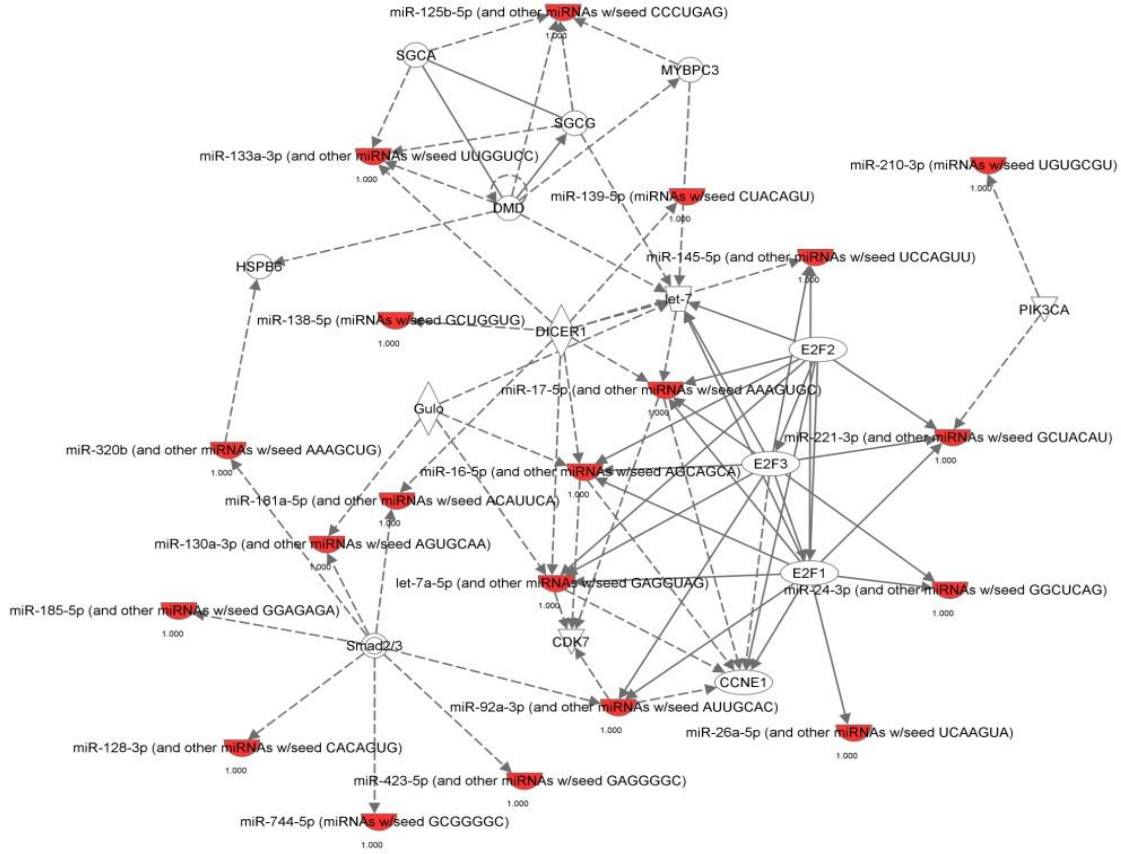
Figure 4.10 The network of interactions of up-regualted miRNAs predicted using the IPA Software in the whole blood of BCG infected NET treated mice.
Network of miRNAs predicted to be involved in a biological network 21 days after infection in uninfected C57BL/6 mice. Data is representative of 2 independant experiments and the pooled RNA of five mice per experiment. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.

4.4.4 MiRNA expression profiles in mouse lung tissue differ between uninfected, BCG infected and BCG infected and MPA or NET mice.

In lungs of BCG infected mice an up-regulation of the majority of miRNAs of the assay used was observed (Figure 4.11). The BCG infected and MPA treated mice had an expression profile that differed from the BCG infected mice. The BCG infected NET-treated mice showed a group of miRNAs that were up-regulated and some that had no change in the level of expression. The expression of individual miRNAs was investigated next to determine how their expression differs in mice infected with BCG and treated with MPA or NET.
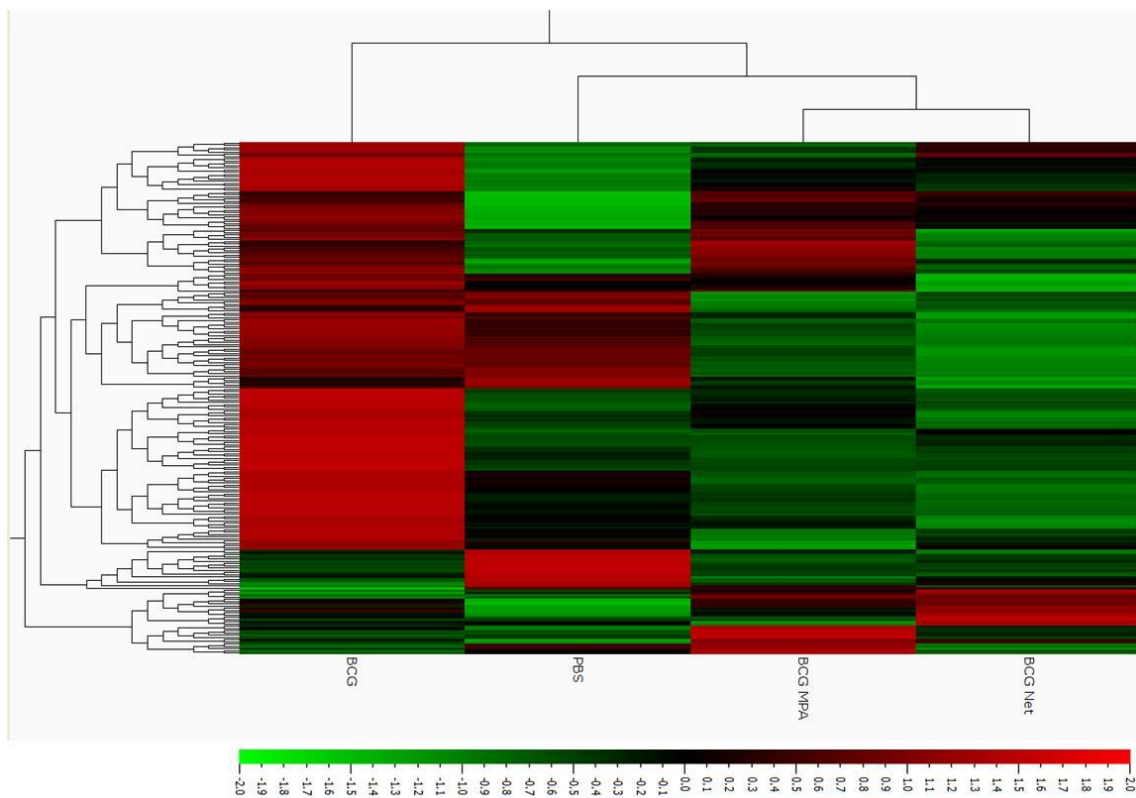
Figure 4.11 Differential expression of miRNAs in the RNA of lung tissue of mice infected with BCG and treated with MPA or NET 21 days after BCG infection.
 Over-expressed miRNAs are shown in red and under-expressed miRNAs in green. LS means were used to for the unbiased clustering analysis. A heatmap was generated using the Qlucore omics explorer software. Experiments were done in duplicate and each group for each experiment consisted of the pooled total RNA of 5 mice. Variations in miRNA expression was determined using a variance to the mean approach. The colour red indicates a variance of 2+ from the mean and the colour green a variance of 2- from the mean.

4.4.5 Expression of individual miRNAs in lung tissue differs between uninfected, BCG infected, BCG infected and MPA or NET treated mice.

The expression of miRNAs is known to be very tissue specific and was investigated at the site of infection, because this is where they most likely exert their function by inhibiting their targets directly. The expression profile for mice infected with BCG and treated with MPA or NET differed from each other and for this reason the expression of each individual miRNA assessed.

4.4.5.1 The expression of miRNAs up-regulated in BCG infected mice.

The BCG infected mice showed a significant increase in the expression level of mmu-miR-1 compared to the uninfected (PBS) (p=0.022) mice (Figure 4.12a). The BCG-induced up-regulation in mmu-miR-1 expression was suppressed by MPA (p=0.019). NET did not cause the same suppression in expression of mmu-miR-1.

In BCG infected mice the mmu-miR-130b level was significantly increased compared to uninfected (p=0.0007) mice (Figure 4.12b). The up-regulation seen in BCG infected mice was partially reversed by MPA (p=0.004) and NET (p=0.004). The expression of mmu-miR-130b in the BCG infected MPA treated (p=0.02) and BCG infected NET treated (p=0.02) mice were up-regulated compared to uninfected mice.

Expression of mmu-miR-138 was significantly increased in the BCG infected mice compared to uninfected (p=0.049) mice (Figure 4.12c). The BCG-induced expression of mmu-miR-138 was suppressed by MPA (p=0.007) to below basal levels. NET suppressed the BCG-induced expression of mmu-miR-138 to basal level, but the suppression did not reach significance.

BCG infected mice showed an induction of mmu-miR-202-3p (p=0.013) compared to uninfected mice (Figure 4.12d). The BCG-induced expression of mmu-miR-202-3p was suppressed by MPA (p=0.03) and NET (p=0.012).

The expression of mmu-miR-1, mmu-miR-130b, mmu-miR-138 and mmu-miR-202-3p was induced in the lungs of mice infected with BCG. The BCG-induced expression of mmu-miR-1 and mmu-miR-138 was suppressed by MPA but not NET. The expression of mmu-miR-130b and mmu-miR-202-3p was suppressed by both MPA and NET.
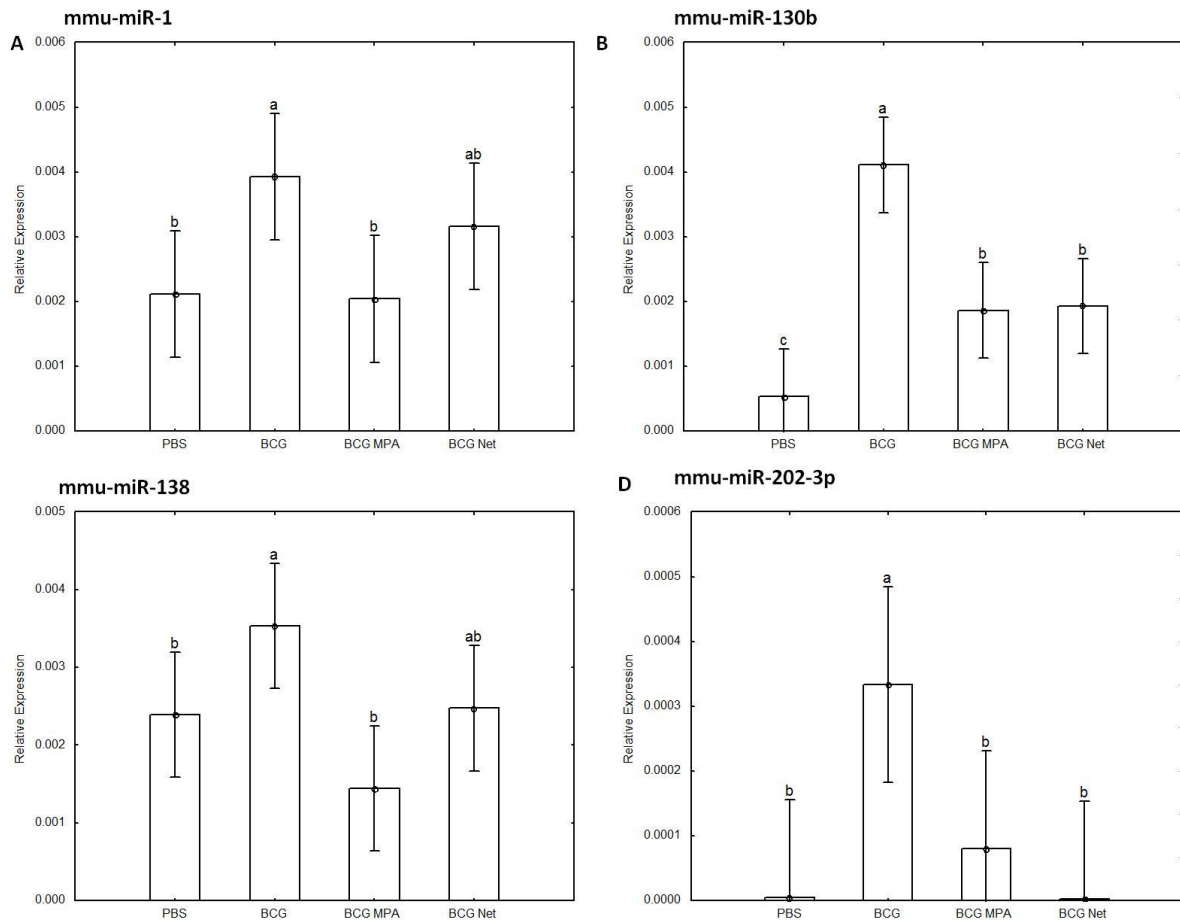
Figure 4.12 The relative expression of miRNAs up-regulated in mice infected with BCG compared to uninfected mice. Relative expression levels of mmu-miR-1(a), mmu-miR-130b (b), mmu-miR-138 (c) and mmu-miR-202-3p (d) in RNA extracted from the lung tissue of C57BL/6 mice infected intranasally with 2×10^6 CFUs in 20μl sterile PBS and injected weekly with 50μl of either 20mg/ml MPA or 26mg/ml NET. Samples were collected 21 days post infection with BCG. Experiments were done in duplicate using the pooled RNA from 5 mice. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a, b and c indicate statistical significance, values with the same letter are not statistically different from each other.

The expression of mmu-miR-20a was induced in mice infected with BCG (p=0.02) compared to uninfected mice (Figure 4.13a). MPA did not alter the BCG-induced production of mmu-miR-20a. NET caused suppression of mmu-miR-20a, but did not return the expression to basal levels.

Mmu-miR-301b expression was induced by BCG infection (p=0.039) compared and suppressed to basal levels by MPA and NET (Figure 4.13b).

The expression of mmu-miR-410 was induced in mice infected with BCG (p=0.006) compared to uninfected mice (Figure 4.13c). MPA (p=0.006) and NET (p=0.011) suppressed the level of mmu-miR-410 to basal level.

Mmu-miR-449a was induced in BCG infected mice (p=0.01) compared to uninfected mice (Figure 4.13d). The BCG-induced expression was also seen in mice infected with BCG and treated with

MPA. Mmu-miR-449a levels in mice infected with BCG and treated with NET was suppressed but the suppression did not reach significance.

The expression of mmu-miR-20a, mmu-miR-301b, mmu-miR-410 and mmu-miR-449a was induced by BCG infection. The BCG-induced expression of mmu-miR-20a and mmu-miR-449a was also seen in mice infected with BCG and treated with MPA, while mice treated with NET had expression levels that did not differ significantly from either the uninfected or infected mice. The BCG-induced expression of mmu-miR-410 was suppressed in mice infected with BCG and treated with MPA or NET. The expression of mmu-miR-301b was induced in mice infected with BCG, the expression of this miRNA in mice treated with MPA or NET did not differ from infected or uninfected mice.
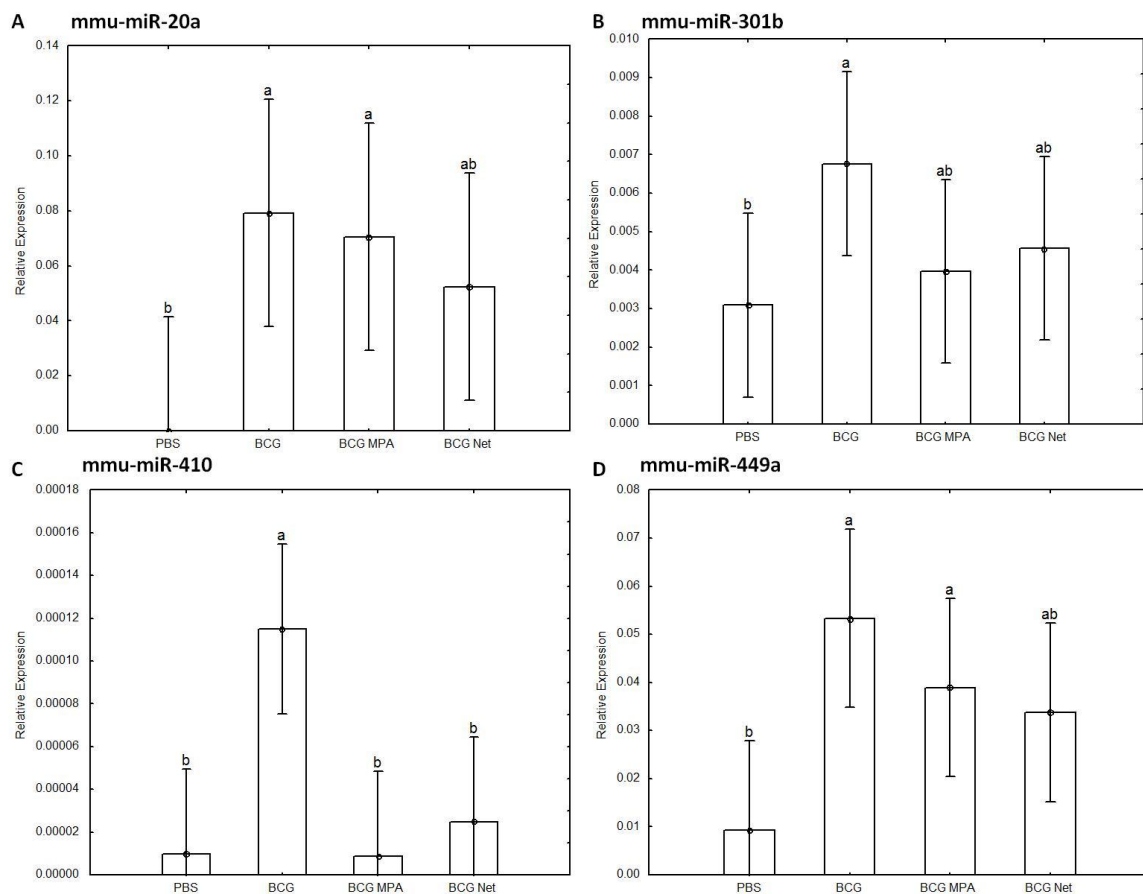
Figure 4.13 The relative expression of miRNAs up-regulated in mice infected with BCG compared to uninfected mice. Relative expression levels of mmu-miR-20a (a), mmu-miR-301b (b), mmu-miR-410 (c) and mmu-miR-449a (d) in RNA extracted from the lung tissue of C57BL/6 mice infected intranasally with $2x10^6$ CFUs in 20µl sterile PBS and injected weekly with 50µl of either 20mg/ml MPA or 26mg/ml NET. Samples were collected 21 days post infection with BCG. Experiments were done in duplicate using the pooled RNA from 5 mice. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a and b indicate statistical significance, values with the same letter are not statistically different from each other.

4.4.5.2 The BCG-induced expression of miRNAs that are down-regulated by MPA or NET.

The level of mmu-miR-132 was suppressed in mice infected with BCG and treated with MPA (p=0.049) compared to BCG infected mice (Figure 4.14a). NET also suppressed the production of mmu-miR-132, but this did not reach significance.

Mmu-miR-133a and mmu-miR-200b expression was suppressed in mice infected with BCG and treated with NET compared to BCG infected mice (p=0.043 and p=0.022 respectively) (Figure 4.14b, d). The level of mmu-miR-133a and mmu-miR-200b in mice infected with BCG and treated with MPA was also suppressed but was not significant.

The production of mmu-miR-16 was suppressed in BCG infected MPA treated (p=0.036) and NET treated mice (p=0.036) compared to BCG infected mice (Figure 4.14c). MPA and NET suppressed the level of mmu-miR-16 to below the basal level.

MPA (p=0.043) and NET (p=0.048) suppressed the BCG-induced production of mmu-miR-24 compared to mice infected with BCG only (Figure 4.15a).

Mmu-miR-379 showed a trend of being induced by BCG infection (p=0.07) compared to uninfected mice (Figure 4.15b). This induction of mmu-miR-379 expression by BCG was suppressed by MPA (p=0.01) and NET (p=0.018).

The expression of mmu-miR-411 was suppressed in BCG infected mice treated with MPA (p=0.032) compared to BCG infected mice (Figure 4.15c). The level of mmu-miR-411 in BCG infected NET treated (p=0.064) showed a trend of being suppressed compared to expression in BCG infected mice.

The BCG-induced expression of mmu-miR-132, mmu-miR-133a, mmu-miR-16 and mmu-miR-200b was suppressed by treatment with either MPA, NET or in both. Mmu=miR-132 was down-regulated in mice treated with MPA. MmumiR-133a and mmu-miR-200b was suppressed in mice treated with NET compared to infected mice. The expression of mmu-miR-16 was suppressed in mice treated with MPA or NET. Mmu-miR-24 and mmu-miR-379 was suppressed in mice treated with MPA or NET. The expression of mmu-miR-411 was suppressed in mice treated with MPA and a trend of suppression was seen in mice treated with NET.
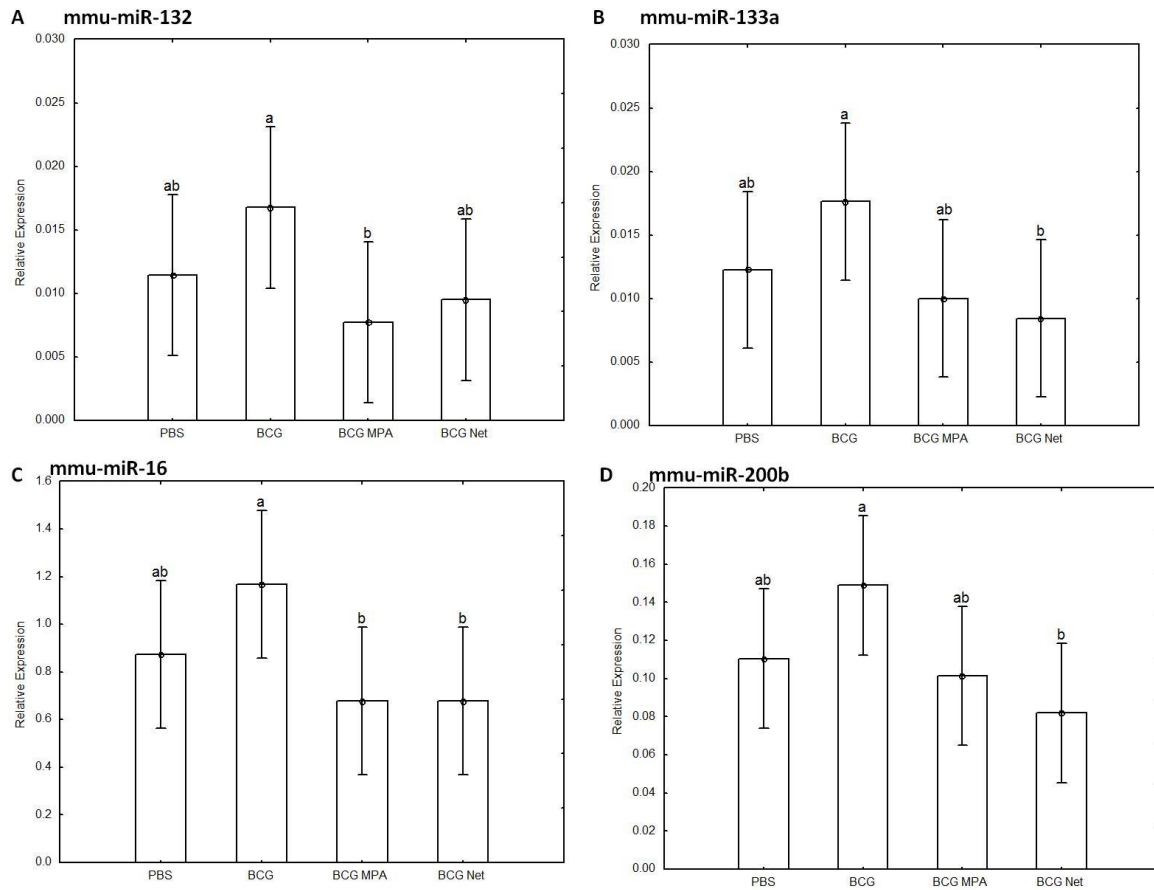
Figure 4.14 The relative expression of miRNAs up-regulated in mice infected with BCG compared to BCG infected and hormone treated mice.

Relative expression levels of mmu-miR-132 (a), mmu-miR-133a (b), mmu-miR-16 (c) and mmu-miR-200b (d) in RNA extracted from the lung tissue of C57BL/6 mice infected intranasally with $2x10^6$ CFUs in 20μl sterile PBS and injected weekly with 50μl of either 20mg/ml MPA or 26mg/ml NET. Samples were collected 21 days post infection with BCG. Experiments were done in duplicate using the pooled RNA from 5 mice. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a and b indicate statistical significance, values with the same letter are not statistically different from each other.
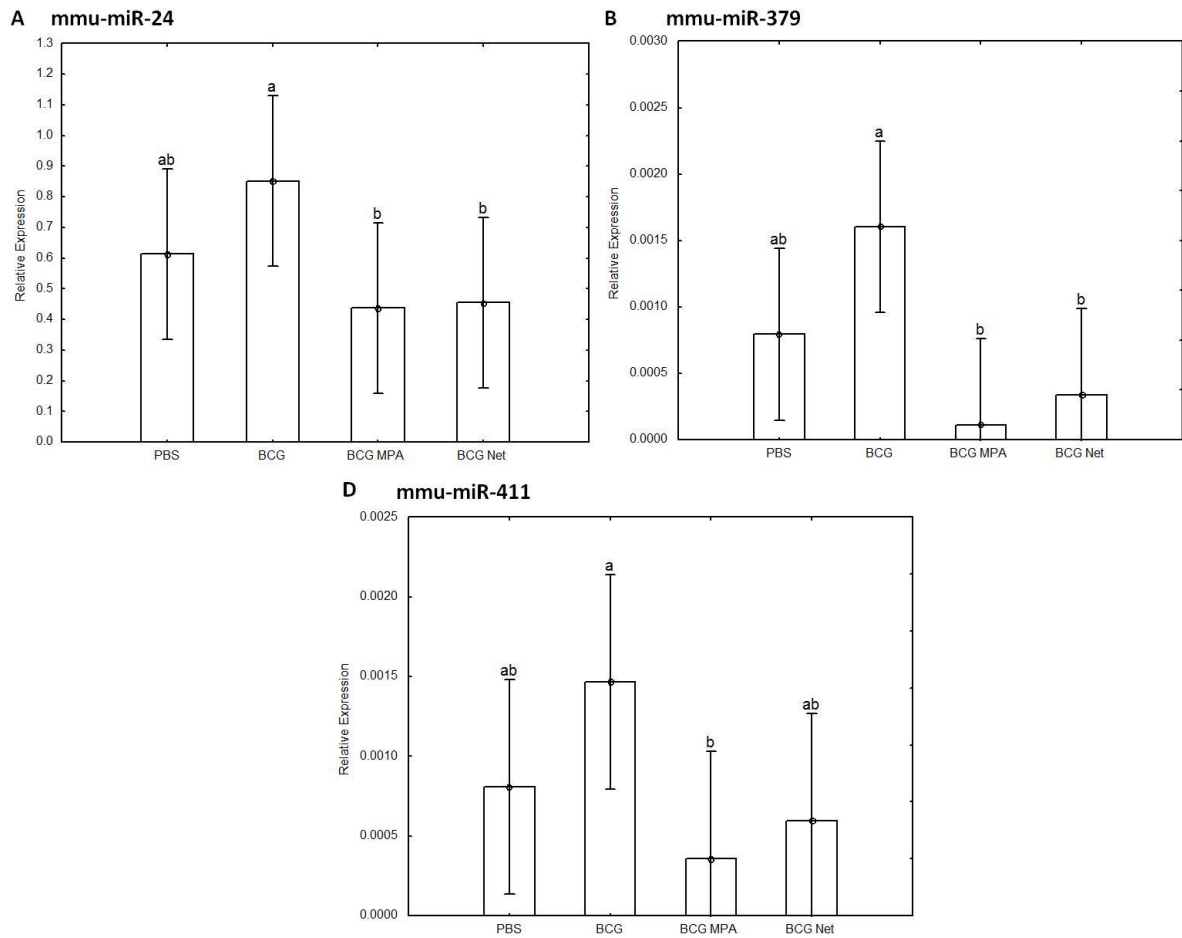
Figure 4.15 The relative expression of miRNAs up-regulated in mice infected with BCG compared to BCG infected and hormone treated mice.

Relative expression levels of mmu-miR-24 (a), mmu-miR-379 (b) and mmu-miR-411 (c) in RNA extracted from the lung tissue of C57BL/6 mice infected intranasally with $2x10^6$ CFUs in 20μl sterile PBS and injected weekly with 50μl of either 20mg/ml MPA or 26mg/ml NET. Samples were collected 21 days post infection with BCG. Experiments were done in duplicate using the pooled RNA from 5 mice. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a and b indicate statistical significance, values with the same letter are not statistically different from each other.

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4.4.5.3 BCG infection causes the down-regulation of mmu-miR-134, mmu-miR-190, mmu-miR-292-3p, mmu-miR-383, mmu-miR-467e and mmu-miR-495 in BGC infected mice or BGC infected and MPA or NET treated mice.

Mmu-miR-134 was down-regulated in mice infected with BCG and treated with MPA (p=0.009) or NET (p=0.014) compared to uninfected mice (Figure 4.16a). Mice infected with BCG showed a trend for mmu-miR-134 to be suppressed (p=0.07).

The expression of mmu-miR-190 was suppressed in BCG infected mice (p=0.0018) compared to uninfected mice (Figure 4.16b). MPA induce the expression of mmu-miR-190 (p=0.015). This induction by MPA still led to levels of mmu-miR-190 that is lower than the basal level. Treatment with NET did not alter the BCG-induced suppression of mmu-miR-190.

Mmu-miR-292-3p was suppressed in mice infected with BCG (p=0.013) compared to uninfected mice (Figure 4.16c). MPA or NET did not change the BCG-induced suppression of mmu-miR-292-3p.

The levels of mmu-miR-383 and mmu-miR-476e was suppressed in BCG infected mice compared to uninfected mice (p=0.02) (Figure 4.17a-b). MPA and NET treatment had no effect on the BCG-induced suppression of mmu-miR-383 and mmu-miR-476e.

The expression of mmu-miR-495 was suppressed in BCG infected mice (p=0.039) compared to uninfected mice (Figure 4.17c). MPA treatment did not alter the BCG-induced suppression of mmu-miR-495. Treatment with NET led to slight increase in the level of mmu-miR-495. The level of mmu-miR-495 in BCG infected NET treated mice did not differ significantly from uninfected or BCG infected mice.

The expression of mmu-miR-292-3p, mmu-miR-383 and mmu-miR-467 was suppressed in mice infected with BCG compared to uninfected mice. The hormones MPA and NET did not affect the expression of these miRNAs as mice infected with BCG and treated with MPA or NET showed the same level of suppression as BCG infected mice. The expression of mmu-miR-134 showed a trend of suppression in BCG infected mice, but was only significantly down-regulated in mice infected with BCG and treated with MPA or NET. This could indicate that the hormones have a further suppressive effect on the level of this miRNA. Mmu-miR-190 was suppressed in BCG infected mice. The same level of suppression was seen in mice infected with BCG and treated with NET. Mice infected with BCG and treated with MPA had an up-regulation in the level of mmu-miR-190 compared to BCG infected mice, although the induction did not reach the basal level. This could indicate that the presence of MPA partially inhibited the functioning of molecules that suppress the expression of

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mmu-miR-190, and leads to a partially restored level of this miRNA. Mmu-miR-495 was suppressed in BCG infected mice. MPA had no effect on the BCG-induced suppression of mmu-miR-495.
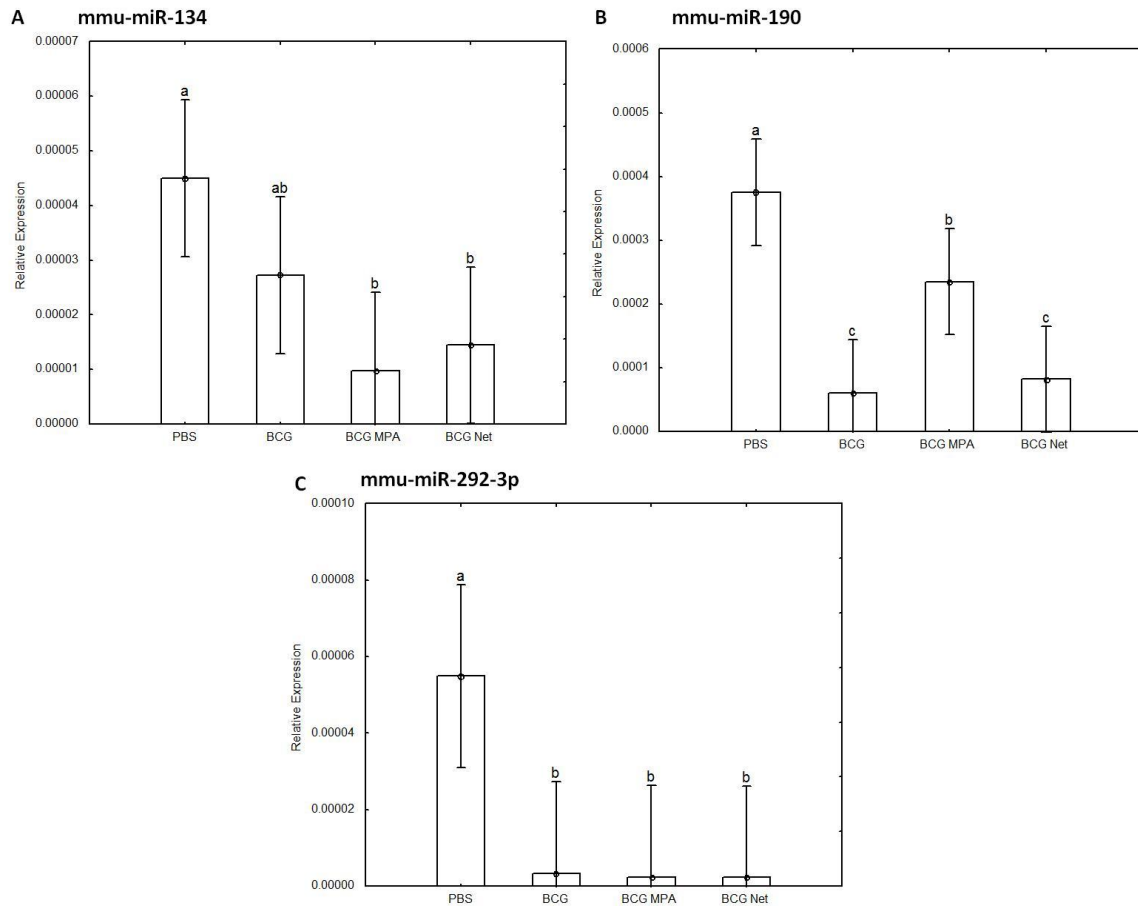
Figure 4.16 The relative expression of miRNAs down-regulated in mice infected with BCG, infected and MPA or NET treated mice compared to uninfected mice.
Relative expression levels of mmu-miR-134 (a), mmu-miR-190 (b) and mmu-miR-292-3p (c) in RNA extracted from the lung tissue of C57BL/6 mice infected intranasally with $2x10^6$ CFUs in 20µl sterile PBS and injected weekly with 50µl of either 20mg/ml MPA or 26mg/ml NET. Samples were collected 21 days post infection with BCG. Experiments were done in duplicate using the pooled RNA from 5 mice. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a, b and c indicate statistical significance, values with the same letter are not statistically different from each other.
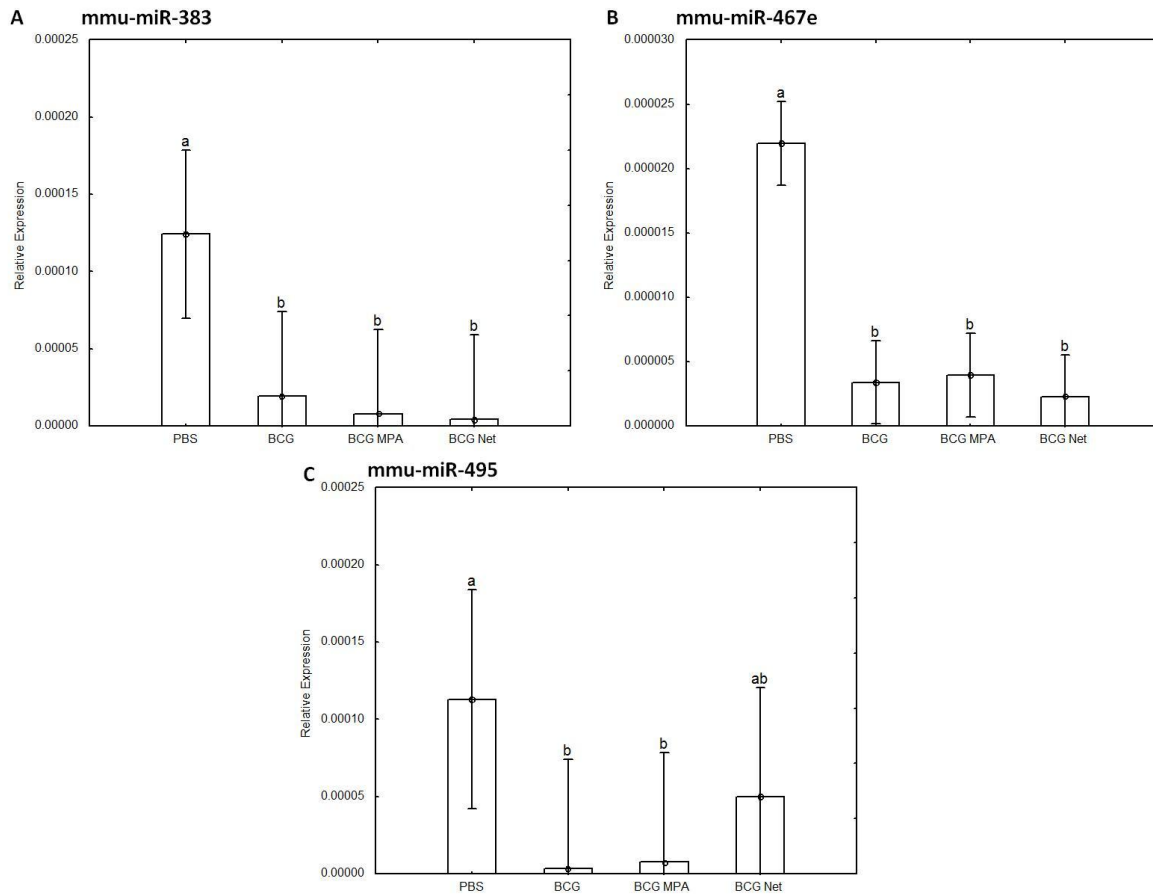
Figure 4.17 The relative expression of miRNAs down-regulated in mice infected with BCG, infected and MPA or NET treated mice compared to uninfected mice.

Relative expression levels of mmu-miR-383 (a), mmu-miR-467e (b) and mmu-miR-495 (c) in RNA extracted from the lung tissue of C57BL/6 mice infected intranasally with $2x10^6$ CFUs in 20µl sterile PBS and injected weekly with 50µl of either 20mg/ml MPA or 26mg/ml NET. Samples were collected 21 days post infection with BCG. Experiments were done in duplicate using the pooled RNA from 5 mice. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a and b indicate statistical significance, values with the same letter are not statistically different from each other.

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4.4.5.4 The expression of miRNAs up-regulated by MPA or NET in BGC infected mice.

Mmu-miR-384-5p was up-regulated in BCG infected MPA treated mice (p=0.00002) compared to BCG infected mice (Figure 4.18a). The level of mmu-miR-384-5p did not differ between uninfected and BCG infected mice. Treatment with NET did not alter the expression if mmu-miR-384-5p.

The expression of mmu-miR-467c did not differ between uninfected and BCG infected mice (Figure 4.18b). The mmu-miR-467c level was increased in BCG infected NET treated mice (p=0.04) compared to BCG infected mice. MPA did not cause a significant increase in the level of this miRNA.
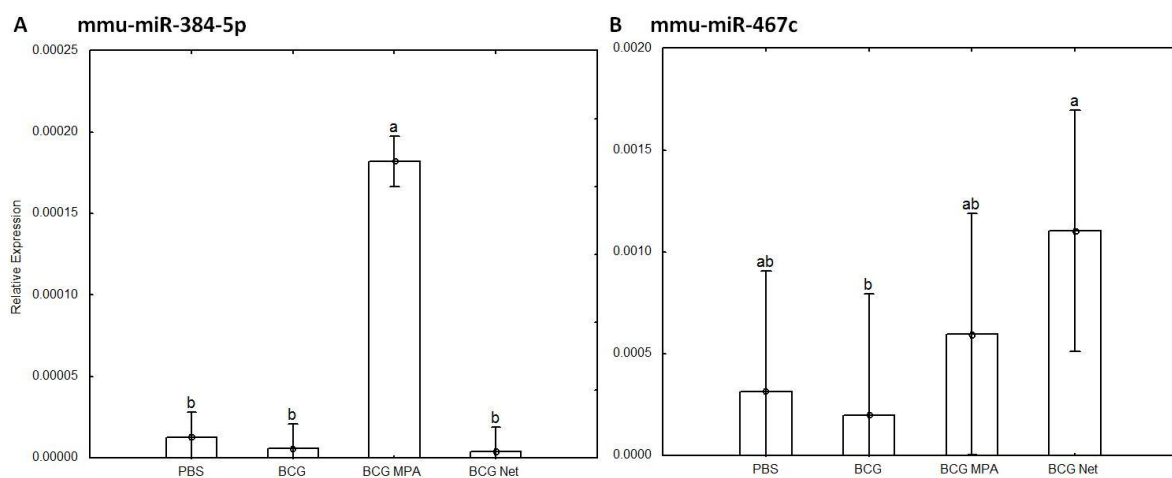
Figure 4.18 The relative expression of miRNAs up-regulated in mice infected with BCG and MPA or NET treated compared to BCG infected mice.

Relative expression levels of mmu-miR-384-5p (a) and mmu-miR-467c (b) in RNA extracted from the lung tissue of C57BL/6 mice infected intranasally with 2×10^6 CFUs in 20μl sterile PBS and injected weekly with 50μl of either 20mg/ml MPA or 26mg/ml NET. Samples were collected 21 days post infection with BCG. Experiments were done in duplicate using the pooled RNA from 5 mice. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a and b indicate statistical significance, values with the same letter are not statistically different from each other.

4.4.6 Ingenuity Pathway Analysis of miRNAs in lung tissue

The relative abundance of miRNAs expressed in the lung of mice infected with BCG and their predicted cellular functions predicted using the IPA software (Figure 4.19). The miRNAs expressed were predicted to have functions involving cellular development, cellular growth and profileration, cell death and the cell cycle.
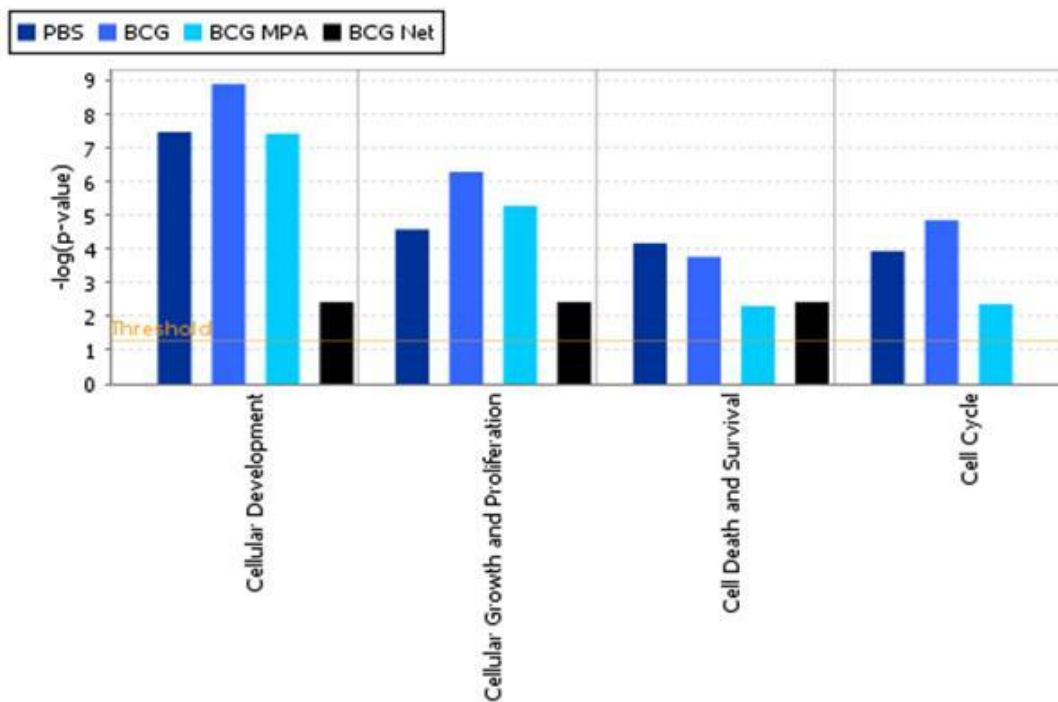
Figure 4.19 Relative abundance of miRNAs linked to the immune system in the different stimulation conditions.
The Ingenuity Software was used to depict the relative abundance of miRNAs expressed in lung tissue of uninfected, BCG infected and BCG infected and MPA or NET treated mice that have predicted functions in cellular procesess.

The network generated for the miRNA expression in the lung of uninfected mice showed the expression of cyclin D1 and CDK2 is predicted to be regulated by the miRNAs expressed in these mice (Figure 4.20). Both these molecules are needed for cells to progress through the G1/S transition of the cell cycle. The expression of the transcription factors RUNX2 and Myc2 are also hypothesised to be regulated by miRNAs up-regulated in this network. The expression of miRNAs in the lung of BCG infected mice is predicted to regulate the expression of the receptor for the cytokine colony stimulating factor (CSF) (Figure 4.21). Signalling through this receptor regulates the function and differentiation of macrophages. The regulation of this receptor by miRNAs could lead to the activation of more macrophages during infection. The transcription factor RUNX2 was predicted to be regulated together with the pro-inflammatory cytokine TNF-α. Both of these molecules are important during the immune response to *M.tb* infection. The nuclear factor NFATC2, is part of the network, this molecule is needed to activate T cells. In the BCG infected and MPA treated mice the expression of CCL9 was part of the network. This chemokine is expressed by macrophages and

attracts DCs (Figure 4.22). The expression of TNF-α and IL-6 was predicted to be regulated. Both are pro-inflammatory cytokines and it is possible that the presence of MPA cause the down-regulation of the cytokines due to its glucocorticoid activity. The receptor of CSF is part of the network and the regulation of this receptor could lead to an alteration in the differentiation and function of macrophages in these mice. The network generated for miRNAs up-regulated in mice infected with BGC and treated with NET included Ago2 and DICER (Figure 4.23). Expression of the transcription factor Myc2 and CDK7 was also part of the network. The protein NROB2 is predicted to directly regulate the expression of 7 miRNAs, this protein can interact with the estrogen receptor and inhibit its function.

Figure 4.20 The network of interactions of up-regualted miRNAs predicted using the IPA Software in the lung of uninfected mice.

Network of miRNAs predicted to be involved in a biological network 21 days after infection in uninfected C57BL/6 mice. Data is representative of 2 independant experiments and the pooled RNA of five mice per experiment. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.

Figure 4.21 The network of interactions of up-regualted miRNAs predicted using the IPA Software in the lung of BCG infected mice.

Network of miRNAs predicted to be involved in a biological network 21 days after infection in uninfected C57BL/6 mice.

Data is representative of 2 independant experiments and the pooled RNA of five mice per experiment. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.
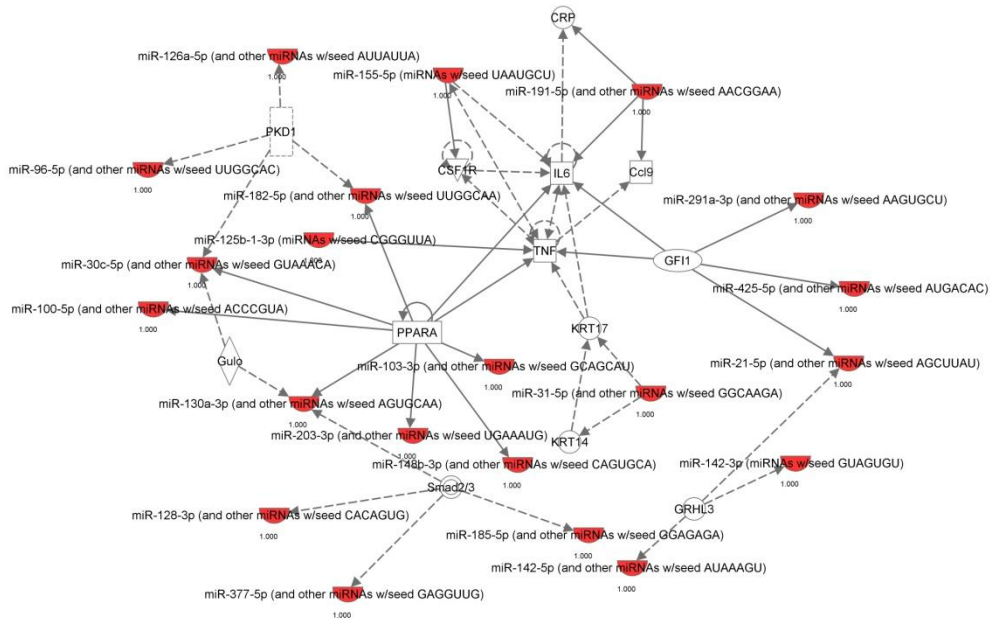
Figure 4.22 The network of interactions of up-regualted miRNAs predicted using the IPA Software in the lung of BCG infected MPA treated mice.

Network of miRNAs predicted to be involved in a biological network 21 days after infection in uninfected C57BL/6 mice.

Data is representative of 2 independant experiments and the pooled RNA of five mice per experiment. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.
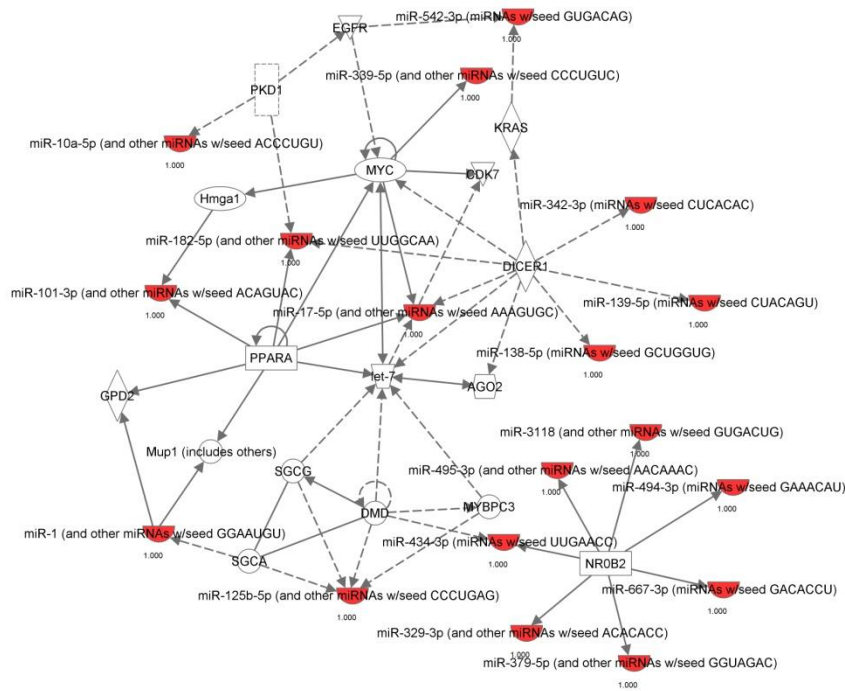
Figure 4.23 The network of interactions of up-regualted miRNAs predicted using the IPA Software in the lung of BCG infected NET treated mice.
Network of miRNAs predicted to be involved in a biological network 21 days after infection in uninfected C57BL/6 mice.

Data is representative of 2 independant experiments and the pooled RNA of five mice per experiment. A solid line indicates a direct interaction while a dotted line indicates an indirect interaction.

4.5 Summary

The effect of MPA and NET on the immune response to BCG in a whole organism model was investigated. The bacterial burden in the spleen of BGC infected and MPA treated mice 21 days after infection was higher than BCG infected mice and BCG infected and NET treated mice. This indicates that there is more extensive dissemination of disease and that mice treated with MPA have an impaired ability to control the bacterial growth.

The miRNA expression patterns in the whole blood and lungs of BCG infected and BCG infected and MPA ot NET treated mice each have distinct patterns. The expression of specific miRNAs also varies between the blood and lung of mice infected with BCG and treated with MPA or NET. This could indicate that the expression of miRNAs are tissue specific. This could also indicate that the synthetic progestins regulate the expression of miRNAs in a tissue specific manner.

The effect that MPA and NET have on the miRNA expression in both the blood and lungs of these mice suggests that they are modulating the immune response to BCG at transcriptional level.

Chapter 5: Discussion

5.1 Impact of MPA and NET on cytokine secretion and miRNA expression in PBMCs

5.1.1 Regulation of cytokine production by glucocorticoids and progesterone

The immunosuppressive and anti-inflammatory effects of GCs on immune cells are mediated mainly by cytosolic GR (106). When GCs bind to the GR they undergo a conformational change. The GC-GR complex forms a dimer with another GC-GR complex and translocates to the nucleus where they can bind to GREs of glucocorticoid responsive genes (73). Binding of the GC-GR homodimer complex can then lead to the activation of transcription of these genes (when bound to a GRE) or block the transcription of a gene (when bound to a nGRE) (106). A GC-GR monomer can also bind to transcription factors such as NFκB or AP-1 directly and prevent them from activating the transcription of their target genes (106).

Spies *et al.* found that T cells treated with 10^{-5} M dexamethasone (Dex) and then treated with anti-CD3/CD28 had lower levels of IL-2, IFN-γ and TNF-α present in the supernatant after 24 hours (106). The same results were found in our work where the expression of these three cytokines was lower in cells treated with cortisol and MPA after stimulating the cells for 24 hours with BCG. The effect was blocked when they added the GC antagonist RU486 to the cells at the same concentration. Kleynhans *et al.* found that the BCG-induced expression of IFN-γ was abolished by cortisol and MPA after 72 hours and that progesterone did not show this suppression (87). My data support the observation that cortisol and MPA also strongly suppressed the expression of IFN-γ, whereas NET and progesterone repressed IFN-γ production to a lesser extent.

In my data IL-10 was induced by BCG but the hormones had no effect after six and 24 hours. 72 hours after stimulation the expression of IL-10 was lower in the presence of cortisol and MPA.This contrasts with the previous findings in our group where the expression of IL-10 was not influenced by the presence of these hormones at 72 hours post-infection (87). In mice it was found that the secretion of the anti-inflammatory cytokine IL-10 was induced in monocytes and DCs after GC treatment (107). The reason for the decrease in the BCG and cortisol or MPA stimulated cells after 72 hours might be due to the individual variation seen between people or a pro-inflammatory cytokine response was launched in response to the BCG stimulation and this blocked the IL-10 response.

Our group has also previously shown that the secretion of IL-1α, IL-2 and IL-6 are down-regulated by cortisol and MPA after 72 hours and that progesterone did not induce the same down-regulation (87).

The expression IL-1α was indeed down-regulated by MPA and cortisol after 24 and 72 hours. However, in my experiment the expression if IL-2 was not induced after 72 hours, but at 24 hours BCG-induced expression was seen and this expression was repressed by MPA and cortisol. The secretion of IL-6 was induced at all time points measured, but the hormones had no effect on the expression of this cytokine. Gustol *et el.* found that stimulation T cells with 10^{-5}M Dex for 48 hours led to decreased levels of IL-2 (108). Also correlating with our findings Huang *et al.* found that Dex inhibits the LPS-induced expression of IL-6 in a dose-dependent manner (109).

GCs suppress the expression of IL-4 (110), in this study it was found that IL-4 expression was not induced by BCG six and 24 hours after stimulation. IL-4 expression was only induced after 72 hours and this induction was suppressed by cortisol and progesterone but not by MPA and NET. This suggests that in this case the two synthetic progestins act the same and neither one mimic the action of cortisol or progesterone. It could be that MPA and NET induces this effect by activating the androgenic receptor (AR).

MPA users have lower levels of IL-1α and IL-12p40 and the authors hypothesised that this might suggest that they are more susceptible to *M.tb* infection (87). My results confirm that the levels of IL-1α, IL-β, IL-12p40 and IL-12p70 are lower in cells stimulated with BCG and treated with MPA and cortisol after 24 and 72 hours. The expression of IL-1β and IL-12p70 was also lower in the presence of NET and progesterone after 72 hours. The expression of IL-12p40 was repressed in the presence of progesterone but not NET after 72 hours. Consistent with the results of this study Ma *et al.* found that Dex suppresses the expression of IL-12p40 in monocytic cells stimulated with LPS (111). This suppression is mediated by the down-regulation of the transcription factors AP-1 and NFκB (111). The secretion of IL-1β is suppressed by Dex in a dose dependent manner in THP-1 cells (112).

Taken together these results suggest that the expression of cytokines in PBMCs, after BCG stimulation show distinct time dependent expression patterns when MPA, NET, cortisol and progesterone are added. The expression of IFN-γ, IL-1α, IL-12p40, IL-12p70, IL-13 and TNF-α was suppressed by MPA and cortisol 24 and 72 hours after stimulation but not 6 hours after stimulation. The BCG-induced expression of IL-1β was suppressed by MPA and cortisol 72 hours after stimulation. These results indicate that MPA mimics cortisol rather than progesterone in the inhibition of cytokine expression.

5.1.2 The effect of MPA, NET and cortisol on BCG-induced miRNA expression in peripheral blood mononuclear cells.

To our knowledge no one has investigated the effect of GCs on miRNA expression during mycobacterial infection. There are studies that observed that miRNAs are regulated by GCs and that miRNA regulate the expression of the GR (97,98). Very little data are available on the effect of

miRNAs on the function of progesterone in the context of infectious diseases. In recent years studies were done on the effect of miRNAs during mycobacterial infection and also the effect that mycobacterial infection has on the expression of miRNAs and the possible use of miRNAs as biomarkers for the diagnosis of disease (56).

Hong *et al*. showed that resistance to GC treatment in DCs correlated with the down-regulation of hsa-miR-29 (103). Hsa-MiR-29b and hsa-miR-29c was shown to induce apoptosis by directly targeting Mcl-1 Bcl-2 in DCs, but could not induce it to the same level as Dex alone (103). Mice deficient in mmu-miR-29 have a greater number of Th1 cells and higher levels of IFN-γ supporting the notion that these miRNAs could promote TLR-inhibited GC-induced apoptosis in DCs (103).

MiR-124a was found to reduce the number of GRs by binding to the 3'UTR of the mRNA of this receptor, as shown in luciferase assays (98). Ledderose *et al*. showed that hsa-miR-124 is induced by GC treatment and that this induction down-regulates GR-α via the direct targeting of GR-α by hsa-miR-124 (99). Due to the targeting of GR-α, the anti-inflammatory effects of GCs are limited. Hsa-miR-130b can bind to the 3'UTR of GR-α in multiple myeloma cells and repress the expression of this protein (97). This possibly indicates that by altering the abundance of GR available, miRNAs play a role in the responsiveness of tissues to GCs.

Hsa-miR-98 is up-regulated by GCs and has an anti-inflammatory effect by suppressing the pro-inflammatory cytokine, IL-13 (100). In our experiments we saw that 24 and 72 hours after addition of hormones the BCG –induced level of IL-13 was suppressed by cortisol and MPA. Although we did not detect the presence of hsa-miR-98 during my experiments, it is possible that the lower levels of IL-13 seen could have been mediated by this miRNA.

Certain miRNAs have also been shown to be involved during infection with *M.tb* and TB disease. Mmu-miR-29 targets the 3'UTR of IFN-γ and this miRNA is down-regulated in NK cells, CD4 T cells and CD8 T cells in mice infected with BCG (55). This miRNA promotes the assembly of the miRISC complex by promoting the binding of Ago2 to the mRNA of IFN-γ (56). MiR-29 was not detected in this study, but as BCG did induce the expression of IFN-γ of stimulated PBMCs, the expression of miR-29 is expected to be down-regulated in these cells. Mmu-miR-147 attenuates the expression of TNF-α and IL-6, suggesting that this miRNA has an anti-inflammatory function (41). Although miR-147 was not detected in our work, we know that the expression of TNF-α and IL-6 was induced by BCG. Since this miRNA suppressed the expression of these cytokines it would be expected that miR-147 be down-regulated in cells where the expression of these pro-inflammatory cytokines is induced. Mmu-miR-21 suppressed the expression of IL-12 by targeting the 3'UTR of the IL-12 mRNA, suppressing the Th1 response of the host (29). This miRNA was not detected in our study, but the expression of IL-12p40 and IL-12p70 was only induced after 24 hours of BCG stimulation. It is possible that low levels of miR-21 suppressed the expression of this cytokine six

hours after BCG stimulation. The expression of miR-21 was only up-regulated at a later time point and could possibly suppress expression of IL-12p40 in cells treated with MPA and cortisol in the case of IL-12p40 and MPA, cortisol and progesterone in the case of IL-12p70.

The data from this study found that the expression of hsa-miR-30c was down-regulated in the BCG infected cells, this suppression was not seen in the BCG and cortisol, MPA or NET stimulated cells. Little is known about hsa-miR-30c and its targets but Bockhorn *et al*. found that this miRNA indirectly targets IL-11, an IL-6 family member (113). We measured the IL-6, but not IL-11, expression in this study. IL-6 expression was found to be induced by BCG and not influenced by MPA, NET, cortisol or progesterone. The expression of hsa-miR-30c was significantly lower in BCG stimulated and BCG and MPA stimulated cells compared to unstimulated cells. The level of hsa-miR-30c in PBMCs stimulated with BCG and cortisol or NET was also lower when compared to the unstimulated cells, but this suppression did not reach significance. These results may suggest that hsa-miR-30c could target IL-6 in the same way it targets IL-11.

The expression of hsa-miR-222 was up-regulated in BCG cortisol stimulated cells. BCG stimulated cells had higher levels of expression than BCG NET stimulated cells. The expression of hsa-miR-222 is increased upon LPS stimulation of THP-1 cells. The presence of IFN-γ caused a down-regulation in the expression of hsa-miR-222 (114). Since it is known that GCs shift the immune response from a Th1 to a Th2 response resulting in less expression of IFN-γ the increased expression of hsa-miR-222 seen in the presence of cortisol may be attributed to this shift in the immune response. A decrease in IFN-γ expression, six hours after addition of BCG and hormones, was seen in the supernatant of these PBMCs. The expression of hsa-miR-222 in BCG MPA and BCG NET stimulated cells were the same as the basal level. Suppression in the level of IFN-γ was seen in BCG MPA stimulated cells, but not to the same extent as in the BCG cortisol stimulated cells. This could mean that IFN-γ expression is suppressed by other mechanisms and that the up-regulation of hsa-miR-222 expression leads to a further suppression IFN-γ expression.

The expression of hsa-miR-454 was significantly higher in BCG stimulated cells than in unstimulated cells. Hsa-miR-454 expression was suppressed to basal levels in PBMCs stimulated with BCG and MPA, NET, or cortisol. Haung *et al*. showed that there was an increase in the expression of hsa-miR-454-3p in monocytes four hours after LPS treatment, however during the validation of these results this up-regulation was not found to be significant (115). It is possible that hsa-miR-454 is not only expressed by monocytes and that expression by other cell types present in PBMCs causing the up-regulation in BCG stimulated cells to be significant. Further studies need to be done to determine the targets of this miRNA to be able to better understand the role of miR-454 in the immune response.

In this study hsa-miR-331-3p had an increased level of expression in PBMCs infected with BCG and treated with cortisol. NK cells activated by IL-2 showed decreased levels of hsa-miR-331-3p (116).

IL-2 is a Th1 cytokine and the presence of GCs can lead to lower levels of this cytokine due to the presence of a GRE in the promoter of the IL-2 gene (117). In my data less IL-2 was detected in the cells stimulated with BCG and cells stimulated with BCG and cortisol or progesterone respectively compared to the expression level of IL-2 in cells stimulated with BCG and MPA or NET respectively six hours after stimulation. It is possible that the presence of cortisol could have led to a decrease in IL-2 expression and that this decrease led to a higher level of hsa-miR-331-3p being expressed in these cells. This however does not explain why the level of hsa-miR-331-3p is not also elevated in the BCG infected cells. It is possible that the expression of hsa-miR-331-3p is elevated the BCG MPA and BCG cortisol stimulated cells after 24 hours due to the suppression of IL-2 expression at this time point.

Taken together these results indicate possible roles for hsa-miR-222, hsa-miR-454 and hsa-miR-331-3p in modulating the immune response to BCG. Hsa-miR-222 possibly suppresses the expression of IFN-γ by a different mechanism than the suppression induced by the GCs. Hsa-miR-454 expression seems to be suppressed in BCG stimulated cells by MPA, cortisol and NET. The mechanism is not known, but it is possible that this inhibition is due to signalling through the GR and PR. The expression of hsa-miR-331-3p is induced by cortisol in BCG stimulated cells. This induction could be due to less IL-2 being present or this miRNA could be the cause of the lower levels of IL-2 present in cells treated with cortisol.

5.2 The impact of MPA and NET on bacterial burden and miRNA expression in a murine model.

This study aimed to determine the effect of BCG infection on the miRNA expression profile in a whole organism model. We chose C57BL/6 mice because differences in the bacterial load have previously been reported in these mice infected with *M.tb* and treated with MPA (88). They were injected i.m with 50 μl of 20mg/ml MPA and 26mg/ml NET weekly. 20mg/ml MPA administered weekly was used as it was previously found that this leads to a serum concentration of MPA similar to that found in women using MPA as a contraceptive (88). The dose of NET chosen in this study was calculated to be equivalent to women using NET as a contraceptive. Seven days after the first injection mice were infected intranasally with $2x10^6$ CFUs of BCG in 20 μl. 21 and 56 days after infection the lungs, spleens and blood were harvested from each group. The right upper lobe of the lung and the blood was used to determine the miRNA expression in the respective tissues. The spleen and the remaining lobes of the lung were used for CFU determination. It was decided to investigate the miRNA expression 21 days post infection because the Th1 response develops at this time (118). Unfortunately, due to financial constraints the miRNA expression was not determined 56 days post infection.

5.2.1 Bacterial burden in the lung and spleen

No significant difference in the CFUs in the lung between the groups was observed at day 21 or 56 post-infection. The CFUs in the lung decreased in all groups from day 21 to day 56 post-infection. CFUs in the spleens of BCG MPA mice were significantly higher compared to the BCG only mice, 21 days post-infection. This difference disappeared by day 56. The CFUs observed at day 21 in the BCG only mice were significantly lower than those seen 56 days post infection.

Infection of C57BL/6 mice with BCG did not show significant differences between mice infected with BCG and treated with MPA or NET. Kleynhans *et al*. however, found that upon infecting mice with a low dose of 20 CFU of live *M.tb*, C57BL/6 mice treated with MPA had a significantly higher bacterial load 21 and 56 days post-infection in the lungs compared to *M.tb* only mice (88). It is possible that these differences were not seen in this study due to the fact that BCG is not as pathonogenic as *M.tb* and the mice are able to better control the infection.

5.2.2 MiRNA expression in the whole blood of mice infected with BCG and treated with MPA and NET

To our knowledge the effect of the synthetic progestins, MPA and NET, on the miRNA expression during infection has not been investigated before. It was first determined whether the expression profile generated from all the miRNAs found to be expressed in the whole blood of the mice were different among the groups. In the BCG infected mice some miRNAs were up-regulated when compared to the uninfected (saline treated) mice (Figure 3.28). The miRNA expression profiles were also different when the BCG infected mice were compared to the BCG infected MPA treated and BCG infected NET treated mice. Most importantly the profiles of the BCG infected MPA treated and BCG infected NET treated mice were also different, although some miRNAs were up-regulated in both. The profile of miRNAs expressed in the BCG infected MPA treated mice differed completely from that seen in the BCG mice and also from the BCG infected NET treated mice. There were certain miRNAs that were up-regulated in both the BCG infected MPA treated and BCG infected NET treated mice.

Mmu-miR-100 was up-regulated in the whole blood of BCG infected mice compared to the uninfected mice, BCG infected MPA treated and BCG infected NET treated mice. This miRNA has mainly been researched as a tumour suppressor. In non-small cell lung cancer, hsa-miR-100 directly targets polo-like kinase 1 (PLK1), a kinase that regulates various steps in cell division. Mmu-miR-99a showed increased expression in the BCG infected MPA treated mice compared to the uninfected, BCG infected and BCG infected NET treated mice. MiR-100 and miR-99a together with miR-99b are

part of the miR-99 gene family. The function and targets of miR-100 and miR-99a have not been well studied. However, hsa-miR-99b was found to negatively regulate the expression of TNF-α, IL-6, IL-12 and IL-1β (60). The suppression of this miRNA leads to reduced growth of *M.tb* (60). Mice infected with *M.tb* and treated with MPA have lower levels of TNF-α in their serum 21 days post-infection and lower levels of TNF-α and IL-6 was present in stimulated mediastinal lymph node cells of *M.tb* infected MPA treated mice (88). MMu-miR-99a expression is up-regulated in BCG infected MPA treated mice. This miRNA could function in the same way hsa-miR-99b does and suppress the expression of TNF-α and IL-6 in BCG infected MPA treated mice. The up-regulation of this miRNA in the blood and suppression of TNF-α and IL-6 could be a possible reason for the higher bacterial load in the spleens of BCG infected MPA treated mice compared to BCG infected mice.

Mmu-miR-509-3p was up-regulated in the BCG infected mice compared to the uninfected, BCG infected MPA treated and BCG infected NET treated mice. The only study previously done on this miRNA showed that it induced apoptosis in renal cell carcinoma (119). It is possible that mmu-miR-509-3p induced apoptosis of phagocytic cells during infection, allowing unkilled bacteria to be taken up by other cells for killing.

These results indicate possible mechanism by which miRNAs found in the whole blood of BCG infected mice can alter the immune response and how this response could be affected by treating these mice with MPA or NET.

5.2.3 MiRNA expression in the lung tissue of mice infected with BCG and treated with MPA or NET

5.2.3.1 MiRNAs that are induced in BCG infected mice.

In lung tissue mmu-miR-1 was up-regulated in the BCG infected mice compared the uninfected mice. The BCG infected MPA treated mice showed a down-regulation of mmu-miR-1 compared to BCG infected mice. Expression of mmu-miR-1 in the BCG infected NET treated mice did not differ from the BCG infected or BCG infected MPA treated mice. This miRNA has mainly been investigated for its role in heart failure. Its expression has not been found to be altered during bacterial infections or GC treatment. It was found in cardiomyocytes that Akt negatively regulates hsa-miR-1 expression through the phosphorylation and inactivation of the transcription factor FOXO3A (120). The over expression of forkhead box O3 (FOXO3A) leads to the suppression of TNF-α secretion (121). In PBMCs the expression of TNF-α is induced by BCG stimulation and in the lung of BCG infected mice the expression of mmu-miR-1 is up-regulated. This could mean that FOXO3A is in its inactive phosphorylated form, indicating a possible role for mmu-miR-1 in the BCG-induced expression of TNF-α.

Mmu-miR-130b was up-regulated in BCG infected mice and suppressed in BCG infected MPA treated and BCG infected NET treated mice compared to the BCG infected mice. Hsa-miR-130b directly targets signal transducer and activator of transcription (STAT) 3 (122). STAT3 activation is enhanced by IL-6 and G-CSF cooperatively (123). The expression of IL-6 is induced by *M.tb* infection, this induction is suppressed by MPA (88). The up-regulation of mmu-miR-130b expression in BCG infected mice could be due to a negative feedback loop. The expression of mmu-miR-130b could be induced to suppress the expression of STAT3 to limit the immunopathology caused by the pro-inflammatory cytokine IL-6. The expression of mmu-miR-130b is suppressed by MPA and NET and the two progestins could thereby inhibit this loop.

Mmu-miR-138 was up-regulated in BCG infected mice and down-regulated in BCG infected MPA treated mice compared to the BCG infected mice. This miRNA was shown to be down-regulated in highly invasive ovarian cancers (124). It directly targets SRY-related HMG-box (Sox)-4 and hypoxia-inducible factor-1α (Hif-1α) and suppress the invasion of cancer cells in a mouse model (124). Sox-4 is important in the development of T and B cells (125,126). It is possible that due to the higher bacterial burden seen in the lungs of *M.tb* infected and MPA treated mice (88) and in the spleen of BCG infected MPA treated mice (as shown in this study), the immune system of these animals are compensating by suppressing the expression of mmu-miR-138 leading to higher levels of Sox-4 to launch a more effective immune response against the pathogen. This is supported by the fact that the bacterial burden at day 56 post-infection is not different between BCG infected and BCG infected and MPA or NET treated mice.

Mmu-miR-202-3p was found to be up-regulated in the BCG infected mice compared to the uninfected, BCG infected MPA treated and BCG infected NET treated mice. No literature could be found on possible targets of this miRNA and therefore its function is still unknown. The expression of mmu-miR-410 was shown to be increased in BCG infected mice compared to uninfected, BCG infected MPA treated and BCG infected NET treated mice. Mmu-miR-301b expression was induced in BCG infected mice compared to uninfected mice. The expression of mmu-miR-301b in BCG infected MPA treated and BCG infected NET treated mice did not differ from the uninfected or BCG infected mice.

Mmu-miR-20a was found to have a higher level of expression in the BCG infected and BCG infected MPA treated mice compared to uninfected mice. This miRNA was found to be up-regulated during LPS induced activation of macrophages and directly targets and lowers expression of signal-regulatory protein α (SIRPα) (127). By targeting SIRPα, hsa-miR-20a together with hsa-miR-17 and hsa-miR-106a regulates macrophage infiltration, phagocytosis and the secretion of pro-inflammatory cytokines such as TNF-α and IL-6 (127). The expression of TNF-α and IL-6 is known to be up-regulated in mice infected with *M.tb* (88). PBMCs stimulated with BCG also had BCG-induced

expression of TNF-α and IL-6, although the expression of miR-20a was not detected. MiR-20a could be involved in induction of expression of these cytokines in mice and PBMCs. The suppression of TNF-α by MPA occurs through a mechanism other than miR-20a, most likely through tethering of the GR to NFκB.

Mmu-miR-449a was up-regulated in BCG infected mice compared to uninfected mice. Nemoto *et al*. found that the expression of rno-miR-449a was induced by Dex in the rat pituitary cells (128). MPA did not induce the expression of this miRNA in the lung of mice infected with BCG and treated with MPA. This could indicate that the effect of Dex on the expression of miRNA is tissue specific or that MPA in the regulation of this miRNA acts like progesterone and not like cortisol in lung tissue.

The miRNAs that were up-regulated in BCG infected mice overall function to induce a protective immune response to the pathogen. This is done by regulating phagocytosis, the production of the pro-inflammatory cytokines TNF-α and IL-6 and regulating the development of T and B cells. Many of these functions of miRNAs are indirect and allow for the fine tuning of the inflammatory response.

5.2.3.2 MiRNAs that are suppressed in BCG infected and MPA or NET treated mice compared to BCG infected mice.

Mmu-miR-132 expression was down-regulated in BCG infected MPA treated mice compared to BCG infected mice. This miRNA was shown to be up-regulated in NK cells after treatment with IL-12. This up-regulation correlated with decreased levels of STAT4 and IFN-γ (129). The up-regulation of mmu-miR-132 might be due to higher levels of IL-12 present. IL-12 is also known to be necessary during the immune response to *M.tb* (130). In our PBMC experiments IL-12p40 and IL-12p70 levels were elevated in the BCG infected cells compared to the US and BCG and MPA stimulated cells at 24 and 72 hours post-infection. Correlating with the expression of IL-12 the level of mmu-miR-130b was also suppressed in BCG infected MPA treated mice compared to BCG infected mice.

The level of mmu-miR-133a was down-regulated in the BCG infected NET treated mice compared to the BCG infected mice. Hsa-miR-133a-1 was found to increase the processing and secretion of IL-1β in THP-1 cells (131). The expression of hsa-miR-133a was not detected in BCG stimulated PBMCs. The level of IL-1β in PBMCs stimulated with BCG was higher than the level of IL-1β in cells stimulated with BCG and NET after 72 hours.

Mmu-miR-200b was down-regulated in the BCG infected NET treated mice compared to the BCG infected mice. This miRNA has many putative binding sites in the TLR4 signalling pathway (132) and decreased the expression of NFκB reporter activity in transfected HEK293 cells (132). Monocytic THP-1 cells that were transfected with hsa-miR-200b showed decreased levels of MyD88, while the LPS induced expression of IL-6 and TNF-α were down-regulated (132). The blocking of pro-

inflammatory cytokines by miR-200b could affect the defence against mycobacterial infections. It is possible that MPA mediates its anti-inflammatory effects by causing the up-regulation of this miRNA in the lung tissue of mice treated with MPA.

The expression of mmu-miR-24 was down-regulated in the BCG infected MPA treated and BCG infected NET treated mice compared to BCG infected mice. Hsa-miR-24 targets pro-apoptotic proteins Bim and caspase 9 in hematopoietic cells and promotes cell survival by down-regulating these targets (133). It is possible that the down-regulation of mmu-miR-24 is an attempt by the immune system to counteract the GC-induced apoptosis caused by treatment with MPA.

Mmu-miR-379 was up-regulated in BCG infected mice compared to BCG infected MPA treated and BCG infected NET treated mice. HsamiR-379 down-regulates the expression of ATP-Binding Cassette C2 protein expression after Rifampicin treatment (134). Hsa-miR-379 regulates the expression of cyclin B1 in breast cancer cells (135). Cyclin B1 is a regulator of cell division (136). It is possible that this miRNA is induced in the lung of BCG infected mice as a negative feedback loop, to inhibit the inflammatory response by suppressing the multiplication of pro-inflammatory immune cells, such as T cells.

The expression of mmu-miR-411 was down-regulated in BCG infected MPA treated mice compared to BCG infected mice. Mmu-miR-16 expression was suppressed in mice infected with BCG and treated with MPA or NET compare to BCG infected mice.

MiRNAs that are suppressed in mice treated with MPA or NET generally have an anti-inflammatory effect in the lungs of mice. They achieve this by targeting STAT4, decreasing IFN-γ, TNF-α and IL-6 expression. This indicates that the progestins have a potential immune suppressive effect in mice.

5.2.3.3 MiRNAs down-regulated in BCG infected, BCG infected and MPA or NET treated mice.

The expression of mmu-miR-134 was down-regulated in BCG infected MPA treated and BCG infected NET treated mice compared to uninfected mice. This miRNA is not well studied but plays a role in the differentiation of stem cells by targeting Sox-2 and Nanog (137).

Mmu-miR-190 expression was down-regulated in the BCG infected mice and up-regulated in BCG infected MPA treated mice compared to BCG infected mice. The expression in BCG infected NET treated mice did not differ from BCG infected mice. Little is known about this miRNA and its functioning. Cheng *et al*. found that inhibition of hsa-miR-190 decreased the growth of HeLA cells (138). Hsa-miR-190 was up-regulated in granulocytes of patients with primary myelofibrosis (139). Hsa-miR-190 indirectly induces the expression of Akt (140). It is known that Akt has an anti-apoptotic effect (141). This could explain why mmu-miR-190 expression is suppressed in all mice

infected with BCG compared to uninfected mice. The level of mmu-miR-190 is up-regulated in mice infected with BCG and treated with MPA compared to BCG infected mice. This could be due to a feedback loop inhibiting GC-induced and BCG-induced apoptosis of phagocytic cells in the lungs to prevent excessive immunopathology.

The expression of mmu-miR-292-3p was down-regulated in all mice that were infected with BCG, regardless of hormone treatment, compared to uninfected mice. This miRNA has not been well studied and to our knowledge this is the first time it has been found to be differentially expressed during bacterial infection. The expression of mmu-miR-301b was up-regulated in BCG infected mice compared to uninfected mice.

Mmu-miR-383 was down-regulated in BCG infected, BCG infected MPA treated and BCG infected NET treated mice compared to uninfected mice. Hsa-miR-383 indirectly suppresses the expression of c-Myc (142). During mycobacterial infection the expression of c-Myc is induced. This induction leads to the induction of TNF-α and IL-6 expression and inhibition of mycobacterial growth (143). This study found that the expression of mmu-miR-383 is suppressed in all mice infected with BCG, possibly leading to greater levels of c-Myc being expressed. This could be another mechanism by which expression of TNF-α and IL-6 is induced in mice infected with *M.tb* (88). The hormones play no role in the suppression of mmu-miR-383 and the suppression of TNF-α in mice treated with MPA must be due to other mechanisms.

The expression of mmu-miR-467e was down-regulated in BCG infected, BCG infected MPA treated and BCG infected NET treated mice.

Mmu-miR-495 was down-regulated in BCG infected and BCG infected MPA treated mice. HsamiR-495 is down-regulated in glioblastoma cells (144). This miRNA was found to regulate the expression of Cyclin dependant kinase 6 (CDK6) and is involved with the inhibition of cell growth by arresting the cell cycle at the G1/S transition (144).

MiRNAs down-regulated by BCG infection generally suppress the inflammatory response or to inhibit division of cells. The inhibition of these miRNAs allows the mice to launch a more effective immune response against the pathogen.

5.2.3.4 MiRNAs up-regulated in BCG infected and MPA or NET treated mice compared to BCG infected mice.

Mmu-miR-384-5p was up-regulated in BCG infected MPA treated mice compared to BCG infected and BCG infected NET treated mice. This miRNA has not been investigated in the context of

bacterial infections but has been shown to target signalling molecule PI3K during an ischemic attack in the heart (145).

Mmu-miR-467c was up-regulated in BCG infected NET treated mice compared to BCG infected mice.

Overall the expression of miRNAs in the tissues of mice has an immune-modulatory effect. Depending on their up- or down-regulation, their effect can be advantageous to the host during infection or have a detrimental effect on the immune response. The miRNAs found to be regulated by MPA indicated that this hormone might have an immune suppressive effect on the host's response to BCG infection.

5.3 Conclusion

MPA is a three-monthly contraceptive favoured by many women in developing countries. MPA has previously been shown to have selective glucocorticoid activity unlike NET which does not activate the GR. The effect of NET on cytokine expression during mycobacterial infection has not been investigated before. During my study I investigated if NET is a better alternative to MPA because it does not have glucocorticoid activity and therefore cannot modulate the immune response via the glucocorticoid receptor. How MPA and NET alter the miRNA expression profile during bacterial infection has not been investigated before and I wanted to determine whether MPA induces its immune modulatory effects by altering miRNA expression. I also wanted to investigate how the differential expression of miRNAs and cytokines *in vitro* (PBMC model) compared to *in vivo* (mouse model).

The results of this study suggest that MPA does alter the expression of miRNA during BCG infection and that this alteration could modulate the immune response to the pathogen. NET did not suppress the immune response to BCG infection in the same way that MPA does. The results suggest MPA acts more like cortisol and NET like progesterone during infection. The expression of miRNAs was also altered by both MPA and NET. Some miRNAs showed the same alteration in expression in both MPA and NET treated cells and mice, while the expression of others varied between the two contraceptives. This could suggest that MPA regulates the expression of some miRNAs via the PR and others via the GR or AR.

These results highlight that not all synthetic progestins are pharmologically identical and that each of the compounds has very distinct immune modulatory effects which need to be further investigated in clinical trials.

Appendix A: MiRNAs found to be expressed in PBMCs but the expression not altered by hormones.
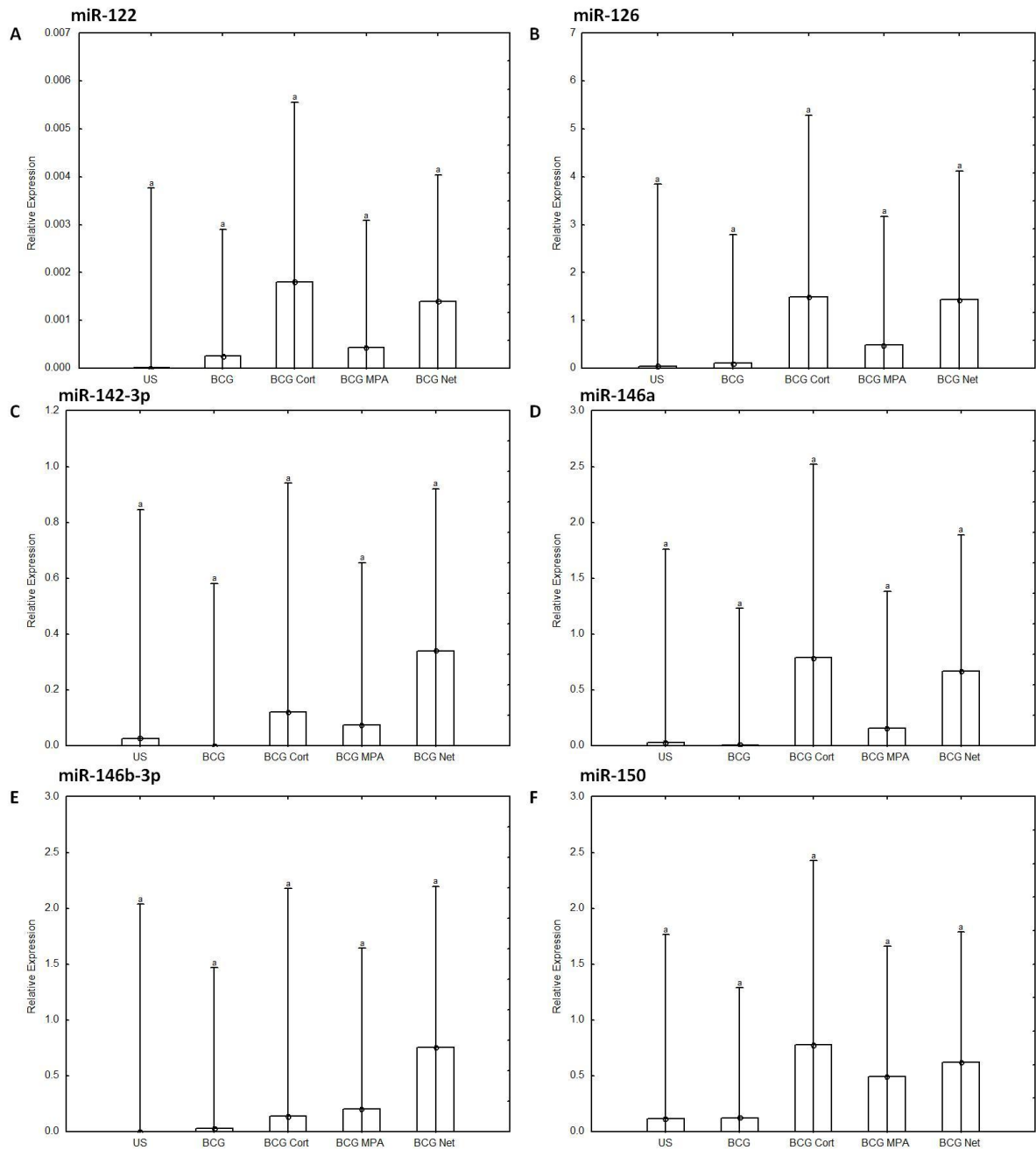
Figure A.2 The relative expression of miRNAs is not different between uninfected, BGC infected, BGC infected and cortisol, MPA or NET treated PBMCs.

Relative expression levels of miR-122 (a), miR-126 (b), miR-142-3p (c), miR-146a (d), miR-146b-5p (e) and miR-150 (f) in PBMCs stimulated with BCG in the presence of cortisol, MPA and NET for 6 hours. Experiments were done in duplicate and whiskers indicate 95% confidence interval. Each condition was done in duplicate. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a, b and c indicate statistical significance, values with the same letter are not statistically different from each other.

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Figure A.3 The relative expression of miRNAs is not different between uninfected, BGC infected, BGC infected and cortisol, MPA or NET treated PBMCs.

Relative expression levels of miR-16 (a), miR-186 (b), miR-191 (c), miR-19b (d), miR-223 (e) and miR-29a (f) in PBMCs stimulated with BCG in the presence of cortisol, MPA and NET for 6 hours. Experiments were done in duplicate and whiskers indicate 95% confidence interval. Each condition was done in duplicate. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a, b and c indicate statistical significance, values with the same letter are not statistically different from each other.

Figure A.40.1 The relative expression of miRNAs is not different between uninfected, BGC infected, BGC infected and cortisol, MPA or NET treated PBMCs.

Relative expression levels of miR-30b (a), miR-335 (b), miR-342-3p (c), miR-342-5p (d), miR-422a (e) and miR-484 (f) in PBMCs stimulated with BCG in the presence of cortisol, MPA and NET for 6 hours. Experiments were done in duplicate and whiskers indicate 95% confidence interval. Each conditi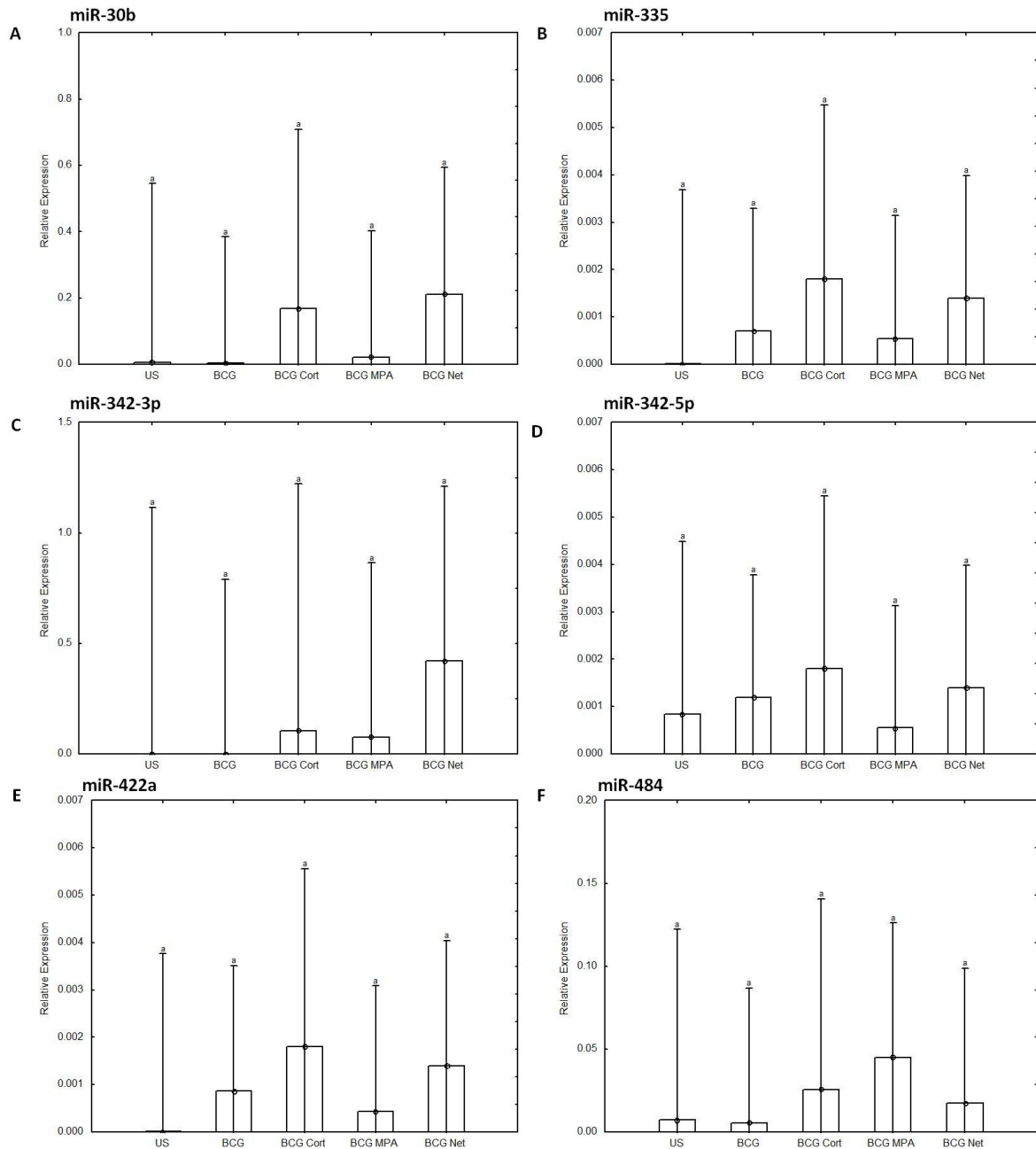on was done in duplicate. Data were analysed by ANOVA (LS means, 95% CI). A p-value of ≤ 0.05 was considered significant. The letters a, b and c indicate statistical significance, values with the same letter are not statistically different from each other.

(1) WHO Tuberculosis report 2013.

(2) Glaziou P, Falzon D, Floyd K, Raviglione M. Global epidemiology of tuberculosis. Seminars in Respiratory and Critical Care Medicine 2013;34(1):3-16.

(3) Tiemersma EW, van der Werf MJ, Borgdorff MW, Williams BG, Nagelkerke NJD. Natural history of tuberculosis: Duration and fatality of untreated pulmonary tuberculosis in HIV negative patients: A systematic review. PLoS ONE 2011;6(4).

(4) Bhatt K, Salgame P. Host innate immune response to Mycobacterium tuberculosis. J Clin Immunol 2007;27(4):347-362.

(5) Salgame P. Host innate and Th1 responses and the bacterial factors that control Mycobacterium tuberculosis infection. Curr Opin Immunol 2005;17(4 SPEC. ISS.):374-380.

(6) Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124(4):783-801.

(7) Killick KE, Ní Cheallaigh C, O'Farrelly C, Hokamp K, Machugh DE, Harris J. Receptor-mediated recognition of mycobacterial pathogens. Cell Microbiol 2013;15(9):1484-1495.

(8) Schlesinger LS, Bellinger-Kawahara CG, Payne NR, Horwitz MA. Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. Journal of Immunology 1990;144(7):2771-2780.

(9) Brightbill HD, Libraty DH, Krutzik SR, Yang R-, Belisle JT, Bleharski JR, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 1999;285(5428):732-736.

(10) Pecora ND, Gehring AJ, Canaday DH, Boom WH, Harding CV. Mycobacterium tuberculosis LprA is a lipoprotein agonist of TLR2 that regulates innate immunity and APC function. Journal of Immunology 2006;177(1):422-429.

(11) Gehring AJ, Dobos KM, Belisle JT, Harding CV, Boom WH. Mycobacterium tuberculosis LprG (Rv1411c): A novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. Journal of Immunology 2004;173(4):2660-2668.

(12) Ernst JD. The immunological life cycle of tuberculosis. Nature Reviews Immunology 2012;12(8):581-591.

(13) Jones BW, Means TK, Heldwein KA, Keen MA, Hill PJ, Belisle JT, et al. Different Toll-like receptor agonists induce distinct macrophage responses. J Leukoc Biol 2001;69(6):1036-1044.

(14) Abel B, Thieblemont N, Quesniaux VJF, Brown N, Mpagi J, Miyake K, et al. Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. Journal of Immunology 2002;169(6):3155-3162.

(15) Fortune SM, Solache A, Jaeger A, Hill PJ, Belisle JT, Bloom BR, et al. Mycobacterium tuberculosis Inhibits Macrophage Responses to IFN-γ through Myeloid Differentiation Factor 88-Dependent and -Independent Mechanisms. Journal of Immunology 2004;172(10):6272-6280.

(16) Buettner M, Meinken C, Bastian M, Bhat R, Stössel E, Faller G, et al. Inverse correlation of maturity and antibacterial activity in human dendritic cells. Journal of Immunology 2005;174(7):4203-4209.

(17) Barry Jr. CE, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn JA, et al. The spectrum of latent tuberculosis: Rethinking the biology and intervention strategies. Nature Reviews Microbiology 2009;7(12):845-855.

(18) Silva Miranda M, Breiman A, Allain S, Deknuydt F, Altare F. The tuberculous granuloma: An unsuccessful host defence mechanism providing a safety shelter for the bacteria? Clinical and Developmental Immunology 2012;2012.

(19) Silva EJR, Queiró DBC, Rodrigues A, Honda L, Avellar MCW. Innate Immunity and Glucocorticoids: Potential regulatory mechanisms in epididymal biology. J Androl 2011;32(6):614-624.

(20) Estaquier J, Idziorek T, Zou W, Emilie D, Farber C-, Bourez - JM, et al. T helper type 1/T helper type 2 cytokines and T cell death: Preventive effect of interleukin 12 on activation-induced and CD95 (FAS/APO-1)-mediated apoptosis of CD4+ T cells from human immunodeficiency virus-infected persons. J Exp Med 1995;182(6):1759-1767.

(21) Castro AG, Silva RA, Appelberg R. Endogenously produced IL-12 is required for the induction of protective T cells during Mycobacterium avium infections in mice. Journal of Immunology 1995;155(4):2013-2019.

(22) Flynn JL, Goldstein MM, Triebold KJ, Sypek J, Wolf S, Bloom BR. IL-12 increases resistance of BALB/c mice to Mycobacterium tuberculosis infection. Journal of Immunology 1995;155(5):2515-2524.

(23) Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. J Exp Med 1992;175(4):1111-1122.

(24) Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, et al. Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. Infect Immun 1997;65(1):298-304.

(25) Hirsch CS, Toossi Z, Othieno C, Johnson JL, Schwander SK, Robertson S, et al. Depressed T-cell interferon-γ responses in pulmonary tuberculosis: Analysis of underlying mechanisms and modulation with therapy. J Infect Dis 1999;180(6):2069-2073.

(26) Fulton SA, Cross JV, Toossi ZT, Boom WH. Regulation of interleukin-12 by interleukin-10, transforming growth factor-β, tumor necrosis factor-α, and interferon-γ in human monocytes infected with Mycobacterium tuberculosis H37Ra. J Infect Dis 1998;178(4):1105-1114.

(27) Rojas RE, Balaji KN, Subramanian A, Boom WH. Regulation of human CD4+ αβ T-cell-receptor-positive (TCR+) and γδ TCR+ T-cell responses to Mycobacterium tuberculosis by interleukin-10 and transforming growth factor β. Infect Immun 1999;67(12):6461-6472.

(28) Lindsay MA. microRNAs and the immune response. Trends Immunol 2008;29(7):343-351.

(29) Kumar R, Halder P, Sahu SK, Kumar M, Kumari M, Jana K, et al. Identification of a novel role of ESAT-6-dependent miR-155 induction during infection of macrophages with mycobacterium tuberculosis. Cell Microbiol 2012;14(10):1620-1631.

(30) O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A 2007;104(5):1604-1609.

(31) Eulalio A, Schulte LN, Voge J. The mammalian microRNA response to bacterial infections. RNA Biology 2012;9(6):742-750.

(32) Chekulaeva M, Filipowicz W. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. Curr Opin Cell Biol 2009;21(3):452-460.

(33) O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. Proc Natl Acad Sci U S A 2009;106(17):7113-7118.

(34) Velu CS, Baktula AM, Grimes HL. Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. Blood 2009;113(19):4720-4728.

(35) Johnnidis JB, Harris MH, Wheeler RT, Stehling-Sun S, Lam MH, Kirak O, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. Nature 2008;451(7182):1125-1129.

(36) Bezman NA, Cedars E, Steiner DF, Blelloch R, Hesslein DGT, Lanier LL. Distinct requirements of microRNAs in NK cell activation, survival, and function. Journal of Immunology 2010;185(7):3835-3846.

(37) Lu TX, Sherrill JD, Wen T, Plassard AJ, Besse JA, Abonia JP, et al. MicroRNA signature in patients with eosinophilic esophagitis, reversibility with glucocorticoids, and assessment as disease biomarkers. J Allergy Clin Immunol 2012;129(4):1064-1075.e9.

(38) Boldin MP, Baltimore D. MicroRNAs, new effectors and regulators of NF-κB. Immunological Reviews 2012;246(1):205-220.

(39) Fontana L, Pelosi E, Greco P, Racanicchi S, Testa U, Liuzzi F, et al. MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. Nat Cell Biol 2007;9(7):775-787.

(40) Taganov KD, Boldin MP, Chang K-, Baltimore D. NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A 2006;103(33):12481-12486.

(41) Liu G, Friggeri A, Yang Y, Park Y-, Tsuruta Y, Abraham E. miR-147, a microRNA that is induced upon toll-like receptor stimulation, regulates murine macrophage inflammatory responses. Proc Natl Acad Sci U S A 2009;106(37):15819-15824.

(42) Tsitsiou E, Lindsay MA. microRNAs and the immune response. Current Opinion in Pharmacology 2009;9(4):514-520.

(43) Taganov KD, Boldin MP, Baltimore D. MicroRNAs and Immunity: Tiny Players in a Big Field. Immunity 2007;26(2):133-137.

(44) Bazzoni F, Rossato M, Fabbri M, Gaudiosi D, Mirolo M, Mori L, et al. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. Proc Natl Acad Sci U S A 2009;106(13):5282-5287.

(45) Hou J, Wang P, Lin L, Liu X, Feng M, An H, et al. MicroRNA-146a feedback inhibits RIG-I-dependent type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. Journal of Immunology 2009;183(3):2150-2158.

(46) Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. Nat Immunol 2008;9(4):405-414.

(47) Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al. Requirement of bic/microRNA-155 for normal immune function. Science 2007;316(5824):608-611.

(48) Rebane A, Akdis CA. MicroRNAs: Essential players in the regulation of inflammation. J Allergy Clin Immunol 2013.

(49) Li Q-, Chau J, Ebert PJR, Sylvester G, Min H, Liu G, et al. miR-181a Is an Intrinsic Modulator of T Cell Sensitivity and Selection. Cell 2007;129(1):147-161.

(50) Chen C-, Li L, Lodish HF, Bartel DP. MicroRNAs Modulate Hematopoietic Lineage Differentiation. Science 2004;303(5654):83-86.

(51) Zhou B, Wang S, Mayr C, Bartel DP, Lodish HF. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. Proc Natl Acad Sci U S A 2007;104(17):7080-7085.

(52) He L, He X, Lim LP, De Stanchina E, Xuan Z, Liang Y, et al. A microRNA component of the p53 tumour suppressor network. Nature 2007;447(7148):1130-1134.

(53) Thai T-, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, et al. Regulation of the germinal center response by MicroRNA-155. Science 2007;316(5824):604-608.

(54) Dorsett Y, McBride KM, Jankovic M, Gazumyan A, Thai T-, Robbiani DF, et al. MicroRNA-155 Suppresses Activation-Induced Cytidine Deaminase-Mediated Myc-Igh Translocation. Immunity 2008;28(5):630-638.

(55) Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, et al. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-γ. Nature Immunology 2011;12(9):861-869.

(56) Harapan H, Fitra F, Ichsan I, Mulyadi M, Miotto P, Hasan NA, et al. The roles of microRNAs on tuberculosis infection: Meaning or myth? Tuberculosis 2013.

(57) Sharbati J, Lewin A, Kutz-Lohroff B, Kamal E, Einspanier R, Sharbati S. Integrated microrna-mrna-analysis of human monocyte derived macrophages upon mycobacterium avium subsp. hominissuis infection. PLoS ONE 2011;6(5).

(58) Lu TX, Hartner J, Lim E-, Fabry V, Mingler MK, Cole ET, et al. MicroRNA-21 limits in vivo immune response-mediated activation of the IL-12/IFN-γ pathway, Th1 polarization, and the severity of delayed-type hypersensitivity. Journal of Immunology 2011;187(6):3362-3373.

(59) Wu Z, Lu H, Sheng J, Li L. Inductive microRNA-21 impairs anti-mycobacterial responses by targeting IL-12 and Bcl-2. FEBS Lett 2012;586(16):2459-2467.

(60) Singh Y, Kaul V, Mehra A, Chatterjee S, Tousif S, Dwivedi VP, et al. Mycobacterium tuberculosis controls MicroRNA-99b (miR-99b) expression in infected murine dendritic cells to modulate host immunity. J Biol Chem 2013;288(7):5056-5061.

(61) Rajaram MVS, Ni B, Morris JD, Brooks MN, Carlson TK, Bakthavachalu B, et al. Mycobacterium tuberculosis lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b. Proc Natl Acad Sci U S A 2011;108(42):17408-17413.

(62) Liu Y, Wang X, Jiang J, Cao Z, Yang B, Cheng X. Modulation of T cell cytokine production by miR-144* with elevated expression in patients with pulmonary tuberculosis. Mol Immunol 2011;48(9-10):1084-1090.

(63) Xu Z, Xiao S-, Xu P, Xie Q, Cao L, Wang D, et al. miR-365, a novel negative regulator of interleukin-6 gene expression, is cooperatively regulated by Sp1 and NF-κB. Journal of Biological Chemistry 2011;286(24):21401-21412.

(64) Steiner D, Thomas M, Hu J, Yang Z, Babiarz J, Allen C, et al. MicroRNA-29 Regulates T-Box Transcription Factors and Interferon-γ Production in Helper T Cells. Immunity 2011;35(2):169-181.

(65) Fu Y, Yi Z, Wu X, Li J, Xu F. Circulating microRNAs in patients with active pulmonary tuberculosis. J Clin Microbiol 2011;49(12):4246-4251.

(66) Banerjee A, Schambach F, Dejong CS, Hammond SM, Reiner SL. Micro-RNA-155 inhibits IFN-γ signaling in CD4+ T cells. European Journal of Immunology 2010;40(1):225-231.

(67) Spinelli SV, Diaz A, D'Attilio L, Marchesini MM, Bogue C, Bay ML, et al. Altered microRNA expression levels in mononuclear cells of patients with pulmonary and pleural tuberculosis and their relation with components of the immune response. Mol Immunol 2012;53(3):265-269.

(68) Li S-, Chan W-, Hu L-, Lai C-, Hsu C-, Lin W-. Identification of homologous microRNAs in 56 animal genomes. Genomics 2010;96(1):1-9.

(69) Weber MJ. New human and mouse microRNA genes found by homology search. FEBS Journal 2005;272(1):59-73.

(70) Newton R. Molecular mechanisms of glucocorticoid action: What is important? Thorax 2000;55(7):603-613.

(71) Kadmiel M, Cidlowski JA. Glucocorticoid receptor signaling in health and disease. Trends Pharmacol Sci 2013;34(9):518-530.

(72) Cao Y, Bender IK, Konstantinidis AK, Shin SC, Jewell CM, Cidlowski JA, et al. Glucocorticoid receptor translational isoforms underlie maturational stage-specific glucocorticoid sensitivities of dendritic cells in mice and humans. Blood 2013;121(9):1553-1562.

(73) Baschant U, Tuckermann J. The role of the glucocorticoid receptor in inflammation and immunity. J Steroid Biochem Mol Biol 2010;120(2-3):69-75.

(74) Busillo JM, Cidlowski JA. The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. Trends in Endocrinology and Metabolism 2013.

(75) Miyaura H, Iwata M. Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. Journal of Immunology 2002;168(3):1087-1094.

(76) Oakley RH, Cidlowski JA. The biology of the glucocorticoid receptor: New signaling mechanisms in health and disease. J Allergy Clin Immunol 2013.

(77) Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids - New mechanisms for old drugs. N Engl J Med 2005;353(16):1711-1723+1658.

(78) Newton R, Holden NS. Separating transrepression and transactivation: A distressing divorce for the glucocorticoid receptor? Mol Pharmacol 2007;72(4):799-809.

(79) Almawi WY, Melemedjian OK. Molecular mechanisms of glucocorticoid antiproliferative effects: Antagonism of transcription factor activity by glucocorticoid receptor. J Leukoc Biol 2002;71(1):9-15.

(80) Cidlowski JA. Glucocorticoids and their actions in cells. Retina 2009;29(SUPPL. 6):S21-S23.

(81) Jantzen H-, Strähle U, Gloss B, Stewart F, Schmid W, Boshart M, et al. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell 1987;49(1):29-38.

(82) Barnes PJ. Therapeutic strategies for allergic diseases. Nature 1999;402(6760 SUPPL. 1):B31-B32.

(83) Löwenberg M, Tuynman J, Bilderbeek J, Gaber T, Buttgereit F, Van Deventer S, et al. Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn. Blood 2005;106(5):1703-1710.

(84) De Oliveira APL, Domingos HV, Cavriani G, Damazo AS, Franco ALDS, Oliani SM, et al. Cellular recruitment and cytokine generation in a rat model of allergic lung inflammation are differentially modulated by progesterone and estradiol. American Journal of Physiology - Cell Physiology 2007;293(3):C1120-C1128.

(85) Lee JH, Lydon JP, Kim CH. Progesterone suppresses the mTOR pathway and promotes generation of induced regulatory T cells with increased stability. Eur J Immunol 2012;42(10):2683-2696.

(86) Dosiou C, Lathi RB, Tulac S, Huang S-J, Giudice LC. Interferon-related and other immune genes are downregulated in peripheral blood leukocytes in the luteal phase of the menstrual cycle. J Clin Endocrinol Metab 2004;89(5):2501-2504.

(87) Kleynhans L, du Plessis N, Black GF, Loxton AG, Kidd M, van Helden PD, et al. Medroxyprogesterone acetate alters mycobacterium bovis BCG-induced cytokine production in peripheral blood mononuclear cells of contraceptive users. PLoS ONE 2011;6(9).

(88) Kleynhans L, Du Plessis N, Allie N, Jacobs M, Kidd M, van Helden PD, et al. The contraceptive depot medroxyprogesterone acetate impairs mycobacterial control and inhibits cytokine secretion in mice infected with Mycobacterium tuberculosis. Infect Immun 2013;81(4):1234-1244.

(89) Hofmeyr GJ, Singata M, Lawrie TA. Copper containing intra-uterine devices versus depot progestogens for contraception. Cochrane database of systematic reviews (Online) 2010;6.

(90) Schwallie PC, Assenzo JR. The effect of depo medroxyprogesterone acetate on pituitary and ovarian function, and the return of fertility following its discontinuation: a review. Contraception 1974;10(2):181-202.

(91) Smit J, Gray A, McFadyen L, Zuma K. Counting the costs: Comparing depot medroxyprogesterone acetate and norethisterone oenanthate utilisation patterns in South Africa. BMC Health Services Research 2001;1:1-9.

(92) Moore NL, Hickey TE, Butler LM, Tilley WD. Multiple nuclear receptor signaling pathways mediate the actions of synthetic progestins in target cells. Mol Cell Endocrinol 2012;357(1-2):60-70.

(93) Koubovec D, Ronacher K, Stubsrud E, Louw A, Hapgood JP. Synthetic progestins used in HRT have different glucocorticoid agonist properties. Mol Cell Endocrinol 2005;242(1-2):23-32.

(94) Bamberger C, Schulte HM. Molecular mechanisms of dissociative glucocorticoid activity. European Journal of Clinical Investigation, Supplement 2000;30(3):6-9.

(95) Smoak KA, Cidlowski JA. Mechanisms of glucocorticoid receptor signaling during inflammation. Mech Ageing Dev 2004;125(10-11 SPEC. ISS.):697-706.

(96) Kotani A, Ha D, Hsieh J, Rao PK, Schotte D, Den Boer ML, et al. miR-128b is a potent glucocorticoid sensitizer in MLL-AF4 acute lymphocytic leukemia cells and exerts cooperative effects with miR-221. Blood 2009;114(19):4169-4178.

(97) Tessel MA, Benham AL, Krett NL, Rosen ST, Gunaratne PH. Role for MicroRNAs in Regulating Glucocorticoid Response and Resistance in Multiple Myeloma. Hormones and Cancer 2011;2(3):182-189.

(98) Vreugdenhil E, Verissimo CSL, Mariman R, Kamphorst JT, Barbosa JS, Zweers T, et al. MicroRNA 18 and 124a down-regulate the glucocorticoid receptor: Implications for glucocorticoid responsiveness in the brain. Endocrinology 2009;150(5):2220-2228.

(99) Ledderose C, Möhnle P, Limbeck E, Schütz S, Weis F, Rink J, et al. Corticosteroid resistance in sepsis is influenced by microRNA-124-induced downregulation of glucocorticoid receptor-α. Crit Care Med 2012.

(100) Davis TE, Kis-Toth K, Szanto A, Tsokos GC. Glucocorticoids suppress T cell function by up-regulating microRNA-98. Arthritis Rheum 2013;65(7):1882-1890.

(101) Zheng Y, Izumi K, Li Y, Ishiguro H, Miyamoto H. Contrary regulation of bladder cancer cell proliferation and invasion by dexamethasone-mediated glucocorticoid receptor signals. Molecular Cancer Therapeutics 2012;11(12):2621-2632.

(102) Yang A, Ma J, Wu M, Qin W, Zhao B, Shi Y, et al. Aberrant microRNA-182 expression is associated with glucocorticoid resistance in lymphoblastic malignancies. Leukemia and Lymphoma 2012;53(12):2465-2473.

(103) Hong Y, Wu J, Zhao J, Wang H, Liu Y, Chen T, et al. miR-29b and miR-29c Are Involved in Toll-Like Receptor Control of Glucocorticoid-Induced Apoptosis in Human Plasmacytoid Dendritic Cells. PLoS ONE 2013;8(7).

(104) Zheng Y, Xiong S, Jiang P, Liu R, Liu X, Qian J, et al. Glucocorticoids inhibit lipopolysaccharide-mediated inflammatory response by downregulating microRNA-155: A novel anti-inflammation mechanism. Free Radical Biology and Medicine 2012;52(8):1307-1317.

(105) Daigneault M, de Silva TI, Bewley MA, Preston JA, Marriott HM, Mitchell AM, et al. Monocytes regulate the mechanism of T-cell death by inducing Fas-mediated apoptosis during bacterial infection. PLoS Pathogens 2012;8(7):30.

(106) Spies CM, Gaber T, Hahne M, Naumann L, Tripmacher R, Schellmann S, et al. Rimexolone inhibits proliferation, cytokine expression and signal transduction of human CD4+ T-cells. Immunol Lett 2010;131(1):24-32.

(107) Ahmed MAE. Effect of glucocorticoids on surface IL-10 receptors and intracellular IL-10 in mouse splenocytes, thymocytes, monocytes, and dendritic cells: Immunofluorescence, confocal microscopy, and electrophoresis study. Egyptian Journal of Histology 2012;35(2):236-246.

(108) Gutsol AA, Sokhonevich NA, Seledtsov VI, Litvinova LS. Dexamethasone effects on activation and proliferation of immune memory T cells. Bulletin of Experimental Biology and Medicine 2013;155(4):474-476.

(109) Huang C-, Acevedo EO, Mari DC, Randazzo C, Shibata Y. Glucocorticoid inhibition of leptin- and lipopolysaccharide-induced interlukin-6 production in obesity. Brain Behav Immun 2013.

(110) Barnes PJ. Th2 cytokines and asthma: An introduction. Respiratory Research 2001;2(2):64-65.

(111) Ma W, Gee K, Lim W, Chambers K, Angel JB, Kozlowski M, et al. Dexamethasone Inhibits IL-12p40 Production in Lipopolysaccharide-Stimulated Human Monocytic Cells by Down-Regulating the Activity of c-Jun N-Terminal Kinase, the Activation Protein-1, and NF-κB Transcription Factors. Journal of Immunology 2004;172(1):318-330.

(112) Nehmé A, Edelman J. Dexamethasone inhibits high glucose-, TNF-α- And IL-1β-induced secretion of inflammatory and angiogenic mediators from retinal microvascular pericytes. Invest Ophthalmol Visual Sci 2008;49(5):2030-2038.

(113) Bockhorn J, Dalton R, Nwachukwu C, Huang S, Prat A, Yee K, et al. MicroRNA-30c inhibits human breast tumour chemotherapy resistance by regulating TWF1 and IL-11. Nature Communications 2013;4.

(114) Graff JW, Dickson AM, Clay G, McCaffrey AP, Wilson ME. Identifying functional microRNAs in macrophages with polarized phenotypes. J Biol Chem 2012;287(26):21816-21825.

(115) Huang H-, Yu H-, Huang L-, Huang H-, Chen R-, Lin I, et al. MiRNA-125b regulates TNF-a production in CD14+ neonatal monocytes via post-transcriptional regulation. J Leukoc Biol 2012;92(1):171-182.

(116) Liu X, Wang Y, Sun Q, Yan J, Huang J, Zhu S, et al. Identification of microRNA transcriptome involved in human natural killer cell activation. Immunol Lett 2012;143(2):208-217.

(117) Bamberger CM, Else T, Bamberger A-, Beil FU, Schulte HM. Regulation of the human interleukin-2 gene by the a and ß isoforms of the glucocorticoid receptor. Mol Cell Endocrinol 1998;136(1):23-28.

(118) Jung Y-, LaCourse R, Ryan L, North RJ. Evidence inconsistent with a negative influence of T helper 2 cells on protection afforded by a dominant T helper 1 response against Mycobacterium tuberculosis lung infection in mice. Infect Immun 2002;70(11):6436-6443.

(119) Zhai Q, Zhou L, Zhao C, Wan J, Yu Z, Guo X, et al. Identification of miR-508-3p and miR-509-3p that are associated with cell invasion and migration and involved in the apoptosis of renal cell carcinoma. Biochem Biophys Res Commun 2012;419(4):621-626.

(120) Kumarswamy R, Lyon AR, Volkmann I, Mills AM, Bretthauer J, Pahuja A, et al. SERCA2a gene therapy restores microRNA-1 expression in heart failure via an Akt/FoxO3A-dependent pathway. Eur Heart J 2012;33(9):1067-1075.

(121) Xia M, Zhu Y. FOXO3a involvement in the release of TNF-a stimulated by ATP in spinal cord astrocytes. Journal of Molecular Neuroscience 2013;51(3):792-804.

(122) Zhao G, Zhang J-, Shi Y, Qin Q, Liu Y, Wang B, et al. MiR-130b Is a Prognostic Marker and Inhibits Cell Proliferation and Invasion in Pancreatic Cancer through Targeting STAT3. PLoS ONE 2013;8(9).

(123) Yan B, Wei J-, Yuan Y, Sun R, Li D, Luo J, et al. IL-6 cooperates with G-CSF to induce protumor function of neutrophils in bone marrow by enhancing STAT3 activation. Journal of Immunology 2013;190(11):5882-5893.

(124) Yeh Y-, Chuang C-, Chao K-, Wang L-. MicroRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1a. International Journal of Cancer 2013;133(4):867-878.

(125) Schilham MW, Moerer P, Cumano A, Clevers HC. Sox-4 facilitates thymocyte differentiation. Eur J Immunol 1997;27(5):1292-1295.

(126) Smith E, Sigvardsson M. The roles of transcription factors in B lymphocyte commitment, development, and transformation. J Leukoc Biol 2004;75(6):973-981.

(127) Zhu D, Pan C, Li L, Bian Z, Lv Z, Shi L, et al. MicroRNA-17/20a/106a modulate macrophage inflammatory responses through targeting signal-regulatory protein a. J Allergy Clin Immunol 2013;132(2):426-436.e8.

(128) Nemoto T, Mano A, Shibasaki T. MiR-449a contributes to glucocorticoid-induced CRF-R1 downregulation in the pituitary during stress. Molecular Endocrinology 2013;27(10):1593-1602.

(129) Huang Y, Lei Y, Zhang H, Hou L, Zhang M, Dayton AI. MicroRNA regulation of STAT4 protein expression: Rapid and sensitive modulation of IL-12 signaling in human natural killer cells. Blood 2011;118(26):6793-6802.

(130) Cooper AM, Magram J, Ferrante J, Orme IM. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. J Exp Med 1997;186(1):39-45.

(131) Bandyopadhyay S, Lane T, Venugopal R, Parthasarathy PT, Cho Y, Galam L, et al. MicroRNA-133a-1 regulates inflammasome activation through uncoupling protein-2. Biochem Biophys Res Commun 2013;439(3):407-412.

(132) Wendlandt EB, Graff JW, Gioannini TL, McCaffrey AP, Wilson ME. The role of MicroRNAs miR-200b and miR-200c in TLR4 signaling and NF-κB activation. Innate Immunity 2012;18(6):846-855.

(133) Nguyen T, Rich A, Dahl R. MiR-24 Promotes the Survival of Hematopoietic Cells. PLoS ONE 2013;8(1).

(134) Haenisch S, Laechelt S, Bruckmueller H, Werk A, Noack A, Bruhn O, et al. Down-regulation of ATP-binding cassette C2 protein expression in HepG2 cells after rifampicin treatment is mediated by microRNA-379. Mol Pharmacol 2011;80(2):314-320.

(135) Khan S, Brougham CL, Ryan J, Sahrudin A, O'Neill G, Wall D, et al. miR-379 Regulates Cyclin B1 Expression and Is Decreased in Breast Cancer. PLoS ONE 2013;8(7).

(136) Vella LA, Yu M, Fuhrmann SR, El-Amine M, Epperson DE, Finn OJ. Healthy individuals have T-cell and antibody responses to the tumor antigen cyclin B1 that when elicited in mice protect from cancer. Proc Natl Acad Sci U S A 2009;106(33):14010-14015.

(137) Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 2008;455(7216):1124-1128.

(138) Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res 2005;33(4):1290-1297.

(139) Guglielmelli P, Tozzi L, Pancrazzi A, Bogani C, Antonioli E, Ponziani V, et al. MicroRNA expression profile in granulocytes from primary myelofibrosis patients. Exp Hematol 2007;35(11):1708.e1-1708.e12.

(140) Beezhold K, Liu J, Kan H, Meighan T, Castranova V, Shi X, et al. miR-190-mediated downregulation of PHLPP contributes to arsenic-induced Akt activation and carcinogenesis. Toxicological Sciences 2011;123(2):411-420.

(141) Persson A, Blomgran-Julinder R, Eklund D, Lundström C, Stendahl O. Induction of apoptosis in human neutrophils by Mycobacterium tuberculosis is dependent on mature bacterial lipoproteins. Microb Pathog 2009;47(3):143-150.

(142) Yin M, Lü M, Yao G, Tian H, Lian J, Liu L, et al. Transactivation of microRNA-383 by steroidogenic factor-1 promotes estradiol release from mouse ovarian granulosa cells by targeting RBMS1. Molecular Endocrinology 2012;26(7):1129-1143.

(143) Yim HCH, Li JCB, Pong JCH, Lau ASY. A role for c-Myc in regulating anti-mycobacterial responses. Proc Natl Acad Sci U S A 2011;108(43):17749-17754.

(144) Chen S-, Chen H-, Chen S-, Huang C-, Chen P-, Wu TWE, et al. MicroRNA-495 inhibits proliferation of glioblastoma multiforme cells by downregulating cyclin-dependent kinase 6. World Journal of Surgical Oncology 2013;11.

(145) Bao Y, Lin C, Ren J, Liu J. MicroRNA-384-5p regulates ischemia-induced cardioprotection by targeting phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta (PI3K p110d). Apoptosis 2013;18(3):260-270.