INVESTIGATING THE IMMUNE MODULATORY PROPERTIES OF KISSPEPTIN: IMPLICATIONS FOR PREGNANCY

Stefan Marc Botha

THESIS SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In fulfilment of the requirements for the degree

MASTER OF SCIENCE IN MEDICINE

MSc (Med) Medical Biochemistry

February 2020

Division of Medical Biochemistry, Faculty of Health Sciences,

Department of Integrative Biomedical Sciences,

Institute of Infectious Disease and Molecular Medicine,

University of Cape Town

Department of Obstetrics and Gynaecology

University of Cape Town, Groote Schuur Hospital

Cape Town

Date of submission:

Supervisor[s]: Professor Arieh Katz

Co-supervisor[s]: Associate Professor Mushi Matjila

Declaration

I, Stefan Marc Botha, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

This thesis/dissertation has been submitted to the Turnitin module (or equivalent similarity and originality checking software).

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Signature: ……

Date: 9 February 2020

Acknowledgements

I feel that this section may be longer than the thesis itself as I owe the completion and submission of this thesis to so many people who have helped me along this intense, long but rewarding journey. This was an extremely tough time and I learnt so much in the process. Over the past two years I have made many friendships, discovered myself, learnt the true value of independence and most importantly I learnt the most valuable lesson, I learnt how to fail forward.

This section will never be enough to express the magnitude of my gratitude but I hope to express it in some part. I would thus like to thank the following people and institutions for making this MSc possible.

Firstly, I would like to thank my supervisor Professor Arieh Katz and my co-supervisor Associate Professor Mushi Matjila. They have provided me with exceptional advice not only with regards to my thesis and research but also with regards to my career and life as a scientist. They have taught me how to approach research/work, think independently and how to own your project.

Dr. Georgia Schäfer for her wisdom and guidance, especially when I would approach her out of the blue. Your patience is appreciated.

Dr Aron Abera for his patience and guidance in helping me with protocols and technical issues. Steven Makatsa for his generosity in helping me establish the whole blood assay during his busy PhD studies.

Graham Christians, Roshan Ebrahim and Xolani Nonzinyana for always being there for me and helping me within the lab. They are all responsible for running the lab and lay the foundations for us to conduct our research.

My lab colleagues: Sinead Carse (a.k.a. bestie), Melissa Blumenthal, Nonku, Alda and Melissa Marx for your unconditional support and help in my desperate times, despite you all being so busy with your own research. I truly appreciate all your help and just willingness to listen and this project could not have been completed without you. I value the friendship established over the time spent with you.

Dr. Leanie Kleynhan-Cornelissen and the immunology research group team at Tygerberg hospital. You taught me so much in terms of techniques and research. I cannot thank you enough for your help and for treating me as one of your own. The squash days, end of year function and braais, I thank you for allowing me to conduct research at your institution and for making me a part of your family.

Dr. Nasiema Allie and Ilze Louw for P3 training and spending so many hours teaching me important techniques which will definitely aid me in my future as a scientist.

The level six lab: Associate Professor Zenda Woodman, Bianca, Humeira, Jamie, Alessandra, Caryn, Monds and Zweni. Another group of people who were essential to the completion of this thesis. They allowed me to take a break from the lab life and gave me scope which allowed me to approach my work from a different perspective. I appreciate all the advice, conversations, pizza nights and game nights. It is a pleasure to call you my friends.

The faculty squash club, you gave me a break from the lab and kept me on my feet with your competitive edge which definitely aided my work. Stanford, Hlumani, Carmelita, Rodney, Ben, Ian and Munya, I loved playing against you guys. Till the next time when I come for the 3-0.

I must thank Hana Faulds who has possibly been the greatest discovery made during this time. Your love, tough love, advice, guidance, support, motivation and patience was so important for me in finishing this project, especially during the last few months. You were there when I needed the most support and I can't thank you enough. Always…

My family: Andre, Melanie, Jacques, Emma and Ethan. Thank you for all your support over the years both emotionally and financially. One could go on about family, but the term speaks for itself. I love you and I could not have done this without you.

I would like to say a massive thank you to Sister Anne Hoffman and Sister Lynne Keck. Without your help getting samples and without your patience, none of this would have been possible. Thank you!

The University of Cape Town and the National Research Foundation (NRF) for their financial support and for affording me the opportunity to conduct research at such a world class institution.

Abstract

Pregnancy is dependent on the development of maternal immune tolerance to the genetically foreign fetus. During pregnancy the mother's immune reactivity and energy metabolism undergoes significant changes and the levels of certain hormones in peripheral blood are significantly increased. Hormones are important regulators of the functional activity of the immune system and immune cells within. Hormones secreted by the placenta, protect the fetus from the maternal immune response of the mother, emphasizing their immunomodulatory effects. Therefore, hormonal regulation is essential for the functional activity of immune cells.

There is evidence that the hormone, kisspeptin, plays a role in the development of immune tolerance during pregnancy based on its role in the regulation of the adaptive T regulatory (aTreg)/T-helper 17 (Th17) cells, induction of the enzyme indoleamine 2,3-dioxygenase (IDO) and regulation of monocyte function during pregnancy. In addition, kisspeptin has been implicated in the regulation of specific cytokines during pregnancy. It is crucial to maintain an appropriate cytokine balance at the maternal–fetal interface as well as in circulation. Several pregnancy-related disorders have been associated with a variation in Th1/Th2/Th17 cytokines and aTreg cell subsets. Kisspeptin has been implicated in regulating cytokines IL-10 and IL-17A as well as aTreg and Th17 cells which are significant role players in immune tolerance during pregnancy. However, its effect on other pro- and anti-inflammatory cytokines remain unknown. Therefore, more research is required to better understand the role of kisspeptin in the development of immune tolerance during pregnancy. The hypothesis of this study is that kisspeptin alters the expression of anti-and pro-inflammatory cytokines and may thus influence the establishment of immune tolerance in pregnancy. To test this hypothesis, we used a previously established *in vitro* peripheral blood mononuclear cell (PBMC) *Mycobacterium tuberculosis* (Mtb) infection assay model as well as a newly established *in vitro* infection model using lipopolysaccharide (LPS)-stimulated whole blood.

Protein expression analysis of selected pro- and anti-inflammatory cytokines was performed on PBMC infected with Mtb and on whole blood cells stimulated with LPS in the absence and presence of kisspeptin-10 for different times. The cytokines levels were measured by luminex multiplex assay and sandwich ELISA, respectively. Results from the PBMC infection assay showed a varied but not statistically significant effect of kisspeptin-10 on selected pro- and anti-inflammatory cytokine expression at 2 hours post-infection. However, there was a suggestion of an inhibitory effect of kisspeptin-10 on selected pro- and anti-inflammatory cytokine expression, macrophage inflammatory protein (MIP)-1α, MIP-1β, tumour necrosis factor (TNF)-α, granulocyte macrophage colony stimulating

factor (GM-CSF) and interleukin (IL)-10, after 24 hours which was not observed at 6 days post-infection. Results from the whole blood stimulation assay suggested an inhibitory effect of kisspeptin-10 on selected LPS-induced pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) whilst generally not having an effect on selected anti-inflammatory cytokines (IL-10). Overall this study suggests, based on the lack of statistically significant data, a potential immunomodulatory effect of kisspeptin-10 based on the observed inhibition of pro-inflammatory cytokines.

Investigating and developing an understanding of key regulators and mechanisms of maternal immune tolerance may help researchers understand the pathophysiological mechanisms underlying certain pregnancy-related disorders. This was a pilot study aimed at characterising the effect of kisspeptin stimulation on cytokines and chemokines responses. Manipulation of regulatory hormones such as kisspeptin could represent a potentially novel approach in the treatment of various pregnancy-related disorders including preeclampsia and unexplained recurrent miscarriage.

Table of Contents

Abbreviations and Symbols

Listed here are all abbreviations used in this document multiple times

ANOVA	analysis of variance
APC	antigen presenting cells
Arg-Phe-NH_2	arginine-phenylalanine-amide (RF-amide)
aTreg	adaptive T-regulatory cell
BMI	body mass index
$(Ca^{2+})i$	cytoplasmic calcium
cAMP	cyclic adenosine monophosphate
CAP18	cationic antimicrobial protein of 18 kDa
cDNA	complementary deoxyribonucleic acid
$CD4^+$	cluster of differentiation 4
$CD8^+$	cluster of differentiation 8
$CD56^+$	neural cell adhesion molecule and cluster of differentiation 56
CFU	colony forming units
cm^3	centimetres cubed
CO_2	carbon dioxide
CREB	cAMP response element-binding protein
CXC3C	chemokine receptor CXC3C
CXCL14	chemokine CXC ligand 14
^oC	degrees Celsius
DAG	diacylglycerol
DC	dendritic cell
dH_2O	distilled water
DMSO	dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EG-VEGF	endocrine gland derived vascular endothelial growth factor
ELISA	enzyme-linked Immunosorbent Assay
EVT	extravillous trophoblast
FOXP3	Forkhead box P3
FSH	follicle stimulating hormone
g	grams

G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GnRH	gonadotropin-releasing hormone
GPR54	G-protein receptor 54 (hOT7T175, KiSS1R or AXOR12)
Gαq	heterotrimeric G protein q-alpha
HBP/CAP37	heparin-binding-protein
HGF	hepatocyte growth factor
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IGFBP-1	insulin-like growth factor-binding protein 1
IL	interleukin
IL-1	interleukin-1
IL-2	interleukin-2
IL-3	interleukin-3
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-11	interleukin-11
IL-13	interleukin-13
IL-15	interleukin-15
IL-17	interleukin-17
IL-17A	interleukin-17α
IL-27	interleukin-27
IP10	interferon gamma induced protein
IP_3	inositol 1,4,5-triphosphate
IRM	idiopathic recurrent miscarriage
iTreg	inducible T regulatory cell
IUGR	intrauterine growth restriction
Kiss1	kisspeptin
Kiss1R	kisspeptin receptor
KNDy	kisspeptin-neurokinin B-dynorphin

Kp-10	kisspeptin-10
Kp-14	kisspeptin-14
Kp-54	kisspeptin-54
Kp-145	kisspeptin-145
LH	luteinising hormone
LIF	leukemia inhibitory factor
LPS	lipopolysaccharides
M	molar
M-CSF	hematopoietic growth factor
MCP-1	monocyte chemoattractant protein-1
mg	milligram
MIP1-A	macrophage inflammatory protein 1 alpha
MIP1-B	macrophage inflammatory protein 1 beta
mL	millilitre
MOI	memorandum of incorporation
mRNA	messenger ribonucleic acid
Mtb	mycobacterium tuberculosis
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [methylthiazolyl tetrazolium]
NaHep	sodium-Heparin
ng	nanogram
NK	natural killer cells
nm	nanometers
OD	optical density
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
pg	pictogram
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PKA	protein kinase A
PLC	phospholipase C
pNK	peripheral natural killer cells
P3	biosafety level 3 laboratory
p234	peptide 234
p356	peptide 356

RANTES	regulated upon activation normal T cell expressed and secreted
RF amides	neuropeptide family
RORyt	retinoic acid receptor-related orphan receptor gamma
RPL	recurrent pregnancy loss
RPMI-HEPES	RPMI medium containing N-2-hydroxyethylpiperazine-N-ethanesulfonic acid
RSM	recurrent spontaneous miscarriage
RT-PCR	Reverse transcription polymerase chain reaction
SAv-HRP	streptavidin-horseradish peroxidase
SDS	sodium dodecyl sulfate
TGF-B	transforming growth factor beta
TGF-B1	transforming growth factor beta 1
Th	T helper
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17 lymphocytes
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor alpha
TNF-B	tumour necrosis factor beta
uNK	uterine natural killer cell
VEFG-C	vascular endothelial growth factor C
vs	versus
µg	microgram
µL	microliters

Chapter One General introduction

1.1 Introduction

Pregnancy is a fascinating event that researchers, to this day, do not yet fully understand. A unique and significant experience that is cherished around the world, pregnancy exists as a life changing event for many. However, not all pregnancies succeed and some progress uneventfully. Pregnancy-related complications may arise in the form of miscarriage, preeclampsia, intrauterine growth restriction (IUGR), preterm birth and unexplained recurrent spontaneous miscarriage (RSM) [1]. Causes of pregnancy loss include poor placentation, autoimmune disease, chromosomal abnormalities, exaggerated maternal immune response towards the fetus and environmental factors. Globally, a significant portion of maternal and perinatal morbidity and mortality is due to such complications, especially in low and middle income countries [1,2,3]. The 2014-2016 *Saving Mothers Report* (*Sixth report on the Confidential Enquiries into Maternal Deaths in South Africa*) reported the top three causes of maternal deaths to be hypertensive disorders in particular preeclampsia (leading cause of direct maternal death in South Africa), obstetric haemorrhage and non-pregnancy related infections [4]. Researchers today are still unwinding the fascinating physiological and biological tapestry of pregnancy in order to understand the mechanisms underlying gestation and pregnancy-related complications.

One of the fundamental requisites for a healthy pregnancy involves the development of physiological tolerance of the maternal immune system to the semiallogenic (sharing some but not all genes i.e. sharing genes of the father and mother) fetus [5,6]. The fetus presents similar susceptibility to rejection by the maternal immune system to that of a transplanted organ (xenograft), however it is not rejected throughout its development [6,7]. It is essential for certain changes in the maternal immune system to occur for the mother to tolerate fetal antigens during pregnancy.

Previously described mechanisms associated with recurrent pregnancy loss (RPL) include: poor placentation, autoimmune disease, human lymphocyte antigen status, uterine abnormalities, hormonal problems, chromosomal abnormalities, an elevated BMI and infection [9,10,11]. However, in less than 50% of RPL, the aetiological mechanisms remain unknown [10,11,12]. It has been suggested that one underlying cause for the remaining proportion of idiopathic RPL or idiopathic recurrent miscarriage (IRM) may be attributed to a lack of maternal immune tolerance towards the fetus [13]. Understanding the mechanisms that drive gestation as well as the physiology of pregnancy has become a global effort, with the hope of elucidating novel interventions for the treatment of

pregnancy-related disorders. Investigating mechanisms behind the development of maternal immune tolerance is crucial for our understanding of pregnancy and pregnancy-related complications.

1.2 Mechanisms of immune tolerance formation and the immunology of pregnancy

It is essential for certain changes in the maternal immune system to take place for the mother to tolerate fetal antigens during pregnancy. There are several mechanisms which play an integral role in the development of immune tolerance including the induction of the enzyme indoleamine 2,3-dioxygenase (IDO) by antigen presenting cells (APC) which is responsible for the increase in the number of adaptive/inducible T regulatory cells (aTreg/iTreg). The increase in aTreg/iTreg results in the suppression of the immune response, the shift from T-helper lymphocyte (Th)1 cytokine subset to Th2 and the significant decrease in the expression of interleukin-17 (IL-17) from Th17 cellsIL-17 is responsible for stimulating cytotoxic reactions [5,6,14,15,16].

During pregnancy the mother's body undergoes significant changes to immune reactivity and energy metabolism, largely related to significant increases in peripheral blood hormones such as the steroid hormones; oestrogen and progesterone, and the protein hormones; kisspeptin, human chorionic gonadotropin and gonadotropin releasing hormone amongst many others [7]. It is known that hormones are important regulators of the functional activity of the immune system and immune cells [5,6,7], for example: progesterone previously been demonstrated to increase the cytokines produced by Th2 cells, resulting in the maintenance of pregnancy. Th2 cells are dominant within the decidua in early pregnancy in humans. The Th2 derived cytokines, IL-4 and IL-6, induce the release of hCG from trophoblasts and the hCG stimulates progesterone production from corpus luteum in pregnancy. Shirshev et al reported that the hormones secreted by the placenta, protect the fetus from the aggressive maternal immune response of the mother, emphasizing the immunomodulatory effects of these hormones [16].

Cellular immunity is mediated by specific effector cells in concert with the cytokines they produce. Two functional subsets of Th cells are responsible for inducing different effector responses. Th1 cells induce several pro-inflammatory responses including cell-mediated cytotoxic and inflammatory reactions through the release of IFN-γ, TNF-α and IL-2 [8,17,18]. In contrast Th2 cells, which have been associated with anti-inflammatory responses and successful pregnancy outcomes, enhance the production of B cell antibodies through the release of cytokines: IL-4, IL-5, IL-6 and IL-10 [17,18]. It has been

acknowledged that Th1 dominant milieu has a deleterious effect in pregnancy based on the fact that some Th1-dependent effector mechanisms are significant role players in acute allograft rejection [10,24,25]. In contrast, the production of Th2-type cytokines seems to be central for the induction and the maintenance of allograft tolerance [17,19,20,21,22]. The development of maternal immune tolerance towards fetal alloantigens is thought to be secondary to the dominance of Th2-type immunity over Th1-type immunity during pregnancy [23]. Th1-type immunity has previously been associated with RSM [24,25].

A recent study investigated macrophages and villous tissue isolated from placentas of normal pregnancies in the first and third trimesters [26]. The study established two groups of cytokine responses., The first was characterised by relatively low basal expression levels of IL-1,IL-6, IL-8, IL-10, and TNFα (which are major regulators of the inflammatory response) under normal physiological conditions whilst being highly inducible by bacterial endotoxin such as LPS [26]. It was suggested that, in normal pregnancy, the cytokines mainly ensure protective responses to bacteria from placental macrophages [26]. The cytokines of the second group (IL-11, IL-17A, IL-17F, TGF-β, VEGF) were relevant to this study as they relate to spontaneous miscarriage and preterm birth [32,33]. Increased levels of TNF-α, IL-1β, IL-6, and IL-8 in the amniotic fluid [27,28,29] and cervicovaginal fluid [27,30,31] have been associated with preterm birth.

There are several role players in the establishment of maternal immune tolerance and it is important to understand the mechanisms and factors regulating its development. Understanding the intricacies involved in maternal immune tolerance tenders an opportunity for better understanding of pregnancy-related disorders.

1.2.1 Cytokine production

Trophoblastic and lymphomyeloid cells responsible for producing cytokines are present within the decidua. The main types of cells present include: T lymphocytes ($CD8^+$), macrophages and natural killer (NK) ($CD56^+$) cells [10,32]. Modulation and regulation of cytokine expression i.e. the type and/or quantity is as a result of intercellular communication. An alteration in, T cell produced, cytokine expression pattern may play an important role in the development of immunological tolerance and/or immune activation. T-helper (Th) cells can be grouped into classes: Th1 cells, which produce pro-inflammatory cytokines, interleukin (IL) 2, tumor necrosis factor (TNF) α and interferon (IFN) γ, which play a role in cell-mediated immunity, and Th2 cells, which produce anti-inflammatory cytokines, IL-4, IL-5 and IL-13 and are involved in humoral immunity [33]. As reviewed by Jamieson et al, it was

suggested that the suppression of cell-mediated immunity whilst maintaining humoral immunity, proved necessary for the development of maternal immune tolerance for fetal antigens [8]. Adaptive immunity comprises two types of immunity i.e. humoral immunity and cell mediated immunity. During adaptive immunity an antigen-specific immune response is generated. Humoral immunity is triggered by B cells while cell mediated immunity is triggered by T cells. The main difference between humoral and cell mediated immunity is that antigen-specific antibodies are produced in humoral immunity whereas antibodies are not produced in cell mediated immunity, whereby T cells destroy foreign molecules through phagocytosis.

Stimulation of trophoblast cells and the influence of gestational hormones leads to, amongst others, the transformation of the endometrium to the decidua which through this same modulation becomes receptive to implantation. It is believed that development of maternal immune tolerance for the genetically foreign fetus is dependent on the interactions of a range of cytokines secreted at the site of implantation by maternal and fetal cells [8,10]. Development of the decidua from the endometrium is dependent on the presence of certain gestational hormones and stimulation of trophoblast cells which is partly regulated by kisspeptin. Communication between the decidua and trophoblast cells is mediated by cytokines and cell surface receptors [8,10]. Figure 1.1 higlights both pro- and anti-invasive paracrine factors during placentation.

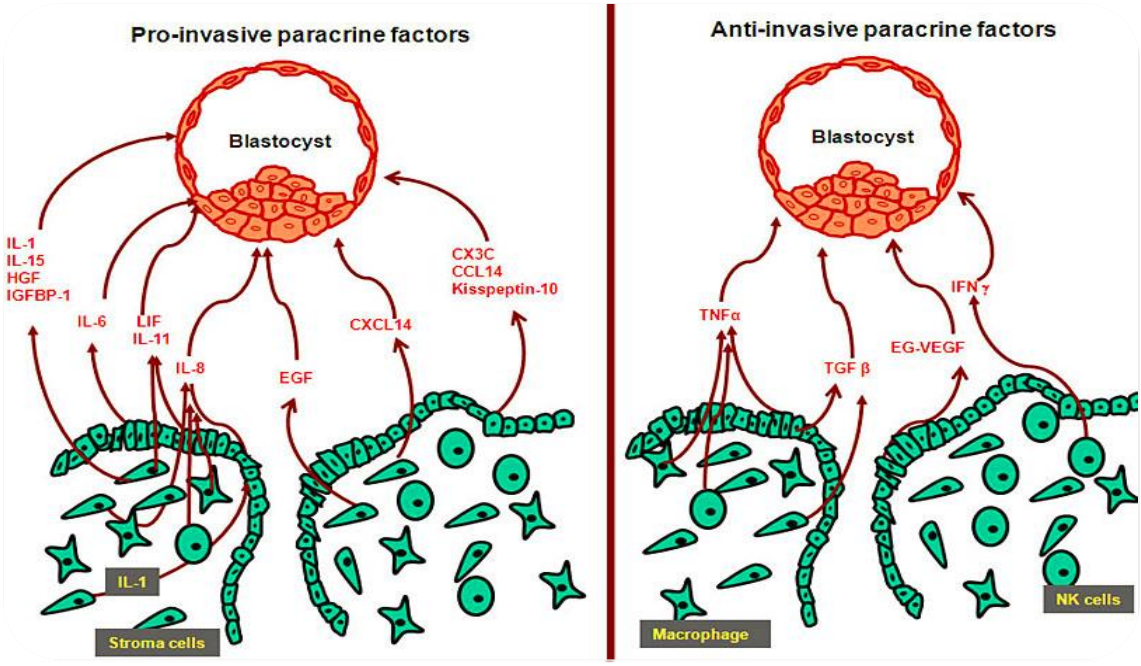

Figure 1.1: Illustration of pro-invasive and anti-invasive paracrine factors essential for the regulation of implantation [34]. Abbreviations: IL-1 – interleukin - 1; IL-15 – interleukin - 15 ; HGF – hepatocyte growth factor ; IGFBP-1 – insulin-like growth factor binding protein - 1 ; IL-6 – interleukin - 6; LIF - ; IL-11 – interleukin - 11 ; IL-8 – interleukin - 8; EGF – epidermal growth factor ; CXCL14 – chemokine ligand 14; CXC3C – fractalkine; CCL14 – chemokine lgand 14 ; TNF-α – tumor necrosis factor alpha ; TGF-β – transforming growth factor beta ; EG-VEGF – endocrine derived vascular epidermal growth factor; IFN-γ – interferon gamma.

It has become evident that the cytokines associated with unsuccessful pregnancy outcomes include: IFN-γ, TNF-α and IL-2 which fall under T helper 1 (Th1) cells. Two functional subsets of Th cells include Th1 and T helper 2 (Th2) cells which are responsible for inducing different effector responses. Th1 cells induce several responses including cell-mediated cytotoxic and inflammatory reactions through the release of IFN-γ, TNF-α and IL-2 [8,17,18]. Th2 cells are conducive to pregnancy through primarily enhancing production of B cell antibodies through the release of cytokines: IL-4, IL-5, IL-6 and IL-10 [17,18]. Therefore, during pregnancy there remains a Th2-bias, and a shift in the ratio towards Th1 potentially results in adverse outcomes such as unexplained miscarriage. This bias was further validated by Raghupathy et al in the circulation of pregnant women, however it remains to be seen whether this immunological shift is present at the maternal-fetal interface [17]. It has been shown that the effects of cell-mediated immunity have deleterious consequences on the fetus during pregnancy. Yui et al demonstrated the effects of tumor necrosis factor (TNF-α) which induces apoptosis in human primary villous trophoblast cells whilst interferon (IFN)-γ enhances TNF-mediated cytotoxicity of these cells [17,35]. Supplementing this finding was that both the cytokines were shown to inhibit human trophoblast outgrowth *in vitro* [17,36]. An increased production of interleukin-2 (IL-2) and IFN-γ by peripheral blood mononuclear cells (PBMC)s along with a reduction in IL-10 have been associated with spontaneous miscarriage in humans [17,37]. In addition, it has been reported that in women with a history of unexplained recurrent spontaneous miscarriages, trophoblast antigens induce the release of embryotoxic cytokines (IFN-γ and TNF-α) by maternal PBMCs [17,38]. Moreso, et al showed that injection of IFN-γ or TNF-α or IL-2 induced abortion in normal pregnant mice [17,39].

A previous study has illustrated in recurrent spontaneous miscarriage (RSM) that the altered immunity is attributed to the Th1/Th2 hypothesis [40]. The fetus escapes maternally derived T-cell responses through alteration of Th1 differentiation toward Th2 pathway [40]. As a result, the pro-inflammatory effects of Th1-type immunity are suppressed/inhibited [10,41,42,43]. In contrast, there is an increased chance of RSM if Th0 differentiation is biased towards the Th1 pathway. However, it has been shown that immunity with a Th2 dominance has also been reported in cases of RSM therefore establishing that the Th1/Th2 balance is not singularly sufficient to explain the mechanism underlying the development of maternal immune tolerance for the fetus [44,45]. Previously it has been suggested that observed differences in cytokine production may be due to factors other than the presence of foreign fetal antigens such as infections during pregnancy, as infectious agents will alter the cytokine balance based on the resultant immune response [46].

Another cellular subset, the CD4+ T lymphocytes (Th17 cells), secrete IL-17, a pro-inflammatory cytokine. Cells expressing IL-17 may induce inflammation during pregnancy and subsequently lead to miscarriage[14,47]. aTreg cells and Th17 are two individual lymphocytes subsets which functionally act in contrast to each other. Autoimmune responses are suppressed by aTreg cells and play a role in preventing fetal rejection. In contrast, Th17 cells promote transplant rejection and autoimmunity, and when numbers are elevated, may play a role in recurrent spontaneous miscarriage [47,48]. In contrast, autoimmune responses are suppressed by aTreg cells and play a role in preventing fetal rejection. aTreg cells play significant roles in immunoregulation and induction of immune tolerance [10]. Previous studies have shown that aTreg cells inhibit cytokine production and proliferation of both $CD4^+$ and $CD8^+$ T cells, immunoglobulin production by B cells, cytotoxic activity of natural killer (NK) cells, and maturation of dendritic cells (DCs), which in turn may result in the development of immune tolerance [49,50]. Unexplained recurrent miscarriage may be a variation of Th1/Th2/Th17 and aTreg cell subsets [17,47,48]. Cytokine imbalances have profound effects on the Th1/Th2/Th17 balance.

Robertson et al demonstrated the importance of IL-10 for pregnancy maintenance, presenting mice with an IL-10 knockout mutation that were more susceptible to inflammation-induced abortion [51]. Th17 induced inflammation is essential for successful placentation and may play a role in the protection of the uterine cavity against extracellular microbes, however, an excessive inflammatory response may lead to fetus rejection [52]. Together with TGF-β, IL-6 induces the development of Th17 cells from undifferentiated T cells and additionally inhibits TGF-β-induced aTreg differentiation [47]. Interleukin-6, is multifunctional and plays a significant role in the context of this study i.e. immune adaptations which are necessary for the development of immune tolerance. Therefore, potential effects of kisspeptin on IL-6 warrant further investigation.

Cytokines are imperative to the regulation of the immune system during pregnancy. It is crucial to maintain an appropriate cytokine balance at the maternal–fetal interface as well as in circulation. It has become evident that, during pregnancy, cytokines play a role in the control of immune response against fetal antigens. Unexplained recurrent miscarriage can potentially be a consequence of variation of Th1/Th2/Th17 and aTreg cells subsets [17,47]. These variations may underlie hypothesised mechanisms in pregnancy loss such as infections and autoimmune disorders.

Based on the numerous roles played by selected cytokines in the development of maternal immune tolerance during pregnancy, it is important to investigate the effects of kisspeptin on all cytokines (pro- and anti-inflammatory) as this may have implications for understanding adverse pregnancy outcomes. Given the critical role of cytokines in altering the balance between Th1/Th2/Th17 cells and Treg cells,

should kisspeptin prove to be a major regulatory factor of these cytokines, this could provide the foundation for further investigation into its use as a potential intervention for various pregnancy-related disorders, including preeclampsia and unexplained recurrent miscarriage. Kisspeptin-mediated effects on selected cytokines have been previously described as the inhibition of pro-inflammatory cytokine expression (IL-1β, IL6 and IL-17) whilst inducing the expression of anti-inflammatory cytokines (IL-10) [16]. However, the effect of kisspeptin on several immunomodulatory cytokines remains to be investigated.The study presented here investigated the effects of kisspeptin on the expression of cytokines induced by a pathogen and lipopolysaccharides (LPS) in order to establish the immunomodulatory characteristics of the hormone (if any) and subsequently gain some insight into kisspeptin-mediated effects on the previously described Th1/Th2/Th17 cytokine ratio.

The cytokines investigated in this study are: interferon (IFN)γ, interleukin-10 (IL-10), interleukin-17A (IL-17A), interleukin-1β (IL-1β), interleukin-1α (IL-1α), interlukein-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-1α, MIP-1β and granulocyte macrophage colony stimulating factor (GM-CSF). These were chosen based on their role in immune response and immune function during pregnancy. A summary of selected cytokines which possess immunomodulatory characteristics, including those investigated in this study, is presented in Table 1.1.

Table 1.1: Summary of selected cytokines, subtype, source and function. These cytokines possess important immunomodulatory properties. All abbreviations and symbols used in this table can be found in the list of Abbreviations and Symbols. The asterisk (*) denotes the cytokines investigated in this study [53].

Cytokines	Th1/Th2 subtype	Source	Function
IL-1*	Th1	Monocyte, lymphocyte, endothelium	Haematopoiesis, co-stimulation of T cell, fibroblast proliferation, acute phase response
IL-3	Th2	Activated T cell, mast cell, NK cell	Proliferation and differentiation of myeloid progenitor stem cell, prevention of apoptosis induction in macrophages
IL-6*	Th1/Th2	T cell, monocyte, endothelial cells, mast cells	Stimulate B cell for antibody production and T cell growth and CTL differentiation
IL-10*	Th1/Th2	Monocyte, lymphocyte, endothelial cells	Inhibition of pro-inflammatory cytokines by monocyte, granulocytes, inhibition of IL-2 production by T cell, inhibition of antigen specific T cell activation
IL-15	Th1	Activated T cells and natural killer	Regulates activation and proliferation of NK cells and T cells
IL-17*	Th1	Th17 T-cells	Regulation of inflammation
IFN-α	Th2	Leucocyte	Anti-proliferative action, immunoregulatory action
IFN-β	Th2	Fibroblast, epithelial cell, endothelial cell	Antiviral, MHC antigen upregulation, NK cell enhanced cytotoxicity, antimicrobial
IFN-γ*	Th1	Monocyte, macrophage, dendritic cell, T cell, B cell	MHC class II expression, macrophage and NK cell activation, Ig isotype selection
G-CSF*	Th1/Th2	Stromal cell, endothelial cell	Proliferation and differentiation of macrophage progenitor cell
M-CSF	Th2	Fibroblast, endothelial cell, T cell, monocyte, neutrophil	Monocyte proliferation, differentiation and activation
GM-CSF*	Th1/Th2	T cell, macrophage, endothelial cell, B cell	Inhibit apoptosis of target, proliferation, differentiation and activation of granulocyte, macrophage lineage
A chemokines	Th1/Th2	Monocyte, neutrophil, endothelial cell, epithelial cell	Neutrophil chemotaxis and adherence, IL-6 secretion
B chemokines	Th1/Th2	Monocyte, fibroblast, epithelial cells, melanocytes	Monocyte activation, basophil activation
RANTES	Th1	T cell monocyte, NK cell, fibroblast, epithelial cell, endothelial cell	T cell chemotaxis and proliferation, monocytic chemotaxis and activation, NK cell chemotaxis, modulation of macrophages, eosinophils, T cells
TNF-α*	Th1	Macrophages, T cell	Cytotoxic for tumor cell, anti-viral, anti-bacterial, ant-parasitic activity
TNF-β	Th1	Mast cell, platelet, fibroblast	Wound repair, cell growth regulation, tissue remodelling, immunosuppression
MIP1-α/β*	Th1	Neutrophils, eosinophils, basophils, fibroblasts, macrophages	Recruitment of inflammatory cells, inhibition of hematopoietic stem cell proliferation
MCP-1*	Th1/Th2	Monocyte, neutrophil, lymphocyte	Regulation of monocytic/macrophage migration and infiltration

1.2.2 Natural killer cells

Natural Killer (NK) cells are immune cells that, via the release of cytolytic granules containing perforin, granzyme and granulysin, are capable of lysing the target cells. During pregnancy there are two subsets of NK cells: peripheral NK (pNK) cells and NK cells present in the uterine mucosa i.e. uterine NK (uNK) cells [54]. The two subsets are contrasting in function. Unlike their pNK counterparts, uNK cells are not cytotoxic, rather they provide a suitable microenvironment in the decidua, making it stable and accommodating for the growing foetus whilst promoting normal placentation, vascular remodeling and trophoblast infiltration, all of which are critical for a healthy pregnancy [55]. Regulation is essential in order to inhibit the cytolytic function of uNK cells as these cells possess cytolytic activity which lead to the lysis of trophoblastic cells when activated. Their regulation is mediated by a balance between activating and killer inhibitory receptors (KIRs).

Trophoblast invasion is regulated by several factors secreted by uNK cells. *In vitro* studies have reported that interleukin 15 (IL-15) stimulates the process while TNF-α and IFN-γ have the inhibitory roles [56]. According to earlier reports, NK cells are capable of producing Th1 and Th2 cytokines [57].

Given the role played by kisspeptin with regards to regulation of cytokine expression, a connection can be made between the activity and regulation of uNK cells during the early stages of pregnancy and the development of maternal immune tolerance. The effect of cytokine modulation by kisspeptin may effect the functional activity of uNK cells depending on the cytokines effected.

1.3 Failure in the development of maternal immune tolerance: clinical implications

1.3.1 Preeclampsia

An idiopathic condition characterised by the development of new-onset hypertension and evidence of end-organ dysfunction normally after 20 weeks of gestation, preeclampsia, as a maternal disorder, develops in about 10% of pregnancies, and is a leading cause of maternal and perinatal morbidity and mortality [58,59]. As defined by the International Society for the Study of Hypertension in Pregnancy (ISSHP), hypertension is diagnosed at a systolic blood pressure greater than 140 mmHG and a diastolic blood pressure greater than 90 mmHG [58]. Preeclampsia is a significant and severe hypertensive disorder which may complicate pregnancy [58,60], and is one of the leading causes of maternal and

perinatal morbidity and mortality worldwide [61,62]. A defective maternofetal immune response may contribute to the development of pregnancy-related complications, such as bleeding complications during the first trimester, pregnancy-induced hypertension, preeclampsia, or preterm birth. GM-CSF could play a role in the preeclampsia pathogenesis [63]. Of several causes of a defective maternofetal immune response, one significant cause, with relevance to this study would be that there is a higher level of GM-CSF present in decidual cells in patients with preeclampsia if compared with normal pregnancy. The cytokines involved in preeclampsia, TNF-α and IL-1β, upregulate GM-CSF mRNA expression in cultured first-trimester human decidual cells [64,65]. Moreso, GM-CSF levels in blood and the ratio of GM-CSF:total protein levels present within the placenta, are significantly higher in preeclamptic pregnancies than in normal pregnancies [64,66].

1.3.2 Recurrent Spontaneous Abortion (RSM)

One rare pregnancy-related complication includes RSM. An estimated 1-3% of women suffer up to three or more consecutive miscarriages prior to 20 weeks' gestation with these cases being termed as RSM or recurrent pregnancy loss (RPL) [10,67]. The etiology of less than 50% of RSM cases are known [10,68]. Several mechanisms have been previously described as known causes of RSM however, it has been suggested that one underlying cause for the remaining proportion of idiopathic RSM cases may be attributed to an exaggerated maternal immune response towards the fetus [46].

It is known that some cases of idiopathic RSM are characterised by an increased production of pro-inflammatory cytokines such as TNF-α and IFN-γ and decreased levels of anti-inflammatory cytokines such as IL-10 within PBMCs [17,26,38,68]. Cytokine expression by the cells of the immune system (human lymphocytes) in periphery does not correlate with the response of immune cells at the materal-fetal interface (69,70). It is important to investigate the role played by other important immunomodulatory cytokines as this may prove important in determining pregnancy outcome. In addition, this may aid in the determination of whether the hypothesis that RSM occurs as a result of high level expression of pro-inflammatory cytokines is teneble.

1.4 *Kiss1* gene, kisspeptins and GPR54

There is a specific group of peptide hormones characterized by an Arg-Phe-NH2 motif at their carboxyl terminus which bind to G protein-coupled receptors [1,71]. Collectively, the group of peptides has been suitably named RF-amides and these hormones are found in many different species from very

primitive species like crustaceans through to humans. One of the most important RF-amides is kisspeptin (Figure 1.2).

Originally isolated from melanoma cells, the *Kiss1* gene was initially described as a tumour metastasis suppressor gene and, thus, the originally indentified 54 amino acid peptide product of this gene was initially termed metastin. The group of peptides encoded by the Kiss 1 gene have since been renamed as kisspeptins. Several important physiological roles for the kisspeptins have also since been identified. In particular, they have been found to be crucial for normal pubertal development and fertility in humans [71,72].

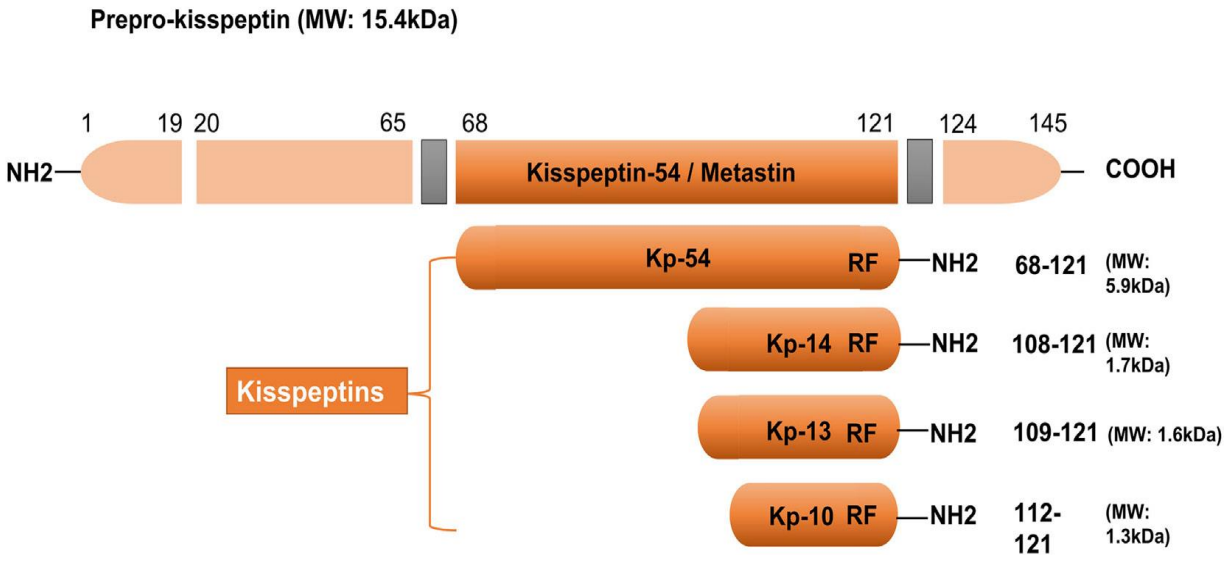

Figure 1.2: Illustration of primary kisspeptin transcript prior to proteolytic procession [73].

The kisspeptin peptide is synthesised as a 145 amino-acid polypeptide prior to posttranslational modifications (Figure 1.2) [1,74]. Proteolytic processing of the primary transcript results in several kisspeptin products which vary in length: kisspeptin-145 (Kp-145), kp-54 (metastin), kp-14, kp-13 and the smallest active cleavage product Kp-10 [75,76,77].

Kisspeptins are the endogenous ligands for the 396 amino-acid G-protein-coupled receptor, GPR54 (also known as hOT7T175, KiSS1R or AXOR12) [75,78,79]. Both *Kiss1* and *GPR54* mRNA are expressed centrally in the brain, spinal cord, and hypothalamus [78,80]. Peripherally, kisspeptins have been found to be expressed in the placenta, pituitary, testes, pancreas, liver, small intestine, skeletal muscle, kidney, and cardiovascular system [78,80,81,82]. Significantly, although molecular localization has

revealed limited kisspeptin expression in both periphery and brain, high expression has been measured in the placenta [72,74,76]. With relevance to this study, GPR54 has been found to be expressed my the cells of the immune system [5,16].

Upon stimulation of GPR54, the receptor activates intracellular Gαq/11 G proteins, which in turn activates the enzyme phospholipase C (PLC)β which catalyzes the hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) to two secondary messenger products; diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) [1,7,75,76]. The production of IP_3 and DAG leads to an increase in the concentration of cytoplasmic calcium (Ca^{2+} ($[Ca^{2+}]i$)) with subsequent activation of protein kinase C (PKC) [83]. In addition, kisspeptin has been shown to increase the concentration of intracellular cyclic adenosine monophosphate (cAMP). The increase in intracellular cAMP is associated with the activation of protein kinase A (PKA) leading to the phosphorylation of cAMP response element-binding protein (CREB). The phosphorylation of CREB results in the transcription of cytokine genes and Forkhead box P3 (*FOXP3*), known to eliminate the suppressive potential of T cells and regulation of the immune response [7,84,85]. This mechanism has been illustrated and summarised in Figure 1.3.

Since its discovery, kisspeptin has been extensively characterized and its role in immune tolerance will be the focus of this study. Amongst kisspeptins, Kp-10 (structure: YNWNSFGLRF-NH2) exhibits the highest conservation amongst several species, is the smallest active cleavage product [86] and has been demonstrated to be most relevant in modifying trophoblast behaviour. This study therefore aims to investigate the potential immunomodulatory effect of Kp-10 by exploring its effect on cytokine responses in pregnancy.

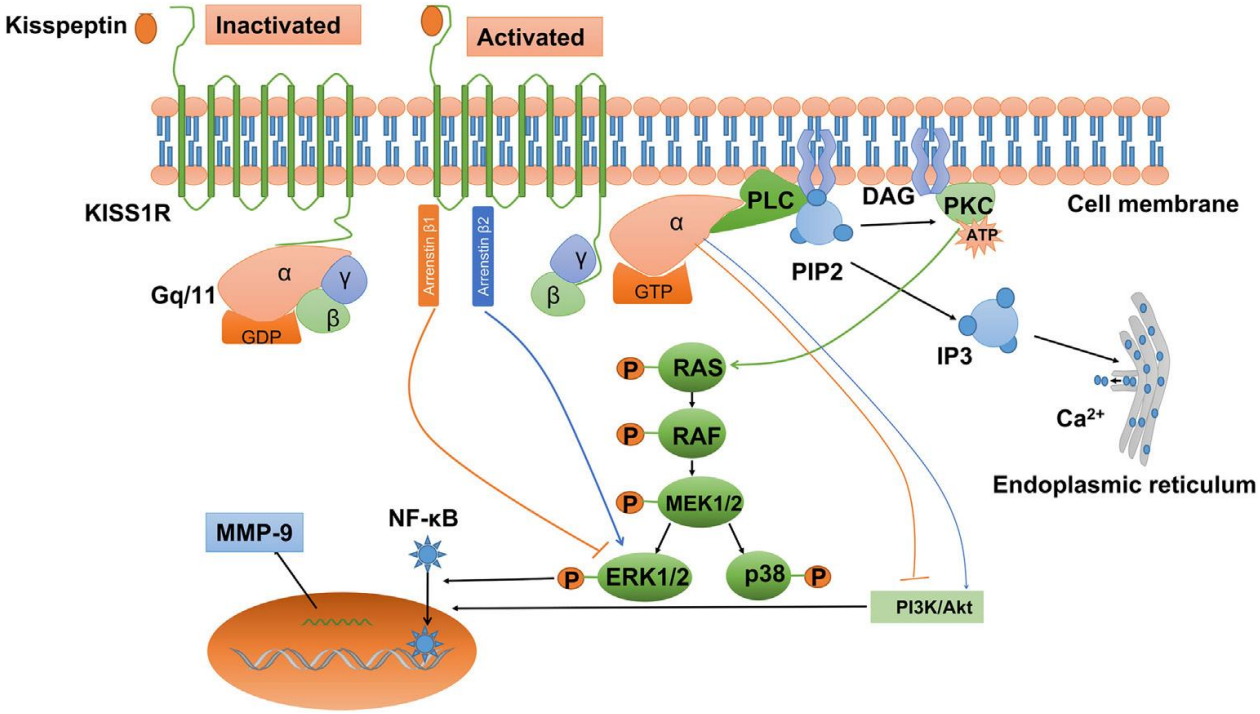

Figure 1.3: Illustration summarising the mechanism of action of kisspeptin upon induction of its receptor GPR54. Kisspeptin/GPR54 signalling has been shown to mediate its effects via intracellular calcium mobilization **[73].** KISS1R is a seven-transmembrane domain, Gq/11-coupled receptor. When kisspeptin binds its receptor, the intracellular portion of KISS1R activates Gq/11. The α-subunit of Gq/11 activates PLC, which subsequently cleaves PIP2 into IP3 and DAG. IP3 promotes intracellular Ca^{2+} release from the endoplasmic reticulum, while DAG activates a signalling by phosphorylating PKC. This activation induces the phosphorylation of ERK1/2 and p38. In addition, activation of KISS1R recruits arrestin- 1 and -2, which down-regulates and up-regulates phosphorylated ERK1/2 levels, respectively. The activation of KISS1R can also stimulate the phosphorylation of PI3K/Akt, and suppress the phosphorylation of PI3K/Akt by blocking the prometastatic chemokine receptor CXCR4 (the receptor for the chemokine stromal cell-derived factor 1) signaling. These signals act on downstream pathways, including NF-κB, MMPs and VEGF, to promote hormone secretion and apoptosis. In addition, these signals inhibit metastasis, migration, angiogenesis and proliferation. Abbreviations are as follows: DAG - diacylglycerol; ERK1/2 - extracellular signal-regulated kinase; IP3 - inositol 1,4,5-triphosphate; PI3K - phosphatidylinositol-3-kinase; MMPs - matrix metalloproteinases; NF-κB - nuclear factor-κB; PIP2 - phosphatidylinositol 4,5-bisphosphate; PKC - protein kinase C; PLC - phospholipase C; VEGF - vascular endothelial growth factor.

1.5 Kisspeptin and the hypothalamic, pituitary and gonadal axis

Other essential functions of kisspeptin and its receptor includes their roles in reproduction, in particular, the regulation and functioning of the gonadotropic axis. This neuroendocrine axis integrates regulatory signals from the hypothalamus, pituitary and gonads comprising; hypothalamic gonadotropin-releasing hormone (GnRH), pituitary gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH) as well as sex steroids and peptides synthesized by the gonads [75,87]. The function of kisspeptin in pubertal development and reproduction is determined by its role in inducing secretion of GnRH in various species [75,88]. When Kp-10 was injected into pregnant rats,

it was able to induce a 27- and 15-fold surge of LH levels on day 13.5 and 20.5 [87]. Several studies have shown that peripheral injection of kisspeptin can potently stimulate gonadotropin secretion in many non-human mammalian species including rats [89,90], mice [91,92], monkeys [93], sheep [94] and humans [95,96,97,98,99,100,101,102]. These observed effects are abolished by pre-administration with a GnRH antagonist [89,90,93] suggesting that kisspeptin-induced gonadotropin secretion is mediated through a GnRH-dependent mechanism. Indeed it has been demonstrated that exogenous kisspeptin stimulates the firing of GnRH neurons *in vitro*. This demonstrates a regulatory effect of kisspeptin in pregnancy based on the regulation of pregnancy-related hormones through GnRH regulation (Figure 1.4).

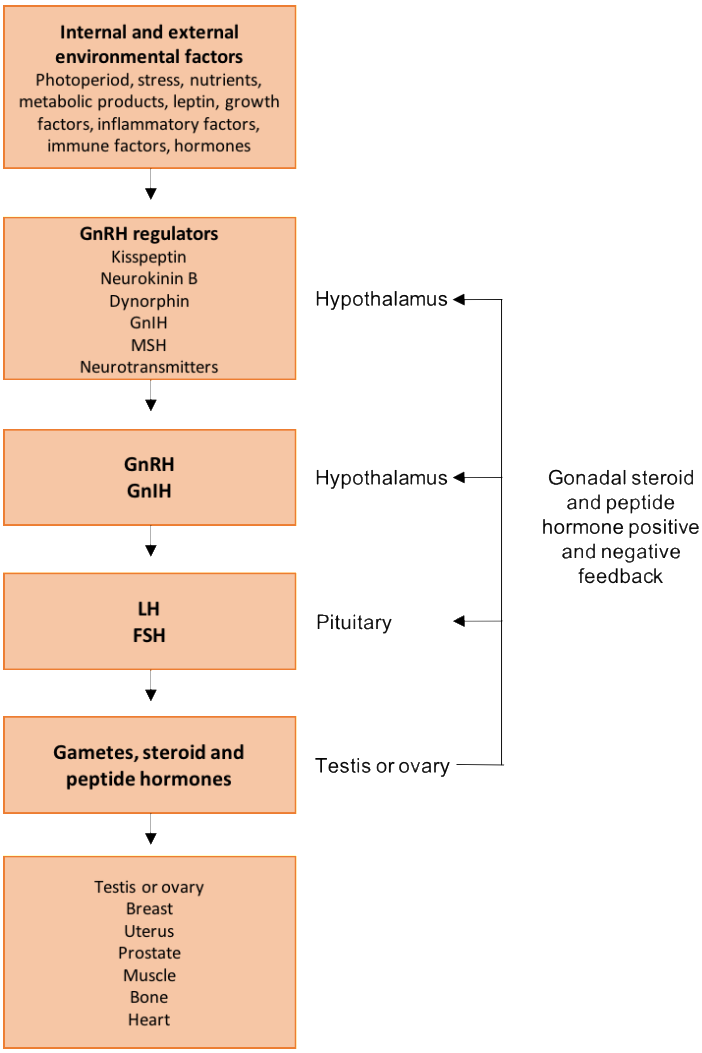

Figure 1.4: A summary of the role of kisspeptin in the regulation of the GnRH axis [103]. Abbreviations: GnRH – gonadotropin releasing hormone; GnIH/RFRP-3 – gonadotropin inhibitory hormone/RF-amide related peptide 3; MSH – melanocyte stimulating hormone; LH – leutinising hormone; FSH - follicle stimulating hormone.

Kisspeptin plays an imperative role in the initiation of puberty, regulation of adult fertility and in placentation during pregnancy [75,104,105]. Puberty and reproduction are regulated by kisspeptin through its effect on GnRH secretion. Kisspeptin, in concert with Neurokinin-B and Dynorphin, are major regulators of GnRH secretion by GnRH neurons in the hypothalamus [72,105,106]. Secretion of gonadotropins (LH and FSH) from the pituitary gland is activated through the direct stimulation of GnRH neurons by kisspeptin-neurokinin B-dynorphin (KNDy) neurons [71,89,107]. In humans, it has previously been shown that mutations in either the *GPR54 or Kiss1* gene, or both, result in gonadal dysfunction, in particular, isolated hypogonadotrophic hypogonadism, infertility and failure to undergo puberty (all brought on through in-activating mutations) [101,108,109,110,111]. In contrast, patients with mutations leading to enhanced activations of the kisspeptin signalling pathway undergo precocious puberty (activating mutations) [112].

1.6 Kisspeptins in pregnancy

Previously, it has been shown that hypothalamic *Kiss1* mRNA increases as pregnancy develops [87,103]. It is important to note that circulatory kisspeptin concentrations increase throughout pregnancy and dramatically decreases post-delivery, which is in stark contrast to kisspeptin expression in placental tissue [113].

From an embryological perspective, the placenta originates from the trophectoderm layer of the blastocyst. The placenta is essential for gaseous exchange and nutrient transport from the mother across to the fetus [114,115]. Additional functions of the placenta include its crucial role in anchoring the developing embryo to the maternal tissues. Healthy pregnancy and fetal development is dependent on effective placentation. The decidua , the endometrial lining during pregnancy, consists of several layers which include: the decidua basalis and capsularis which underlie the placenta and form part of the placental bed and the decidua parietalis. The decidua parietalis is the innermost uterine layer (endometrial layer) in pregnancy. This layer has no overlying placenta and is devoid of trophoblast cells therefore plays little direct role in placentation [1]. Matjila, et al. [104], showed low levels of kisspeptin expression in the decidua basalis, in comparison to the placenta and decidua parietalis. However, they demonstrated that receptor (GPR54) expression was much higher in the maternal tissues, supporting the autocrine/paracrine functional hypothesis proposed by Bilban et al. [86]. Simultaneously peripheral and circulatory kisspeptin concentrations were compared to placental tissue expression levels in the context of preeclampsia [58] and reported that while circulatory levels were diminished, tissue expression was elevated in pregnancies complicated by preeclampsia.

Recently lower circulatory kisspeptin levels in early pregnancy have been associated with an increased risk of spontaneous miscarriage [116]. In addition, circulating kisspeptin concentrations were found to be lower during early stages of pregnancy in women with a history of miscarriage, premature delivery and small-for-gestational-age neonates [116,117]. Additionally, low kisspeptin levels have been described in pregnancies with placental dysfunction such as gestational diabetes, preeclampsia, and intrauterine growth restriction [116].

Cetkovic, et al*,* showed that in pregnancies with type 1 diabetes and chronic hypertension, the mean plasma kisspeptin levels were significantly lower when compared with the healthy control group ($p < 0.001$ in the first and second trimesters and $p < 0.05$ in the third trimester) [118]. The study also showed a decrease in plasma kisspeptin levels in the second and third trimesters which was found in patients with gestational diabetes ($p < 0.001$ in the second and third trimesters) and preeclampsia ($p < 0.001$ in the second trimester and $p < 0.05$ in the third trimester) [118]. In patients with preclampsia and placental dysfunction, they showed that low kisspeptin levels in the third trimester were associated with adverse perinatal outcome. Armstrong, et al., showed that serum kisspeptin levels were significantly lower in those women who subsequently developed preeclampsia than in healthy controls [median (quartile range) 1109 (449) vs 1188 (365) pg/mL, p = 0.029] and in those with intra-uterine growth restriction [1164 (386) vs 1188 (365) pg/mL, p = 0.016] [119].

Further investigation may advance our knowledge on the role of kisspeptin in the establishment of the placenta and knowledge of the mechanisms underlying placentation. It is important to compare the expression levels of kisspeptin in the periphery (serum) during the early stages of pregnancy, comparing healthy pregnancies with pregnancies with a history of RSM or other pregnancy related disorders. This is important when investigating the development of maternal immune tolerance formation during pregnancy.

1.7 Kisspeptin and trophoblast invasion

Placentation can be described as a process involving, amongst others, adequate trophoblast invasion, transformation of the spiral arteries from high resistance low capacity vessels to low resistance high capacity vessels as well as adaptation of the maternal decidua, all of which are crucial for successful pregnancy [104]. Insufficient transformation of the spiral arteries correlates with poor pregnancy outcomes such as preeclampsia and IUGR [120,121]. Spiral arterial transformation is dependent on the efficiency and level of endovascular invasion of extravillous trophoblast (EVT) cells. GPR54 is expressed in EVT cells whereas both kisspeptin and its receptor are expressed in villous trophoblast cells [75,104].

The *Kiss1* gene and GPR54 have been identified to play a major role in trophoblast invasion and migration as reported by several studies [86,104,122].

The importance of kisspeptin and GPR54 in trophoblast invasion was demonstrated by comparing mRNA signatures between the first and third trimesters whereby Bilban et al found abundant expression of *Kiss1* and GPR54 transcripts in the first trimester, a period where invasive capacity and migration efficiency is most important [86]. The study also highlighted that the smallest modified product of the *Kiss1* gene, Kp-10, inhibited trophoblast invasion. In addition to the role kisspeptin plays in invasion inhibition, kisspeptin has been implicated in the regulation of genes involved in matrix degradation and angiogenesis, both being crucial processes in placentation [123,124]. Following implantation, the supply of nutrients and oxygen to growing foetus is essential [125]. Trophoblast and uterine natural killer (uNK) cells [126] promote the transformation of endometrial vessels and spiral arteries into high capacity and low resistance vessels allowing adequate supply of blood and nutrients from mother to foetus [127].

Previously it has been shown that kisspeptin inhibits trophoblast invasion *in vitro* [58,86,128]. In healthy pregnancies the concentration of kisspeptin in serum significantly increase as pregnancy progresses and considerably decreases post-delivery [113,116]. In contrast, expression of both *Kiss1* and GPR54 transcripts in the placenta are highest in the first trimester (suggesting a role for regulation of invading trophoblasts) and lowest at term [129,130]. Decreased serum levels of kisspeptin, during early pregnancy, may be associated with an increased risk of spontaneous abortion [116]. Furthermore, it has been found that during the early stages of pregnancy, circulating concentrations of kisspeptin were found to be lower in women who had miscarriage, preterm delivery, small-for-gestational-age neonates, or intrauterine growth retardation [116,131]. Therefore, it can be suggested that kisspeptin may play a pivotal role in successful placental and fetal development.

1.8 Kisspeptin and maternal immune tolerance formation

Gorbunova et al reported that the recently discovered peptide hormone, kisspeptin, increased the formation of active aTreg cells [7]. In addition, the study found that kisspeptin decreased the expression of the pro-inflammatory cytokine, IL-17, through the inhibition of Th17 cell differentiation and concurrently, decreased IL-17A secretion by CD4+T cells regardless of kisspeptin concentration [5]. Liu et al suggested that human Th17 cells play a major role in rejection of fetal antigens and as a result may be deleterious to pregnancy [14]. The same study maintained that aTreg cells were beneficial to

the outcome of pregnancy, highlighting the importance of the ratio of aTreg/Th17 during pregnancy which plays an important role in the development of maternal immune tolerance to the fetus [7,14]. The shift of ratios of aTreg/Th17 towards aTreg is a major mechanism of immune tolerance stability during pregnancy [47,48]. Therefore, tight and specific hormonal regulation of aTreg cell numbers, functional activity and homeostasis *in vivo* is suggested as one of the most important factors contributing to the mechanisms of developing maternal immune tolerance to the fetus during pregnancy [47].

Cells expressing increased levels of IDO can additionally contribute to the generation of aTreg and the subsequent activation of IDO leads to the death of cytotoxic T lymphocytes due to the absence of sufficient levels of tryptophan [14,16]. Mellor et al reported the reaction catalysed by IDO, whereby tryptophan is consumed and degraded into toxic products such as kynurenine which was shown to effect the activity of cytotoxic T lymphocytes [15]. This is significant as kisspeptin does not only elicit an effect on the ratio of aTreg/Th17, but the hormone is also responsible for the increased induction of IDO by monocytes during the second trimester of pregnancy, the most sensitive period for miscarriage [16]. The increased induction of IDO by kisspeptin results in the suppression of the immune response based on the increase in aTreg cells and decrease in IL-17 producing Th17 cells.

It has been demonstrated that IL-27 may regulate the expression of Th17 and aTreg cells in women with recurrent spontaneous miscarriage [132]. The study showed that IL-27 induced IL-10 production by both CD4+*FOXP3*- and CD4+*FOXP3*+ cells which subsequently promotes the differentiation of IL-10 secreting aTreg cells and inhibits Th17 differentiation [132]. It has been reported that the downregulation and loss of function of aTreg cells including low expression of *FOXP*3, IL-10 and TGF-β leads to an increase in the development of preeclampsia and spontaneous abortion [5,133]. In conjunction with the effects of IL-27, kisspeptin enhances the expression of *FOXP3* (aTreg/CD4+*FOXP3+*) and IL-10 whilst suppressing the expression of IL-17 and *RORγ*t (inhibits production of Th17 cells/CD4+*RORγ*t+ cells) [16]. The role of kisspeptin in cytokine regulation cannot be underplayed as kisspeptin enhances the secretion of IL-10 by CD4+ T lymphocytes primed with transforming growth factor (TGF)-β1 which contributes to the decrease of IL-17A secretion by CD4+ T lymphocytes primed with IL-1β/IL-6 [16].

It is important to note that the inflammatory phenotype of macrophages presented in the early stages of pregnancy is attenuated and increases with the progression of pregnancy [27]. Pavlov et al, reported that the low basal but inducible cytokines levels are possibly modulated by kisspeptin during pregnancy, especially during infection. Cytokines in the second group which are constitutively

expressed and not responsive to LPS stimulation [27], may be regulated by kisspeptin due to their constant expression, however, these findings need further investigation.

It has been shown previously that kisspeptin is an essential regulator of monocyte function during pregnancy [134], therefore the hormone may be critical for the formation of immune tolerance to antigens of the semiallogenic fetus. Based on this, there is adequate evidence suggesting that kisspeptin and its cognate receptor, GPR54, play key roles in immune regulation during pregnancy and these roles are further investigated in this study. If a role of kisspeptin is established based on the findings of this study, this may lead to a better understanding of how maternal immune tolerance is achieved and may therefore lead to novel treatments.

1.9 Significance

Kisspeptin has been well described in terms of its role in regulating puberty onset and trophoblast invasion in pregnancy. There is evidence implicating kisspeptin and its cognate receptor, GPR54, as important role-players in immune regulation during pregnancy and this study aims to extend existing knowledge.

Investigating and developing an understanding of key regulators and mechanisms of maternal tolerance development may help us understand certain pregnancy related disorders and how to improve treatment.

1.10 Hypothesis

This study hypothesised that kisspeptin alters the expression of anti-and pro-inflammatory cytokines and may thus influence the establishment of immune tolerance in pregnancy.

1.11 Aim

To determine the immunomodulatory effects of kisspeptin using peripheral blood mononuclear cells (PBMCs) and whole blood from healthy and non-pregnant, women, using *in vitro* PBMCs and whole blood stimulation models (this is a pilot study in healthy, non-pregnant women prior to engaging in pregnancy investigations).

1.12 Objectives

1. Investigate the regulation of cytokines by kisspeptin in PBMCs of healthy and non-pregnant women using an *in vitro* infection assay.

2. Investigate the regulation of cytokines by kisspeptin in PBMCs of healthy non-pregnant women using an *in vitro* whole blood LPS stimulation model.

These experiments will serve as the basis for an *in vitro* proof-of-concept model for future *in vitro* experiments on pregnant women with a history of idiopathic recurrent miscarriage to determine the immunomodulatory properties of kisspeptin in pathological states.

Chapter Two METHODS AND MATERIALS

2.1 Reagents

Unless otherwise indicated, all chemicals and solvents used in this study were purchased from Sigma Aldrich, Merck or Thermo-Fischer Scientific (South Africa). Kisspeptin-10 (Kp-10) and Peptide 356 (p356) were obtained from EZbiolabs (USA) whilst Lipopolysaccharides (*Escherichia coli* O55:B5, CAT. NUMBER: L4005) was obtained from Sigma-Aldrich.

All solutions prepared in the laboratory are detailed in Appendix A.

2.2 Ethics statement

Initial tests (PBMC infection assay) were conducted in collaboration with the Immunology Research Group, Division of Molecular biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa.

Ethics for this study was approved by the Human Ethics Committee of the Faculty of Health Sciences, University of Cape Town (REF-4412019). Approval for research at Stellenbosch University was granted by the Health Research Ethics Committee of the Faculty of Health Sciences, Stellenbosch University (ref: N13/05/064A). The recruitment procedure and ethics was included in an existing project within the ALERT study of the Immunology Research Group at Stellenbosch University. Recruitment to the study was conducted by members of the research team at the University of Cape Town and Stellenbosch University, respectively.

A written informed consent was obtained from all study participants. The patients were counselled about the study in their preferred language. Patient comprehension of the details of the study was ensured before enrolment and sample collection. Therefore, enrolment was based on the patient's willingness to participate in the absence of any coercion. Refusal to participate in the study did not prejudice further patient care.

2.3 Study participants

Patient *inclusion criteria* for the study were as follows:

Healthy non-pregnant women of reproductive age (18-35 years old) with no history of recurrent miscarriage, previous pregnancy complications or known medical disorders.

Patient *exclusion criteria* for the present study were as follows:

1. Women below the age of 18 and above the age of 35 years.

2. Women with known underlying medical conditions such as autoimmune, endocrine or thrombophilic conditions.

Healthy non-pregnant women were recruited from both community health clinics, Groote Schuur hospital and Tygerberg Hospital.

2.4 Peripheral blood mononuclear cell (PBMC) infection assay

2.4.1. Tissue sampling and collection

Fractionated suspensions of PBMCs were used. PBMC are comprised of monocytes and lymphocytes and included any perioheral blood cell that has a round nucleus. Blood was obtained from 5 healthy non-pregnant women of reproductive age via venepuncture (Tygerberg Hospital, Stellenbosch University, South Africa). 54 ml of blood was collected from each patient in 6 x 9 ml sodium-heparin (NaHep) tubes for subsequent PBMCs isolation.

PBMCs were isolated using Ficoll-Paque Plus (GE Healthcare, Lifesciences, USA) density gradient centrifugation (1.077 g/cm^3). The isolation procedure involved carefully layering (pipetting) 35 ml of blood from NaHep tubes diluted (1:1) in phosphate-buffered saline (PBS) (Appendix A) onto 15 ml of Ficoll-Paque Plus within a 50 ml conical tube. Following this, without disturbing the Ficoll layer, cells were centrifuged at 400 x g for 25 minutes at room temperature (no brake). Once complete, the yellow plasma layer was carefully aspirated and discarded and the thin white PBMC layer was removed and placed into a 50 ml conical tube. Cells were then washed with PBS (made up to 50 ml) and centrifuged

at 600 x g for 4 minutes at 4 °C (no brake). Cells were then washed again with ice cold PBS (made up to 50 ml) and centrifuged at 150 x g for 8 minutes at 4 °C (no brake). The supernatant was carefully removed and cells were resuspended in 5 ml of AIM-V medium (serum-free) (ThermoFischer Scientfic, South Africa) for subsequent cell counting (see Section 2.4.2). Cell viability of each suspension was evaluated employing Trypan blue manual cell counting and only batches with a cell viability between 90%-98% were used for further investigations. Note: if there was a high degree of red blood cell contamination on top of the PBMC pellet, an optional red blood cell lysis step was conducted prior to resuspension in AIM-V media which included: resuspending the pellet in 5 ml of sterile 1X ammonium-chloride-potassium lysis buffer (Appendix A); incubating the pellet for 3-5 minutes; adding 10 ml of ice cold PBS and centrifuging the cells at 400 x g for 10 minutes at room temperature. After isolation and counting, cells were then suspended in AIM-V medium at 1 x 10^6 cells/ml.

2.4.2. Cell counting

For counting purposes, 10 µl of resuspended cells was added to 90 µl Trypan blue (1:10 dilution in PBS), and 10 µl of the resultant mixture was pipetted onto a Neubauer haemocytometer slide (Marienfeld) and covered with a cover slip. Trypan blue allows for the identification of live cells through staining of dead cells, therefore allowing differentiation of live cells from dead cells whereby live cells were counted using a microscope (Nikon TMS).

To determine cell concentration and the volume of cells needed for seeding, the following formulae were used:

$$\textbf{Cell concentration: } Cells/ml = \frac{\#\ cells\ counted\ in\ 4\ squares\ of\ hemocytometer}{4} \times 10 \times 10\,000$$

$$\textbf{Seeding}: Volume\ (ml) = \frac{Number\ of\ cells\ required}{Number\ of\ live\ cells\ (per\ ml)}$$

2.4.3. Mycobacterium tuberculosis (Mtb) preparation

5X H37Rv Mtb stocks were prepared by diluting Mtb (Stellenbosch University, South Africa) in 7H9 medium (Appendix A) to obtain a final concentration of 2.5 x 10^6 Colony Forming Units (CFU)/ml. Thereafter a working stock was prepared by diluting the 5X Mtb stock (2.5 x 10^6 CFU/ml) in AIM-V

medium to obtain a final concentration of 5 x 10^5 CFU/ml (1 x10^5 CFU/well, multiplicity of infection (MOI) of 5:1).

2.4.4. PBMC infection and kisspeptin treatment

This assay was designed to determine the effects of kisspeptin on cytokine expression following PBMC infection/stimulation. Initial stimulation experiments with 5 healthy and non-pregnant patients were conducted at the Division of Molecular biology and Human genetics, Immunology Research Group Stellenbosch University, South Africa. The Biosafety level 3 laboratory (P3) was utilised as well as an existing and optimised model for *Mycobacterium tuberculosis* (Mtb) infection (described below).

PBMCs were seeded at 4 x 10^5 cells and cultured overnight in screw-top (with O-ring) Eppendorf tubes (Lasec, South Africa) at 37°C in 5% CO_2 in the absence or presence of treatment i.e. the positive control (*Mycobacterium tuberculosis* [Mtb] infection only and no hormonal treatment); negative control (no infection and no hormonal treatment); antagonist (alone) p356 at 10^{-6} M; Kp-10 (alone) at 10^{-6} M; Kp-10 + p356 both at 10^{-6} M; Kp-10 (alone) at 10^{-9} M and Kp-10 at 10^{-9} M + p356 at 10^{-6} M. All hormonal treatments were stimulated via Mtb infection. The solvent i.e. distilled water, was used as a control for treatment. Stimulation/infection experiments were conducted following overnight incubation.

Following overnight incubation, 400 µl of PBMC samples were centrifuged at 250 x g for 5 minutes before the media was removed and thereafter washed with RPMI-HEPES media (Sigma Aldrich). Mtb infection took place for 2 hours in the absence or presence of fresh treatment (with or without Kp-10 and antagonist p356). Cytokines were harvested at 3 different time points following infection i.e. 2 hours (immediately after infection), 24 hours (1 day) and 144 hours (6 days). Uninfected and untreated samples were used as the negative control whereby 7H9 media (Appendix A) was used as the control for infection. Cells were incubated for two hours at 37°C in 5% CO_2 in the presence or absence of Mtb at an multiplicitiy of infection (MOI) of 5:1 with treatment.

Processing at the end of each time point was conducted as follows: cells were centrifuged at 250 x g for 5 minutes. Once the cells had been pelleted, the supernatants were collected and centrifuged at maximum speed (600 x g) for two minutes. Harvested supernatants were then filter sterilized using syringe driven Millex-GV filter units and stored at -80°C for subsequent cytokine analysis via Luminex. For the time points, 24 hours and 144 hours, AIM-V medium was replenished containing the respective treatments. Following the 2 hour infection process and samples were incubated at 37°C at 5% CO_2 until

harvesting. Full media (including treatment) was replenished for the 144 hour time point again at 72 hours (day 3) and all samples were re-treated with/without hormone every day from infection until harvest.

2.4.5. Mtb plating procedure, in order to determine Mtb uptake employing colony forming unit (CFU) counts

After the harvesting of supernatants (Section 2.4.4.) the cell pellet was lysed by adding cold sterile dH_2O containing 0.05% sodium dodecyl sulphate (SDS) (Appendix A) for 5 minutes at 4°C. Thereafter cells were transferred to a clean Eppendorf tubes containing 7H9 medium, used to neutralize the effects of SDS. Lysates were plated in serial dilutions (0 = stock; -1 = 10X dilution; -2 = 100X dilution; -3 = 1000X dilution and -4 = 10 000X dilution all made up in 7H9 media) onto 7H11 agar plates (Appendix A) supplemented with BD Difco™ BBL™ Middlebrook OADC Enrichment media (Fishcer Scientific) and sealed for incubation at 37°C with 5% CO_2 for four weeks. The number of viable bacteria was determined after four weeks, post incubation, and represented as CFU/ml.

2.5 MTT cell proliferation/viability assay

PBMC were cultured overnight in screw-top (with O-ring) Eppendorf tubes and treated with the respective test compounds (Kp-10 and p356 or the solvent control (dH_2O)) for the different time points i.e. two hours, 24 hours and 144 hours (Section 2.5). Processing of cells at each time point included the removal of cell culture supernatant and the addition of 40 µL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [methylthiazolyl tetrazolium]) and 360 µL of AIM-V medium for 4 hours of incubation. The MTT and medium was removed following incubation and the remaining formazin crystals were dissolved using 200 µl of dimethyl sulfoxide (DMSO). Thereafter, the absorbance was measured via a spectrophotometer at 595 nm. The fold increase was plotted relative to the solvent control (dH_2O).

2.6 Whole blood stimulation assay

2.6.1. Tissue sampling and collection

Blood was obtained from healthy and non-pregnant women of reproductive age via venepuncture (Tygerberg Hospital, Stellenbosch University and Groote Schuur Hospital, University of Cape Town,

South Africa). 45 ml of blood was collected from each patient in 5 x 9 ml Ethylenediaminetetraacetic acid (EDTA) tubes or in 5 x 9 ml Sodium Heparin (NaHep) tubes.

2.6.2. Optimisation of LPS stimulation model and final assay design

Whole blood obtained from patients was placed into screw-top (with O-ring) Eppendorf tubes at 1ml of whole blood per tube. In order to test the effects of Kp-10 on LPS-induced cytokine expression, the whole blood stimulation model had to be optimised and established. TNF-α was the cytokine measured through the optimisation process via ELISA anaylsis.

Firstly, it was important to establish the best time for LPS stimulation and the optimum LPS concentration needed for sufficient stimulation of cytokine expression. LPS was solubilised in PBS to obtain a working solutions of 10 mg/ml. Further dilutions to 100 µg/ml and 1 µg/ml were made in PBS. Whole blood collected in EDTA tubes was stimulated with LPS for 4 hours, 12 hours and 24 hours using different concentrations of LPS, 10 µg/ml and 100 µg/ml (high concentrations were used to exaggerate a cytokine response). It was decided that 4 hours was the best time for LPS stimulation and the next step in the optimisation process included the use of more appropriate titrations of LPS concentrations (1 µg/ml, 100 ng/ml and 10 ng/ml) and a 6 hour stimulation time point in conjunction with a repeat of the 4 hour stimulation time point for validation purposes. It was ultimately decided to make use of a 4 hour LPS stimulation time at a LPS concentration of 10 ng/ml.

Subsequent optimisations included the optimisation of kisspeptin pre-incubation time. Whole blood was pre-incubated with treatment for 4 hours, 6 hours, 8 hours and 24 hours. Treatment included a treatment of the cells with Kp-10 at 1 µM to exaggerate a kisspeptin-induced response and a Kp-10 titration (1 µM to 1 nM) in order to characterise the effect of Kp-10 at different pre-incubation times. Whole blood was stimulated with LPS at 10 ng/ml for 4 hours following pre-incubation.

In conclusion, the final assay design used in this study was a 8 hour pre-incubation with Kp-10 treatment (a kisspeptin titration from 1 µM to 1 pM) followed by a 4 hour LPS stimulation at 10 ng/ml. Supernatants were then harvested for protein expression analysis.

Different blood collection tubes were tested in order to investigate if it had any influence on the results. Whole blood collected in NaHep tubes was subjected to the previously described assay design as well as the addition of a separate 24 hour pre-incubation experiment which was followed by a 4 hour LPS stimulation at 10 ng/ml. It was decided to do longer pre-incubation time points to enhance cellular reactivity with the Kp-10 treatment based on the results of the intial experiments using NaHep

tubes. Whole blood collected in NaHep tubes was treated with Kp-10 (titration from 1 µM to 1 nM) for 24 hours, 48 hours and 72 hours prior to being stimulated with LPS at 10 ng/ml for 4 hours. Cells were replenished with treatment every 24 hours.

After the indicated treatment times, cells were centrifuged at 1 000 x g for 10 minutes and the supernatant was removed and stored in -80°C for cytokines analysis via ELISA at a later stage.

2.7 Protein expression analysis

2.7.1. Luminex assay

Cell culture supernatants were harvested as previously described in Section 2.4.4. all Once the samples had thawed, supernatants were centrifuged at 600 X g for 10 minutes at 4°C to remove any debris or floating cells that may have been collected.

2.7.2. Luminex assay protocol

The supernatants were assayed to determine the concentrations of the following cytokines: IFNγ, IL-10, IL-17A, IL-1β, IL-1α, IL-6, MCP-1, TNF-α, and MIP-1α, MIP-1β and GM-CSF. The cytokine concentrations present in the supernatant obtained from the PBMC infection assays were assayed using a luminex MilliPlex® 27 plex MAP Human Cytokine/Chemokine Magnetic Bead Panel (cat #: HCTYOMAG-60K, Merck, South Africa) via the Bio-Plex 200 machine (BioRad, South Africa) according to the manufacturer's instructions (Appendix B). Depending on choice, the assay was customized to detect specific cytokines from a minimum concentration of 0.06 ng/ml to 100 ng/ml (Linear range 0.06-0.8 ng/ml) with an inter-assay and intra-assay variability of <15% and <10% respectively. Absorbance was read on a microplate reader at 450 nm. A standard curve constructed from relative absorbance versus concentration of the standard solutions was plotted and the concentrations of cytokines within the various samples were calculated from the standard curve.

2.7.3. Enzyme-linked Immunosorbent Assay (ELISA)

Cell culture supernatants were harvested as previously described in Section 2.6.2. Once the samples had thawed, supernatants were centrifuged at 600 X g for 10 minutes at 4°C to remove any debris or floating cells that may have been collected. The supernatants were assayed for the following cytokines: IL-1β, IL-10, TNF-α and IL-6, using separate sandwich ELISA sets from BD Biosciences (BD OptEIA™, BD Biosciences).

A 96-well high-binding Nunclon Delta treated ELISA plate (Nunc™ Microwell™ 96-well microplates) was coated with 100 µl of capture antibody (1:250) diluted in ELISA Coating Buffer (refer to Appendix A for composition). The plate was sealed and stored at 4°C overnight. Following overnight incubation, the wells were aspirated and washed 3X with ≥ 300 µl ELISA Wash Buffer (Appendix A). The wells/plate were blocked using 200 µl ELISA Assay Diluent (Appendix A) and sealed at room temperature for 1 hour. The plate was then washed 3X with ELISA Wash Buffer. ELISA standards were prepared as 2-fold serial dilutions in Assay Diluent, specific to the cytokine of interest. The following were the ranges of concentrations for each cytokine: 250 pg/ml – 0 pg/ml for *IL-1β*, 500 pg/ml – 0 g/ml for *IL-10* and *TNF-α* and 300 pg/ml – 0 pg/ml for *IL-6*. Blank Assay Diluent served as the zero standard for the assay (0 pg/ml). Eight standards were prepared in total. Wells were then loaded with 100 µl of standard or sample, in duplicate, sealed and incubated for 2 hours at room temperature. The plate was then washed 5X with ELISA Wash Buffer which, 100 µl of Working Detector (Detection Antibody at kit/cytokine-specific concentration and streptavidin-horseradish peroxidase (SAv-HRP) (1:250) reagent diluted in Assay Diluent) was added to each well, sealed and incubated for 1 hour at room temperature. The plate was then washed 7X using ELISA Wash Buffer with each wash consisting of 40 second soak in wash buffer. After the final wash step, 100 µl of Substrate Solution was added to each well (TMB 2-component Microwell Peroxidase substrate, cat.number: 5120-0053, SeraCare) and incubated in the dark for 30 minutes. The enzyme reaction was stopped using 50 µl of ELISA Stop Solution – 2N H_2SO_4 (Appendix A). Once all bubbles were removed, absorbance was measured using a microplate spectrophotometer reader (GloMax® Discover System, Promega) at 450 nm with a lambda correction of 560 nm (within 30 minutes of stopping the reaction). Protein concentration of samples was determined by plotting the concentration of the standards against the corresponding optical density (OD) reading to generate a standard curve.

2.8 Statistical analysis:

A paired *t* test was used to compare baseline cytokine expression to LPS/Mtb stimulation of cytokine secretion with and without kisspeptin and antagonist, in whole blood or PBMC of healthy participants.

A linear regression analysis between the variables was performed and the best fit curve drawn on the data points. One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the differences across all the different participant categories. GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com) was used for all analysis. A $p < 0.05$ defined statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively.

Chapter Three Results

3.1 Determination of the effect of Kp-10 on Mtb induced expression of MIP-1α, MIP-1β, TNF-α and GM-CSF in PBMCs of non-pregnant and healthy women

As this is a pilot study, the first step of this investigation was to establish whether kisspeptin-10 (Kp-10) had an effect on cytokine release in response to an infection, employing human PBMCs infected with Mtb as a model system. The model used was an established *in vitro* system utilising blood taken from 5 healthy (non-diabetic with no history of pregnancy-related complications) and non-pregnant women in sodium heparin (NaHep) blood collection tubes. The patient data in Table 3.1 displays the patient code number, sample collection time, sample date, patient age, date of birth and HbA1C value (average blood glucose levels for past two to three months i.e. indicator of diabetic status). 54 ml of blood was collected from each woman and PBMCs were isolated and subjected to Mycobacterium tuberculosis infection in the absence or presence of Kp-10 and kisspeptin antagonist, peptide 356 (p356) treatment. Following infection, cell culture supernatants were collected at 2 hours, 24 hours and 6 days, and subjected to a Luminex multiplex assay to determine the level of several cytokines.

Table 3.1: Patient criteria as recruited for PBMC infection assay.

Patient ID	Sample Date	Sample Collection Time	D.O.B	Age	Gender	HbA1c (POC)
A386	2018/06/18	8:43	1970/05/19	48	Female	5.6
A387	2018/06/25	8:37	1994/10/19	24	Female	5,9
A388	2018/07/02	8:50	1994/08/19	24	Female	5,4
A389	2018/07/23	8:50	1996/10/11	22	Female	5,5
A390	2018/08/06	8:50	1998/01/27	20	Female	5,4

3.1.1 *in vitro* PBMC infection assay

Protein expression analysis on selected cytokines employing a PBMCs infection assay with Mtb was performed on the 5 patients in order to determine the effect of Kp-10 on Mtb-induced immune response. PBMCs isolated from the women were infected with Mtb (MOI of 5:1) in the absence or presence of treatment with kisspeptin and its antagonist, p356 for the following time points: 2 hours, 24 hours and 6 days (in the case of 6 day treatment, Kp-10 and p356 treatment was replenished every 24 hours). Treatment included: uninfected and untreated negative control, infected and untreated positive control, infected and treated with: Kp-10 alone at 1 µM, kisspeptin antagonist p356 alone at 1 µM, and combination treatments: Kp-10 (1 µM) + p356 (1 µM) and Kp-10 (1 nM) + p356 (1 µM). This was done to form the basis for the continuation of the present study.

At processing of each time point, cell culture supernatants were harvested and subjected to expression analysis of selected cytokines. The cytokines chosen for expression analyses were: interferon (IFN)γ, interleukin-10 (IL-10), interleukin-17A (IL-17A), interleukin-1β (IL-1β), interleukin-1α (IL-1α), interlukein-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-1α, MIP-1β and granulocyte macrophage colony stimulating factor (GM-CSF). These cytokines were chosen based on their immunomodulatory properties as described in Table 1.1. The expression levels of the secreted cytokines were determined employing Luminex multiplex assay. As a result of many proteins being expressed only in response to an inflammatory stimulus, all figures are displayed as percentage protein expression relative to the Mtb-infected and untreated (positive control) samples (set to 100%).

It must be noted that of the 5 patients recruited for this initial investigation, only data for 2 patients was viable, meaning that of the analytes analysed, 2 patients had responses that fell within the observable range of the assay i.e. the standard curve, deeming the data accurate for quantification. The responses/analyte readings for the remaining patients all fell below the observable range, meaning that the response/induction (concentration of analyte) was not sufficient to be measured. Of the 12 analytes investigated using the luminex multiplex assay, only 5 analytes (GM-CSF, TNF-α, MIP-1α, MIP-1β and IL-10) had responses that were within the range of the standard curve (Figure 3.1,Figure 3.2 and Figure 3.3). Not all these analytes were observed at all the time points.

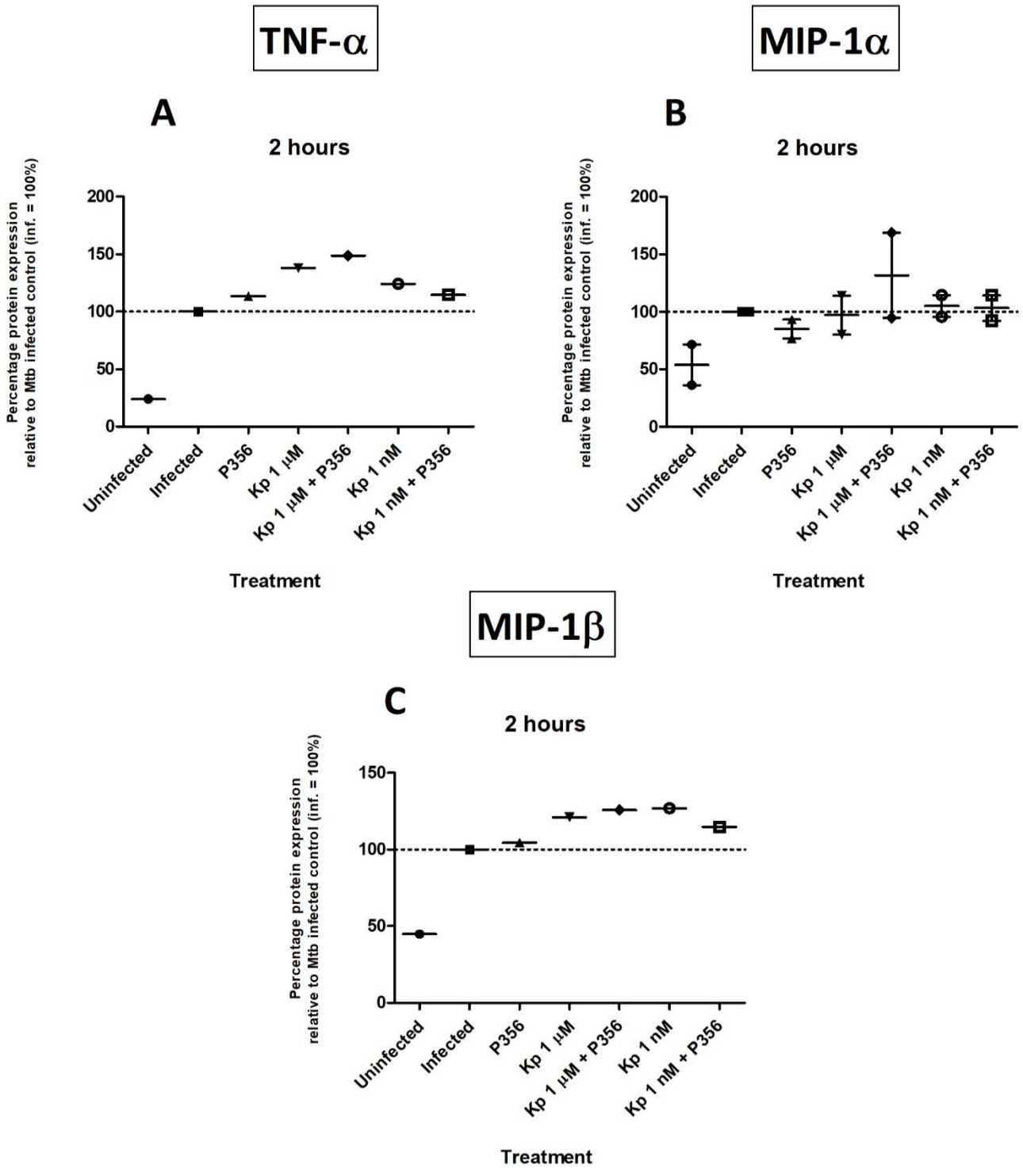

Figure 3.1: The effect of Kp-10 on Mycobacterium tuberculosis induction of tumour necrosis factor-α, macrophage inflammatory protein-1α and macrophage inflammatory protein-1β in PBMCs of non-pregnant healthy women. Cells were pre-incubated with treatment overnight and subjected to Mtb infection (MOI 5:1) for 2 hours the following day in the absence or presence of the respective treatments for the respective time points. Cell culture supernatants were harvested at 2 hours and subjected to luminex analysis. Percentage protein expression (± range) represented as a percentage relative to/plotted against the infected and untreated control (positive control) which was set to 100%. All graphs, where applicable, are representative of pooled data from 5 independent experiments whereby only 2 presented viable results. There were only sufficient responses for one patient (A388 from Table 3.1) for cytokines TNF-α and MIP-1β whereas for MIP-1α, two patients had sufficient responses hence the error bars within the graph. Statistical analysis could not be conducted as the viable sample size was too small.

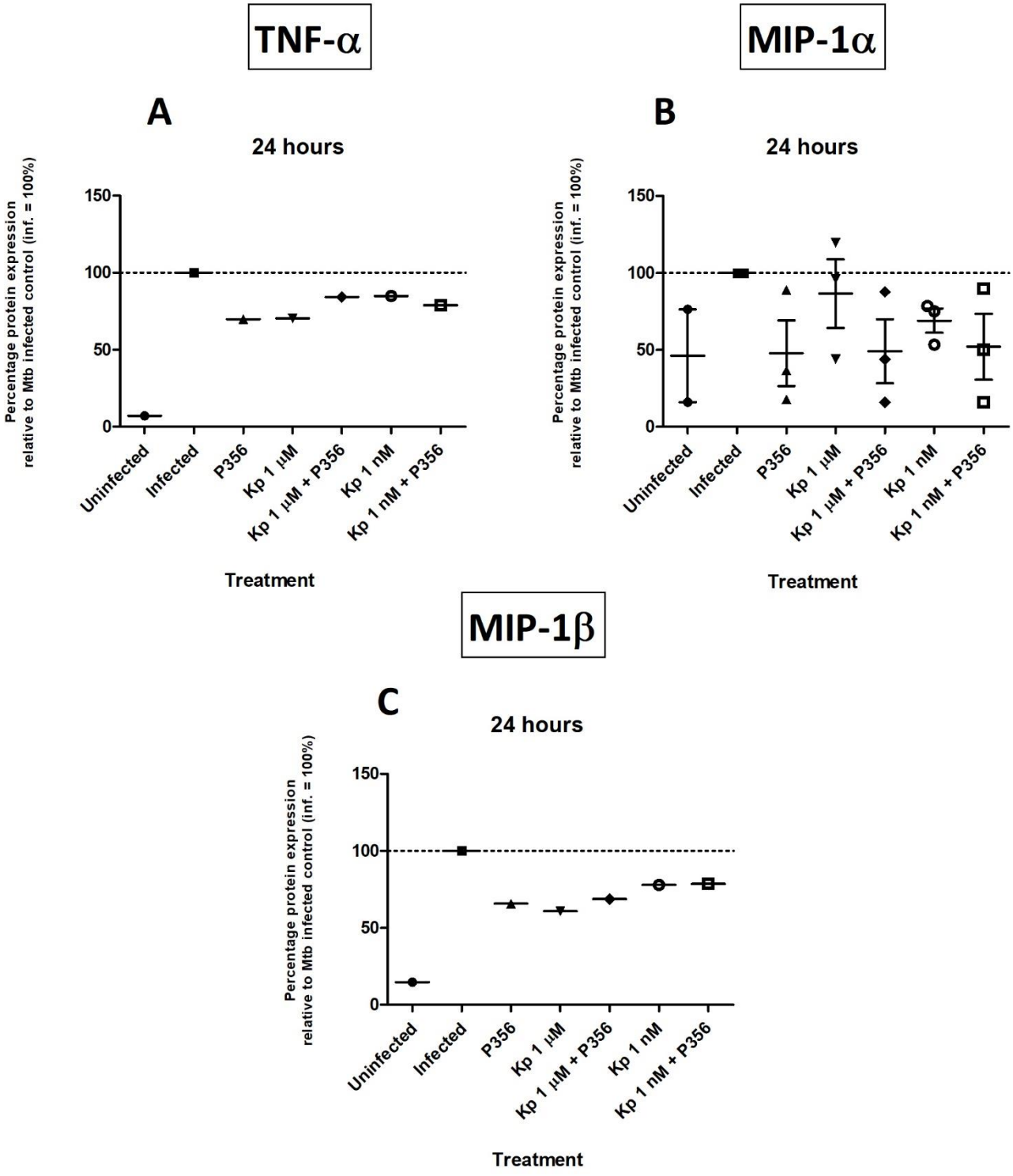

Figure 3.2: The effect of Kp-10 on Mycobacterium tuberculosis induction of tumour necrosis factor-α, macrophage inflammatory protein-1α and macrophage inflammatory protein-1β in PBMCs of non-pregnant healthy women. Cells were pre-incubated with treatment overnight and subjected to Mtb infection (MOI 5:1) for 2 hours the following day in the absence or presence of the respective treatments for the respective time points. Cell culture supernatants were harvested at 24 hours and subjected to luminex analysis. Percentage protein expression (± range) represented as a percentage relative to/plotted against the infected and untreated control (positive control) which was set to 100%. All graphs, where applicable, are representative of pooled data from 5 independent experiments whereby only 2 presented viable results. There were only sufficient responses for one patient (A388 from Table 3.1) for cytokines TNF-α and MIP-1β whereas for MIP-1α, two patients had sufficient responses hence the error bars within the graph. Statistical analysis could not be conducted as the viable sample size was too small.

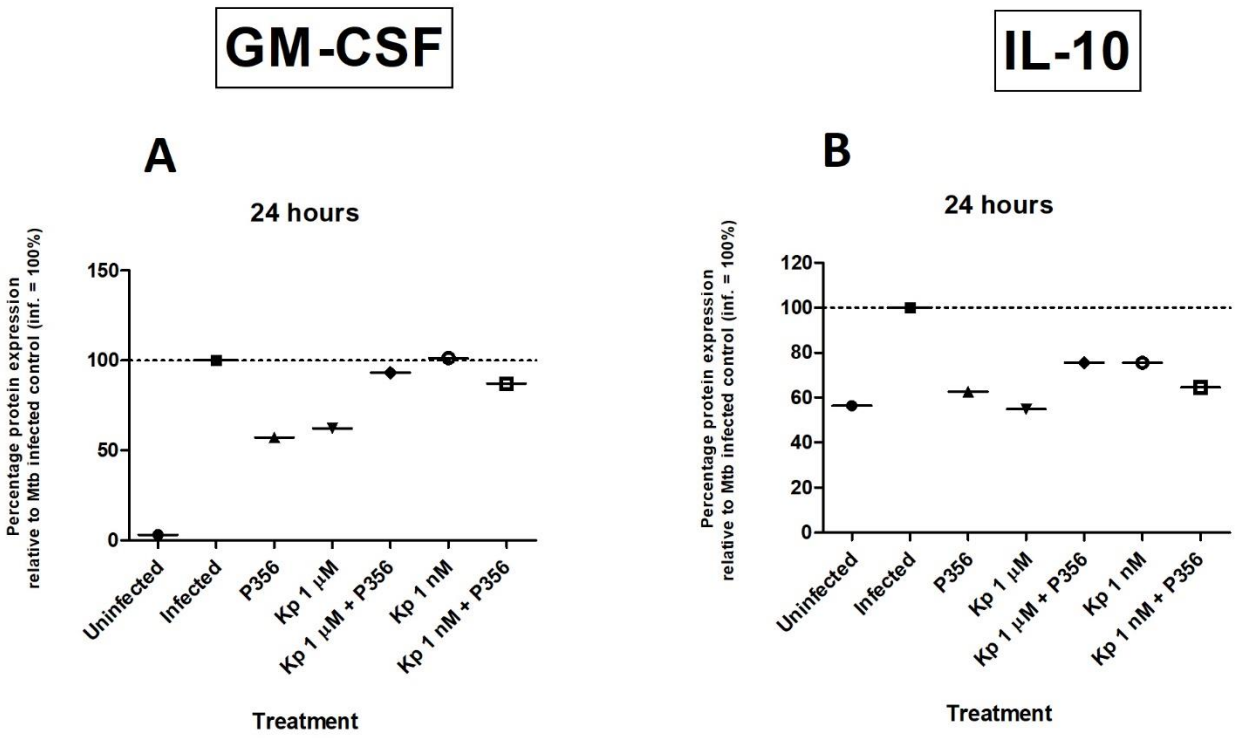

Figure 3.3: The effect of Kp-10 on Mycobacterium tuberculosis induction of granulocyte macrophage colony stimulating factor and interleukin-10 in PBMCs of non-pregnant healthy women. Cells were pre-incubated with treatment overnight and subjected to Mtb infection (MOI 5:1) for 2 hours the following day in the absence or presence of the respective treatments for the respective time points. Cell culture supernatants were harvested at 24 hours and subjected to luminex analysis. Percentage protein expression (± range) represented as a percentage relative to/plotted against the infected and untreated control (positive control) which was set to 100%. All graphs, where applicable, are representative of pooled data from 5 independent experiments whereby only 2 presented viable results. There were only sufficient responses for one patient (A388 from Table 3.1) for cytokines GM-CSF and lL-10. Statistical analysis could not be conducted as the viable sample size was too small.

At 2 hours post Mtb infection a 5 fold increase in TNF-α was observed (Figure 3.1A), there was a slight further induction of TNF-α in response to Kp-10 treatment (1 µM = 138.11% and 1 nM = 124.21%). It must be reiterated that due to the observation of effective responses in only 1-2 patient samples, it was not possible to do statistical analysis. The antagonist p356 had no effect on Mtb-induced cytokine expression for TNF-α (p356 alone at 1 µM = 113.39%). Treatment with Kp-10 (1 µM) + p356 (1 µM) led to an increased induction of TNF-α (148.75%) compared to the negligible increased induction for Kp-10 (1 nM) + p356 (1 µM) (114.57%).

Mtb infection led to a 2 fold induction of MIP-1α at 2 hours (Figure 3.1B) and treatment with Kp-10 alone or together with p356 did not have an effect on the induction of MIP-1α, while treatment with p356 alone slightly reduced the induction level of MIP-1α (85.11% ± 8.19). The mean +/- range is indicative of the difference between the results from the results observed from the two patients. Mtb infection also led to a 2 fold induction of MIP-1β at 2 hours (Figure 3.1C). Treatment with Kp-10 or

p356 alone or together led to an increase in the induction of MIP-1β. Both concentrations of Kp-10 showed similar responses (1 μM = 121.01% and 1 nM = 126.91%). The antagonist had no effect on MIP-1β induction (p356 alone = 104.49%), whereas Kp-10 together with p356 led to a slight increase in MIP-1β (Kp-10 (1 μM) + p356 (1 μM) (126.84%) and Kp-10 (1 nM) + p356 (1 μM) (114.81%). In general, overall treatment with Kp-10 (1 μM) + p356 (1 μM) led to a response greater then Kp-10 alone at both concentrations whereas treatment with Kp-10 (1 nM) + p356 (1 μM) led to a decreased response in induction when compared to Kp-10 at both concentrations.

At 24 hours there was successful induction by Mtb for the cytokines presented. For TNF-α, at 24 hours post Mtb infection, a 10 fold increase was observed (Figure 3.2A). Slight inhibition of TNF-α induction was seen in response to Kp-10 treatment (1 μM = 70.50% and 1 nM = 84.92%). The antagonist, p356, had similar inhibitory effects to that of Kp-10 at 1 μM on Mtb-induced cytokine expression for TNF-α (p356 alone at 1 μM = 69.91%). Combination treatments such as Kp-10 (1 μM) + p356 (1 μM) led to slight inhibition of TNF-α expression (84.31%) as well as for Kp-10 (1 nM) + p356 (1 μM) (78.96%).

For MIP-1α at 24 hours, Mtb infection again led to a 2 fold induction of MIP-1α (Figure 3.2B) and treatment with Kp-10 alone or together with p356 as well as p356 alone had varying effects on the induction of MIP-1α. Whilst both concentrations of Kp-10 showed inhibitory effects on MIP-1α induction, Kp-10 at 1 nM (52.05% ± 21.35) displayed greater inhibitory effects than Kp-10 at 1 μM (86.59% ± 22.32).Treatment with p356 showed a slight decrease in the induction level of MIP-1α (47.89% ± 21.31). Combination treatments displayed similar inhibitory effects to that of p356 showing that p356 enhanced its effects when combined with Kp-10. Both combination treatments were similar as Kp-10 (1 μM) + p356 (1 μM) (49.25% ± 20.87) was similar to Kp-10 (1 nM) + p356 (1 μM) (52.05% ± 21.35).

Mtb infection led to a 5 fold induction of MIP-1β at 24 hours (Figure 3.2C), treatment with Kp-10 or p356 alone or together led to a inhibitory effect of the induction of MIP-1β. The two concentrations of Kp-10 displayed a dose response effect in terms of inhibition of MIP-1β induction (1 μM = 60.77% and 1 nM = 77.90%). The antagonist displayed slight inhibitory effcts on MIP-1β induction when alone (p356 alone = 65.74%) The antagonist slightly decreased the inhibitory effect of Kp-10 at 1 μM when in combination (Kp-10 at 1 μM + p356 at 1 μM = 69.64%) but had no effect when in combination with Kp-10 at 1 nM (Kp-10 at 1 nM + p356 1 μM = 78.60%). Both combination treatments displayed slight inhibitory effects on MIP-1β induction.

With regards to GM-CSF at 24 hours, Mtb infection led to drastic 50 fold induction of GM-CSF (Figure 3.3A). A slight inhibitory effect on GM-CSF induction was seen in response to Kp-10 treatment at 1 μM (62.32%), however Kp-10 at 1 nM had no effect (101.37%). Again the antagonist, p356, had similar

inhibitory effects to that of Kp-10 at 1 μM on Mtb-induced cytokine expression for GM-CSF (p356 alone at 1 μM = 57.25%). Combination treatments such as Kp-10 (1 μM) + p356 (1 μM) led to slight inhibition of GM-CSF expression (93.24%) as well as for Kp-10 (1 nM) + p356 (1 μM) (87.12%).

For IL-10, Mtb infection led to a 2 fold induction at 24 hours (Figure 3.3B). Treatment with Kp-10 or p356 alone or together led to an inhibition effect on the induction of IL-10. The two concentrations of Kp-10 displayed inhibitory effects of IL-10 induction similar to that of MIP-1β at 24 hours (1 μM = 54.87% and 1 nM = 75.48%). The antagonist displayed inhibitory effects of IL-10 induction similar to Kp-10 at 1 μM when alone (p356 alone = 62.61%). The antagonist slightly decreased the inhibitory effect of Kp-10 at 1 μM when in combination (Kp-10 at 1 μM + p356 at 1 μM = 75.49%) and had slightly enhanced the inhibitory effect of Kp-10 at 1 nM when in combination with Kp-10 at 1 nM (Kp-10 at 1 nM + p356 1 μM = 64.52%).

For this timepeoint, treatment with Kp-10 (1 μM) + p356 (1 μM) led to a response greater then Kp-10 alone at both concentrations with exception to MIP-1α (the response was less than both concentrations) and MIP-1β (the response was less than Kp-10 at 1 nM) (Figure 3.2B and Figure 3.2C). Treatment with Kp-10 (1 nM) + p356 (1 μM) led to a varied response in induction when compared to Kp-10 at both concentrations. Induction for combination treatments was lower than Kp-10 (1 nM) and higher than Kp-10 (1 μM) for TNF-α (Figure 3.2A) , lower than both Kp-10 (alone) concentrations for MIP-1α (Figure 3.2B) and higher than Kp-10 (1 μM) but the same for Kp-10 (1 nM) for MIP-1β (Figure 3.2C).

At 6 days post Mtb infection. No cytokines were measured as the response for all 5 patients was negligible and fell outside of the observable range (induction was too low to be quantified) and thus could not be accurately quantified for all analytes.

In summary, Kp-10 treatment increases induction at 2 hours for TNF-α and MIP-1β whereas for MIP-1α, Kp-10 treatment has little effect. At 24 hours, Kp-10 inhibited the Mtb-induction of TNF-α and MIP-1β as well as the Mtb- induction of IL-10 and GM-CSF. The antagonist p356 displayed partial agonistic effects and did not inhibit or reverse the effects of kisspeptin.

3.1.2 Mtb infection and the culturing of PBMCs in AIM-V medium over a prolonged period of time caused a reduction in cell viability

In order to establish the reason for a lack of response at the day 6 time point, the viability of the isolated PBMCs over the allotted time points i.e. 2 hours, 24 hours and 6 days, was investigated using a MTT colorimetric cell viability assay which was performed on the PBMCs of each patient. The assay is based on the reduction of yellow tetrazolium salt (MTT) to purple formazan crystals which are formed within the mitochondria of metabolically active cells, thus allowing for the determination of viable cells. Cell viability was determined as a percentage by measuring relative absorbance at 595 nm and plotting each treatment against the overall uninfected and untreated. The control for these tests was set as the untreated and uninfected control at 2 hours which was set to 100%. It must be noted that there was a decline in absorbances measured for uninfected and untreated controls across the time points: for 2 hours - 0,251, for 24 hours - 0,442 and for day 6 - 0,052. This gave further indication of a loss of cell viability. The MTT showed that from the 2 hour time point (Figure 3.4A) and the 24 hour time point (Figure 3.4B), PBMCs remained viable (on average ±100% viability) whereby treatment and Mtb infection did not have a significant effect on cell viability. However, at day 6, there was a greater than 2 fold reduction in cell viability (Figure 3.4C) especially in the case of both Kp-10 treatments (1 µM = 13.59 ± 1.45 and 1 nM = 21.31 ± 3.19).

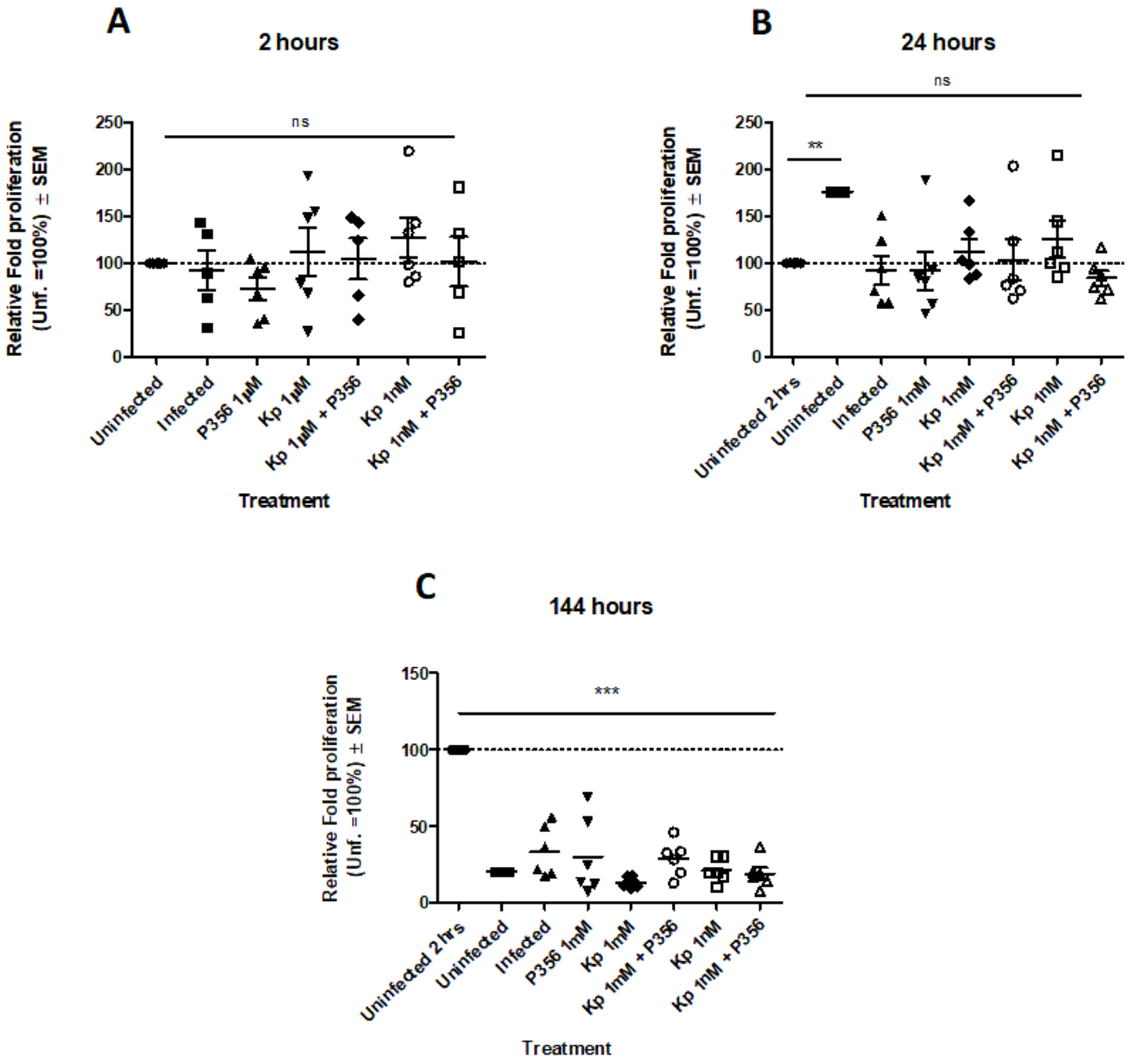

Figure 3.4: Relative cell proliferation/viability of PBMCs over 2 hours, 24 hours and 144 hours with selected treatment. Whole blood was pre-incubated with treatment overnight and subjected to Mtb infection (MOI 5:1) for 2 hours the following day in the absence or presence of the respective treatments for the respective time points (replenished each day). Supernatants were harvested at each time point (2, 24 and 144 hours) and the pellet was subjected to MTT treatment for 4 hours. Relative fold proliferation (±SEM) represented as a percentage relative to/plotted against the overall average uninfected and untreated control which was set to 100. Graphs are representative of pooled data from 5 patient samples. One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the differences across all the treatments. Descriptive statistics was computed using the same software and $p < 0.05$ defines statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively. The symbol "ns" depicts no significance.

3.1.3 Kisspeptin had an affect on Mtb infection based on Colony Forming Unit (CFU) count and Mtb uptake analysis

CFU analysis was conducted on lysed PBMCs following the previously described PBMC infection assay. The analysis was performed on 9 patients in order to determine the effect of Kp-10 on Mtb uptake/infection.

At processing of each time point, cell culture supernatants were removed. After the harvesting of supernatants the cell pellet was lysed and cells were plated in serial and sealed for incubation for four weeks. The number of viable bacteria was determined after four weeks, post incubation, and represented as CFU/ml. Mtb infection/uptake was analysed and statistical analysis was performed on each column. Unfortunately, for the 144 hour (day 6) time point, no infection/uptake was observed for all treatments and patient samples.

The results from the CFU analysis (Figure 3.5) showed no significance difference between the treatments and the infected untreated control for both time points. The assay worked, as there was a significant difference between the untreated and uninfected control and the infected untreated control for both time points.

For the 2 hour time point (Figure 3.5A), Kp-10 had a non-significant affect on the uptake of Mtb. Kp-10 (1 nM) had the lowest CFU/ml count at 2 hours (2,5 X 10^4 CFU/ml ± 1,4 X 10^4 CFU/ml) when compared to the infected and untreated control (4, 38 X 10^4 CFU/ml ± 1,4 X 10^4 CFU/ml). The antagonist, p356, and Kp-10 at 1 µM had a similar affect on Mtb uptake when compared to the infected untreated control (p356 = 3, 40 X 10^4 CFU/ml ± 0,10 X 10^4 CFU/ml and Kp-10 = 3, 35 X 10^4 CFU/ml ± 1,24 X 10^4 CFU/ml). The combination treatments enhanced the effect of the antagonist and Kp-10 alone for both concentrations. Kp-10 (1 µM) + p356 (1 µM) was 3, 38 X 10^4 CFU/ml ± 0,56 X 10^4 CFU/ml and Kp-10 (1 nM) + p356 (1 µM) was 4, 53 X 10^4 CFU/ml ± 0,16 X 10^4 CFU/ml when compared to the infected and untreated control and the antagonist and Kp-10 alone.

For the 24 hour time point (Figure 3.5B), Kp-10 again had a non-significant affect on the uptake of Mtb and the affects were similar to that at 2 hours. Kp-10 (1 nM) + p356 (1 µM) had the lowest CFU/ml count at 24 hours (1,6 X 10^3 CFU/ml ± 0,38 X 10^3 CFU/ml) when compared to the infected and untreated control (1, 98 X 10^3 CFU/ml ± 0,38 X 10^3 CFU/ml). The antagonist, p356, and combination treatment, Kp-10 (1 µM) + p356, had a similar affect on Mtb uptake when compared to the infected untreated control (p356 = 1,8 X 10^3 CFU/ml ± 0,18 X 10^3 CFU/ml and Kp-10 (1 µM) + p356 = 1, 74 X 10^3 CFU/ml ±

0,24 X 10^3 CFU/ml). The combination treatments had opposite affects to the 2 hour timepoint as they decreased the effect of the antagonist and Kp-10 alone for both concentrations. Kp-10 (1 µM) + p356 (1 µM) was 1, 74 X 10^3 CFU/ml ± 0,24 X 10^3 CFU/ml and Kp-10 (1 nM) + p356 (1 µM) was 1,6 X 10^3 CFU/ml ± 0,38 X 10^3 CFU/ml when compared to the infected and untreated control and the antagonist and Kp-10 alone. Kp-10 alone increased Mtb uptake when compared to the combination treatments and the control. Kp-10 (1 µM) was 2, 23 X 10^3 CFU/ml ± 0,25 X 10^3 CFU/ml which was the greatest effect and Kp-10 (1 nM) which was 2, 03 X 10^3 CFU/ml ± 0,50 X 10^3 CFU/ml.

Overall for both timepoints, The data was not significant with regards to statistical analysis and there was a general inconsistency in inter-patient variability which contributed to the lack of significance observed.

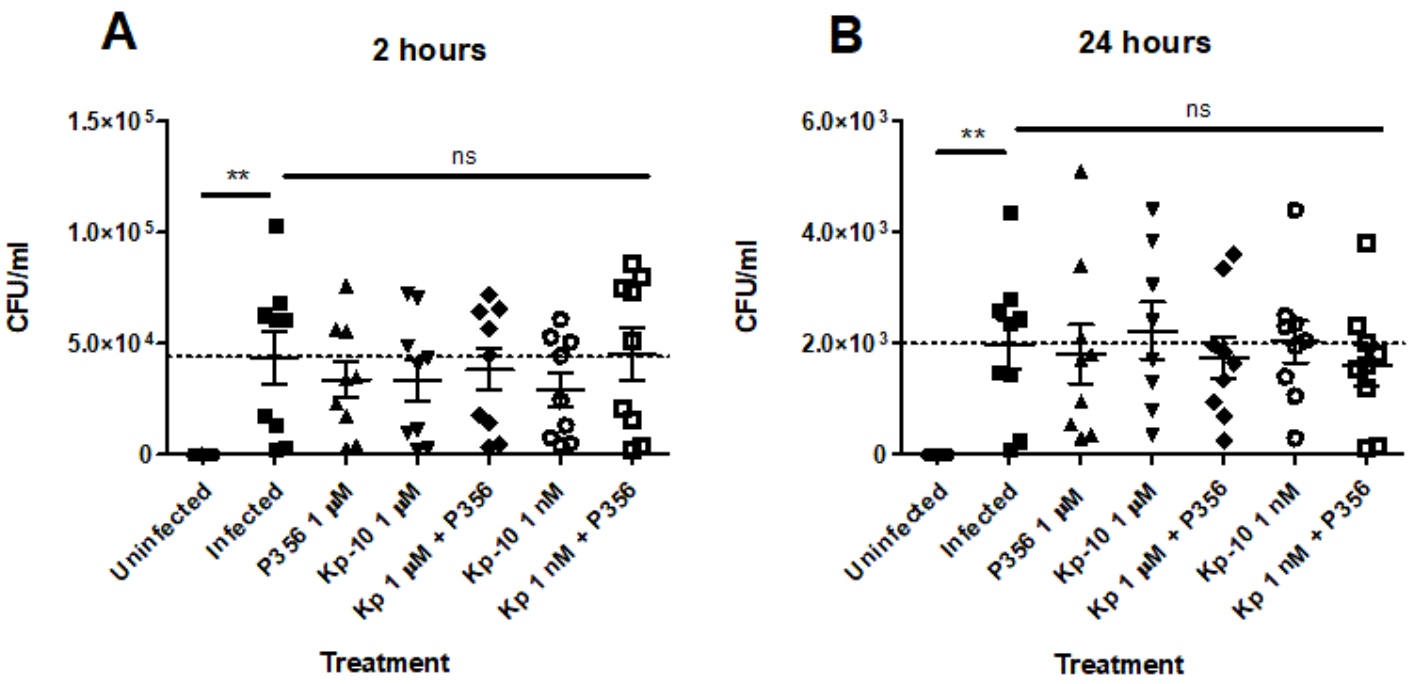

Figure 3.5: CFU/ml count after 4 weeks following infection and 2 hours and 24 hours incubation with selected treatment. Cells were pre-incubated with treatment overnight and subjected to Mtb infection (MOI 5:1) for 2 hours the following day in the absence or presence of the respective treatments for the respective time points (replenished each day). Supernatants were harvested at each time point (2 and 24 hours) and the pellet was subjected to lysing and plating on 7H11 agar plates (n= -1 to n = -4 dilution series) and left ot incubate for 4 weeks at 37 °C before counting. CFU/ml was plotted for each treatment with each column being compared to the infected and untreated control (±SEM). Graphs are representative of pooled data from 9 patient samples. One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the differences across all the treatments. Descriptive statistics was computed using the same software and $p < 0.05$ defines statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively. The symbol "ns" depicts no significance.

3.2 *in vitro* whole blood stimulation assay

The initial experiments of this investigation described in section 3.1 involved infection with Mtb and working in a P3 facility, which is not without any risk and in general the assay was not suitable for the measurements required i.e. it was not sufficient in measuring appropriate cytokine induction in the majority of patients. Therefore, it was decided to establish an alternative model employing lipopoloysaccharides (LPS) and whole blood instead of Mtb and PBMCs. Initially, the LPS *in vitro* model system needed to be optimised. First optimising LPS stimulation time and optimal LPS concentration, thereafter, determining the pretreatment and posttreatment time with Kp-10 and p356. The blood was initially collected in K3 EDTA tubes and thereafter, in NaHep tubes. Based on the fact that p356 displayed partial agonistic effects within the PBMC infection assay model, it was decided to exclude the antagonist from the whole blood stimulation assay and completely characterise the effects of Kp-10.

3.2.1 Establishing a whole blood *in vitro* immune response model using LPS

Pathogen-associated molecular patterns (PAMPs) from foreign microorganisms are recognised by cells of the innate immune system. This recognition results in the stimulation of an immune response towards these PAMPs. A common PAMP includes LPS which is a bacterial endotoxin found within the outer membrane of gram-negative bacteria [135]. It is common to use LPS as a stimulating agent for cultured macrophages, PBMCs and whole blood, which are commonly used for *in vitro* assays in order to study immune responses [136,137,138,139]. The selected concentration of LPS depends on its biological origin, the cell line used, the stimulation time and the appropriate level of inflammatory response desired. It is known that cytokines, chemokines and inflammatory mediators present different expression patterns over time during the inflammatory response [140,141,142]. In this study we used LPS derived from the *E. coli* serotype 055: B5 (Cat. Number: L4005, Sigma-Aldrich/Merck). Concentrations of from 10 µg/ml to 1 ng/ml were tested. For these experiments, the negative control was cells not stimulated with LPS and not treated with Kp-10. Whereas the positive control was cells stimulated with LPS and not treated with Kp-10.

3.2.1.1. Optimising LPS stimulation time and LPS concentration range

Whole blood taken from 4 healthy and non-pregnant women in EDTA tubes was sampled and 1 ml was placed into each tube for culture and LPS induction. Due to its inflammatory properties, high induction upon immune stimulation and time constraints, TNF-α was chosen as a readout for immune response to LPS to determine optimum experimental conditions. TNF-α was measured in harvested cell culture supernatants using sandwich ELISA after testing different LPS stimulation times (4 hours, 12 hours and 24 hours) (Figure 3.6) at an LPS concentration of 10 μg/ml and 100 μg/ml. Figure 3.6A, shows that at 4 hours after LPS stimulation a large induction was observed. This induction was ± 2.5 fold greater for 100 μg/ml LPS (784.65 pg/ml ± 24.01) than 10 μg/ml LPS (303.54 pg/ml ± 23.87). At 12 hours (Figure 3.6B), induction was ± 4 fold greater for 100 μg/ml LPS (829.30 pg/ml ± 2.69) than 10 μg/ml LPS (212 pg/ml ± 30.11). While, at 24 hours (Figure 3.6C), induction was ± 10 fold greater for 100 μg/ml LPS (788.70 pg/ml ± 12.13) when compared to 10 μg/ml LPS (75.61 pg/ml ± 15.98). The induction at 100 μg/ml was consistent across all time points whereas the response to 10 μg/ml decreased at the 24 hour timepoint.

The 4 hour stimulation time (Figure 3.6A) proved sufficient for the experimental design, since, the maximal LPS induction of TNF-α was observed already at this time point for both LPS concentrations. The use of the 4 hour stimulation time point was also supported by literature [145,149,150] in order to measure multiple cytokines and chemokines.

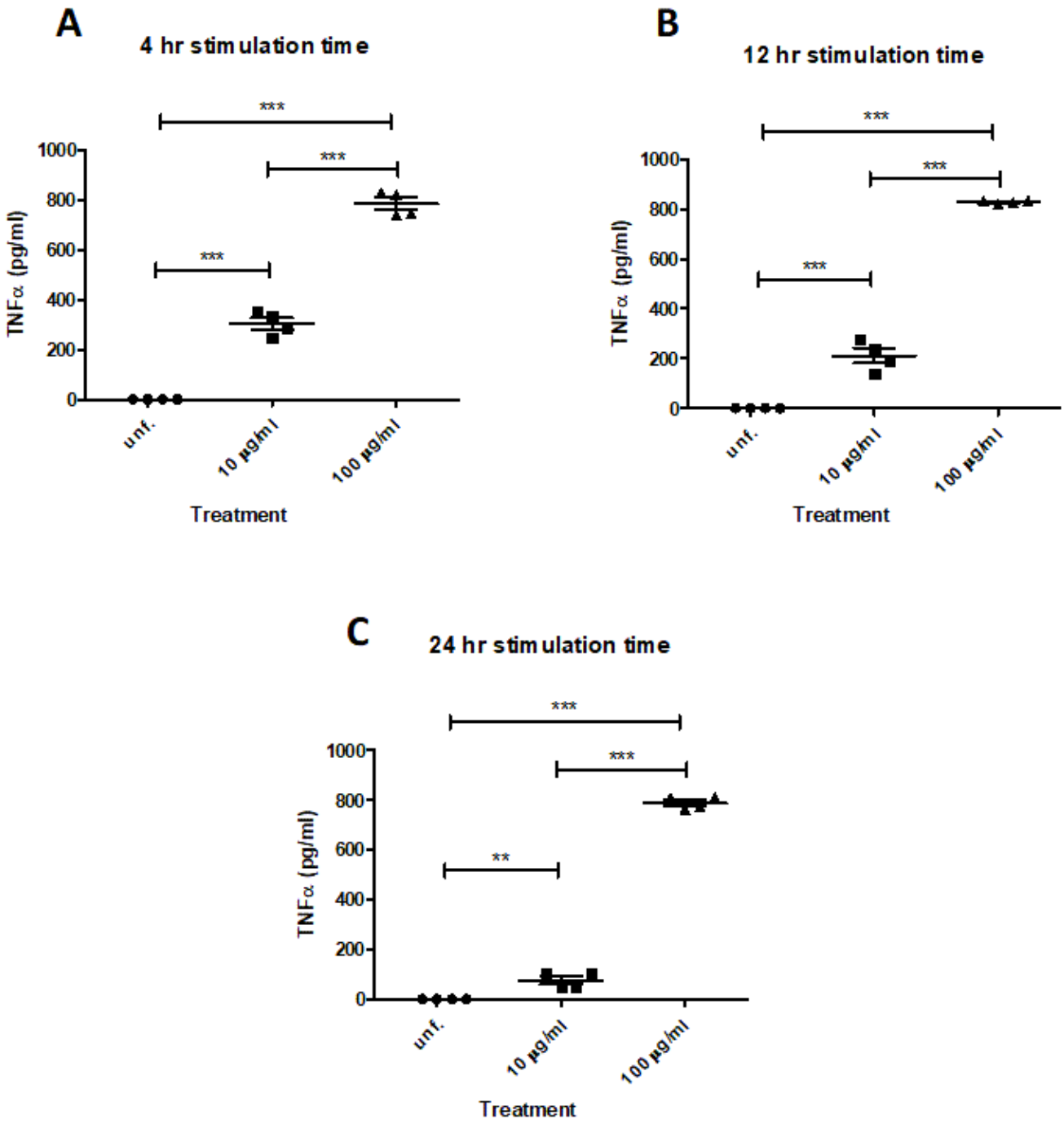

Figure 3.6: Optimisation of stimulation time for a LPS-induced induction of TNF-α expression in whole blood. Whole blood was treated with 10 µg/ml or 100 µg/ml of LPS for 4 (A), 12 (B) and 24 (C) hours. Following stimulation, supernatants were harvested and subjected to protein expression analysis by sandwich ELISA to detect TNF-α. Graphs displayed pooled data, representative of 4 independent samples for each stimulation time (±SEM). One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the differences across the different treatment groups. Descriptive statistics was computed using the same software and $p < 0.05$ defines statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively. The symbol "ns" depicts no significance.

In order test whether lower LPS concentrations will still direct an induction of TNF-α in whole blood the following LPS concentrations (1 µg/ml, 100 ng/ml and 10 ng/ml) were tested. It was decided to use the 4 hour and 6 hours stimulation times.

Following 4 hours of LPS stimulation, TNF-α induction was clearly observed for all concentrations of LPS. The results for TNF-α induction at 4 hours were as follows (Figure 3.7A): 1 µg/ml LPS = 1236 pg/ml ± 49.13; 100 ng/ml LPS = 994.40 pg/ml ± 43.16 and 10 ng/ml LPS = 333.7 pg/ml ± 51.79 (less ± 4 fold than 1 µg/ml LPS and less ± 3 fold than 100 ng/ml LPS). The lowest induction was in response to 10 ng/ml (± 5 fold less than the maximum at 1 µg/ml), the response 100 ng/ml was similar to the maximum induction and the maximum induction was in response to 1 µg/ml LPS. The 6 hour LPS stimulation time (Figure 3.7B) presented similar induction results, highlighting no significant difference between the two time points. The results for the 6 hour stimulation were as follows (Figure 3.7B): maximum induction at 1 µg/ml LPS = 1246 pg/ml ± 49.82; second highest induction at 100 ng/ml LPS = 1002 pg/ml ± 55.60 and lowest induction at 10 ng/ml LPS = 321.60 pg/ml ± 51 (less ± 4 fold then 1 µg/ml LPS and less ± 3 fold then 100 ng/ml LPS).

For subsequent experiments testing the effects of Kp-10 on LPS induced cytokine expression, it was decided to use 10 ng/ml at a 4 hour stimulation time as it proved sufficient in terms of TNF-α induction, since longer stimulation time did not further increase the induction and allowed for a shorter induction time and at a low LPS concentration. Since, pre-treatment/incubation with Kp-10 and/or p356 was required a few hours before LPS stimulation, this allowed maximal pre-incubation time since cell viability with time was a concern.

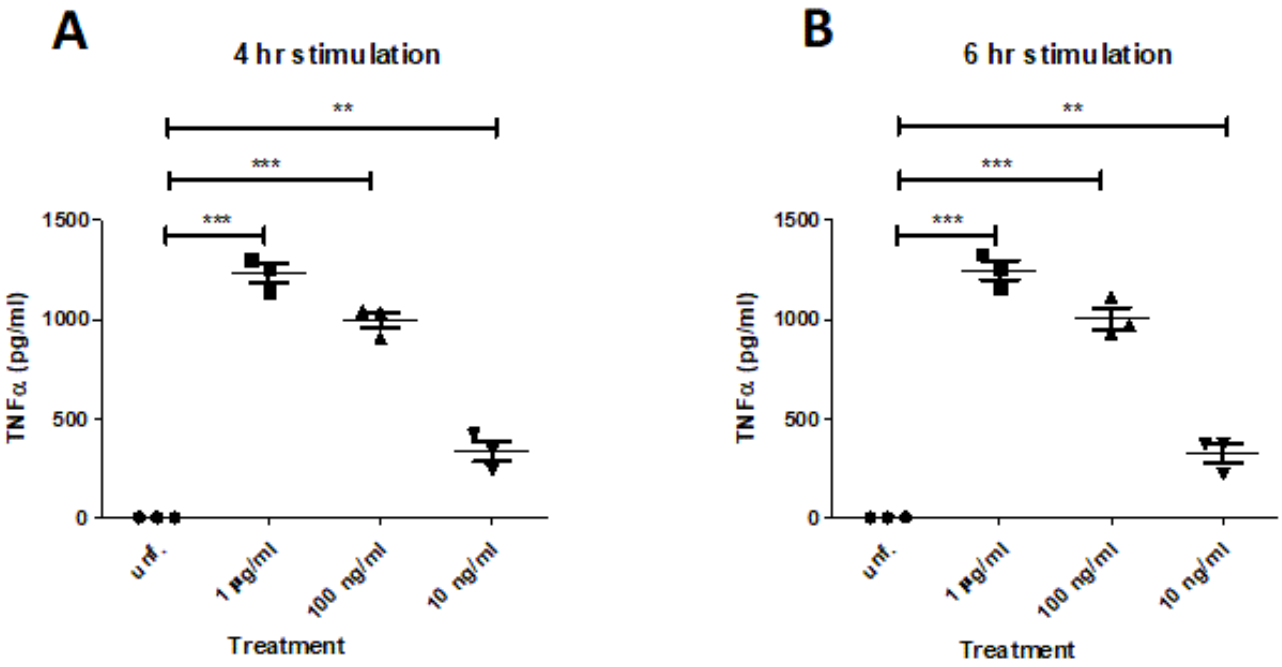

Figure 3.7: Optimisation of stimulation time and concentration for a LPS-induced induction of TNF-α expression in whole blood. Whole blood treated with tirations of LPS (1 µg/ml, 100 ng/ml and 10 ng/ml) for 4 (A) and 6 hours (B). Cell culture supernatants were harvested and subjected to protein expression analysis. Graphs display pooled data, representative of 3 independent samples for each time point (±SEM). One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the difference between treatments. $p < 0.05$ defines statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively. The symbol "ns" depicts no significance.

3.2.1.3. Optimising the time for Kp-10 modulation of LPS-induced cytokine expression

To optimise the Kp-10 pre-incubation time prior to LPS-induction of cytokine expression, whole blood was drawn from 5 healthy and non-pregnant women. Preliminary experiments were performed in order to determine the best time point to harvest cell culture supernatants for protein expression analysis of the selected cytokines.

In order to measure Kp-10 effects on LPS-induced cytokine expression, it was decided to have a pre-incubation time with Kp-10. Therefore, it was required to first optimise the pre-incubation time with Kp-10 prior to the 4 hour stimulation with LPS. Pre-incubation times 4 hours and 24 hours were tested initially to highlight a minimum and possible maximum range since a minimum time of preincubation with Kp-10 is required while a too long incubation may impact on the viability of the cells using a high concentration of Kp-10 (1 µM). Following this, whole blood from healthy non-pregnant women was

pre-incubated with a range of concentrations of Kp-10 (1 nM to 1 µM) treatment for 4 hours and 8 hours.

Following Kp-10 treatment, blood was then treated with 10 ng/ml of LPS for 4 hours. Cell culture Supernatants were collected following LPS stimulation. Figure 3.8 and Figure 3.9 show the protein expression profiles for TNF-α at these time points.

Figure 3.8A shows TNF-α release from blood cells after 4 hours of pre-incubation with Kp-10 and 4 hours of LPS stimulation. The results show that Kp-10 at 1 µM slightly inhibits 10 ng/ml LPS induction (From 588.10 pg/ml ± 37.48 for LPS alone to 491.50 ± 48.83 with Kp-10 at 1 µM). Kp-10 alone with no LPS stimulation had no effect on TNF-α induction (0 pg/ml) and was included as a control (Figure 3.8A). Pre-incubation with Kp-10 for 24 hours followed by a 4 hour LPS stimulation, did not show any induction for any of the treatments (Figure 3.8B).

Since a pre-incubation for 24 hours did not show any LPS-induction it was decided to test an intermediate 8 hour pre-incubation time (Figure 3.9A). Cells were pre-incubated with Kp-10 treatment (Kp-10 titration: 1 µM – 1 nM) for 4 and 8 hours and stimulated with LPS at 10 ng/ml for 4 hours in the presence of fresh treatment. LPS alone induced expression of TNF-α for both pre-incubation times. . The peak in TNF-α protein expression was seen at 4 hours of pre-incubation (Figure 3.9A) whereby for, LPS (alone), induction was ± 2 fold higher (LPS at 10 ng/ml = 620.50 pg/ml ± 22.02) than for the 8 hour pre-inubation time (LPS at 10 ng/ml = 298.30 pg/ml ± 6.012) (Figure 3.9B).

For the 4 hour pre-incubation the results were varying at different concentrations of Kp-10: 1 µM = 628.10 pg/ml ± 14.58 (slight induction); 10^{-7} M = 591.30 pg/ml ± 12.31 (slight inhibition); 10^{-8} M = 645.90 pg/ml ± 52.44 (slight induction) and 1 nM = 591.80 pg/ml ± 76.12 (slight inhibition). Overall no concentration of Kp-10 had any significant effect on LPS-induced TNF-α expression.

For the 8 hour pre-incubation time point, an indication of a dose-dependent Kp-10 inhibitory effect was seen up until Kp-10 at 1 nM and was observed as follows for the various Kp-10 concentrations: 1 µM = 242.60 pg/ml ± 16.46 (slight inhibition); 10^{-7} M = 226.40 pg/ml ± 34.47 (slight inhibition); 10^{-8} M = 220.10 pg/ml ± 16.21 (slight inhibition) and 1 nM = 269.20 pg/ml ± 76.74 (slight inhibition) (Figure 3.9B). As a control, Kp-10 alone at different concentrations did not induce TNF-α expression, therefore Kp-10 as a control was excluded from further investigations as it did not play a role in cytokine induction alone. Kp-10 displayed similar effects for both incubation times however the effect was more pronounced for the 8 hour pre-incubation time (Figure 3.9B). Overall, Kp-10 attenuates the LPS response but a significant dose-dependent effect is not seen.

In summary, Kp-10 had a greater inhibitory effect on LPS-induced expression of TNF-α following 8 hour pre-incubation than compared to 4 hour pre-incubation time. A suggestive (non-significant) dose response was also observed for the 8 hour pre-incubation with Kp-10 time point, whereas for the 4 hour pre-incubation time point, no significant effects were observed.

As we wanted to assess the effects of kisspeptin treatment on LPS-induced expression of multiple cytokines, chemokines and inflammatory mediators, the time point for sampling protein expression levels was chosen to best represent the peaks in expression of the cytokines under investigation in this experiment and from what was seen in literature [143]. In addition, the time point was based on the results of the optimisation as the 8 hour pre-incubation paired with a 4 hour LPS stimulation provided the longest possible pre-incubation with Kp-10 while still ensuring TNF-α (cytokine) induction. Therefore, it was decided that the 8 hour pre-incubation plus the 4 hour LPS stimulation proved optimum for the *in vitro* inflammatory model and this workflow is summarised in Figure 3.10. The concentrations used for the investigation was a Kp-10 titration from 1 µM to 1 pM.

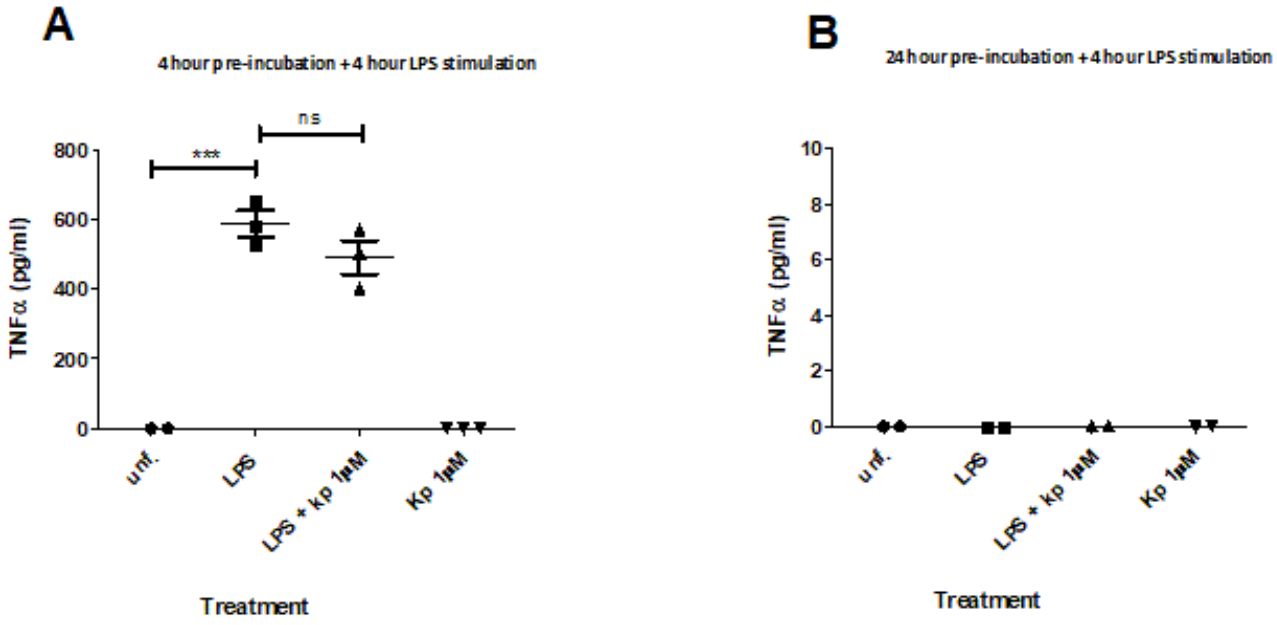

Figure 3.8: Optimisation of stimulation time and concentration for a LPS-induced induction of TNF-α expression in whole blood. Whole Blood was pre-treated for 4 and 24 hours with Kp-10 (1 µM) followed by a treatment with LPS (10 ng/ml) for 4 hours. Stimulation was done in the presence of replenished Kp-10 treatment for respective samples Cell culture Supernatants were harvested and subjected to protein expression analysis via sandwich ELISA to detect TNF-α. Graphs display pooled data, representative of 3 independent samples for each time point (±SEM). One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the difference between treatments. $p < 0.05$ defines statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively. The symbol "ns" depicts no significance.

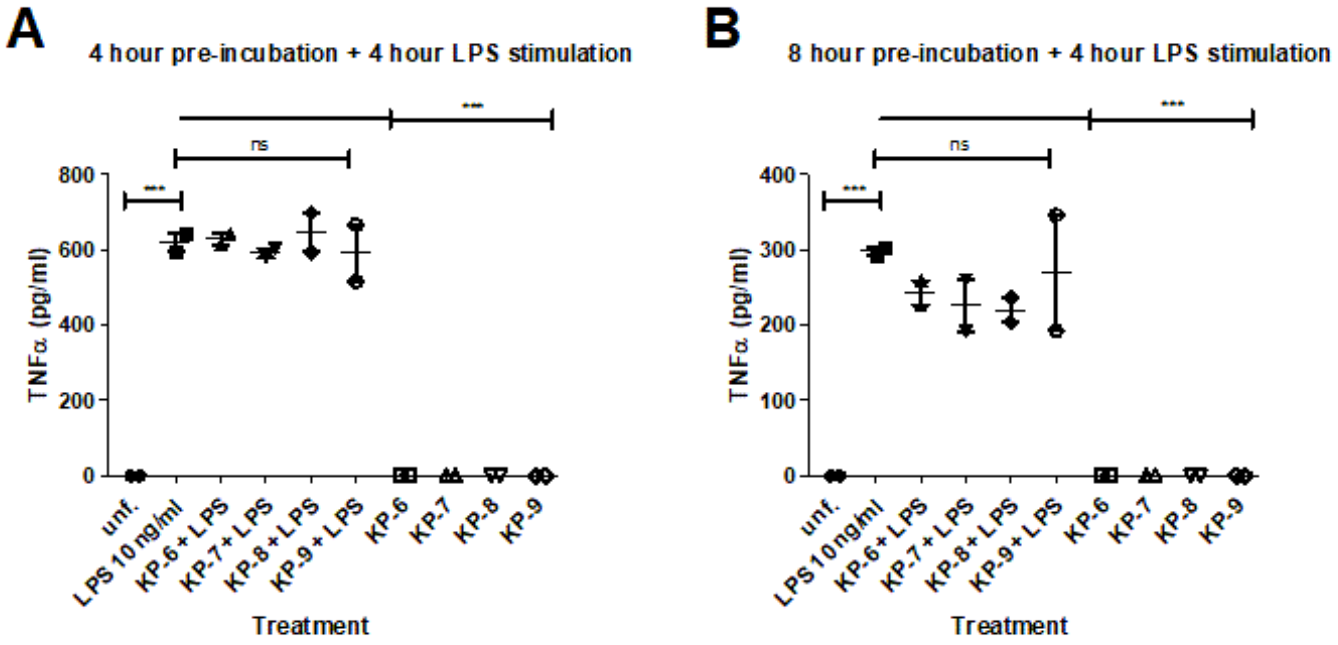

Figure 3.9: Optimisation of stimulation time and concentration for a LPS-induced induction of TNF-α expression in whole blood. Whole blood was pre-treated for 4, 6 (data not shown due to unsuccessful LPS-induction) and 8 hours with Kp-10 (1µM [-6] to 1 nM [-9]) followed by a treated with LPS (10 ng/ml) for 4 hours. Stimulation was done in the presence of replenished Kp-10 treatment for respective samples. Cell culture Supernatants were harvested and subjected to protein expression analysis via sandwich ELISA to detect TNF-α expression. Graphs display pooled data, representative of 2 independent samples for each time point (±SEM). One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the difference between treatments. $p < 0.05$ defines statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively. The symbol "ns" depicts no significance.

3.2.2 Determination of the effect of Kp-10 on IL-1β, IL-10, TNF-α and IL-6 expression in the whole blood of non-pregnant and healthy women using an *in vitro* infection model

The workflow for the established *in vitro* inflammatory model that generated the results seen under this section is summarised in Figure 3.10. Whole blood was taken from 5 healthy and non-pregnant females for this investigation. Only 4 patients had sufficient induction (measurable responses).

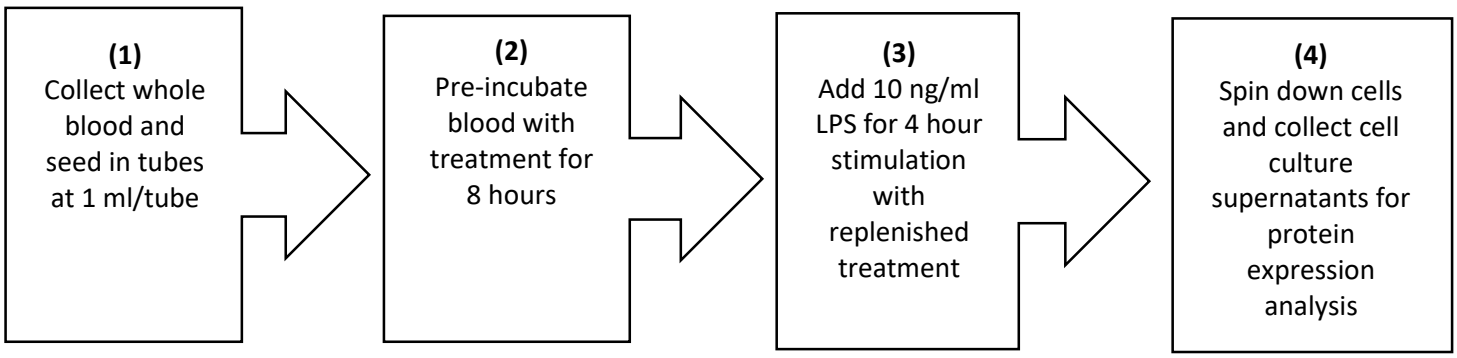

Figure 3.10: Diagram summarising the work flow for the established *in vitro* inflammatory model using whole blood from healthy pregnant women

LPS induced (10 ng/ml) induced TNF-α expression of 233.00 pg/ml ± 68.93 pg/ml (Figure 3.11A). Kp-10 inhibited TNF-α for all concentrations (1 µM – 1 pM) especially at the lower concentrations (10^{-10} M to 1 pM) when compared to the control and were as follows: 10^{-10} M (84.93 pg/ml ± 3.17) (± 3 fold inhibition); 10^{-11} M (87.42 pg/ml ± 0.54) (± 3 fold inhibition) and 1 pM (119.80 pg/ml ± 15.62) (± 2 fold inhibition). The inhibition was varied across the different concentrations with 10^{-10} M, showing the greatest inhibition (Figure 3.11A). The rest of the concentrations for Kp-10 only showed slight inhibition and results for the remaining concentrations were as follows: 1 µM (178.50 pg/ml ± 58.06); 10^{-7} M (220.30 pg/ml ± 78.18) (negligible inhibition); 10^{-8} M (199.00 pg/ml ± 63.76); 1 nM (191.30 pg/ml ± 67.66).

Due to the very little LPS-induction oF IL-1β (10.16 pg/ml ± 5.44) (Figure 3.11B) not much can be observed from the data except that Kp-10 has a negligible effect on IL-1β expression despite the outlier seen at Kp-10 (1 pM) of 58.84 pg/ml ± 21.38.

LPS at 10 ng/ml resulted in a marked induction of IL-6 (420.10 pg/ml ± 15.02). Kp-10 had a small inhibitory effect on LPS-induced expression of IL-6 (23% reduction) (Figure 3.11C) at a concentration 10^{-10} M (323.0 pg/ml ± 54.48), while other Kp-10 concentraations did not affect the level of LPS-induced IL-6 expression.

Reviewing the effect of Kp-10 on IL-10 expression (Figure 3.11D), since, there was no induction by LPS it was not possible to examine the effect of Kp-10 on LPS induction of IL-10 expression. In summary, Kp-10 elicited more of a response at lower concentrations especially 10^{-10} M with the exception of IL-10 whereby there was no induction.

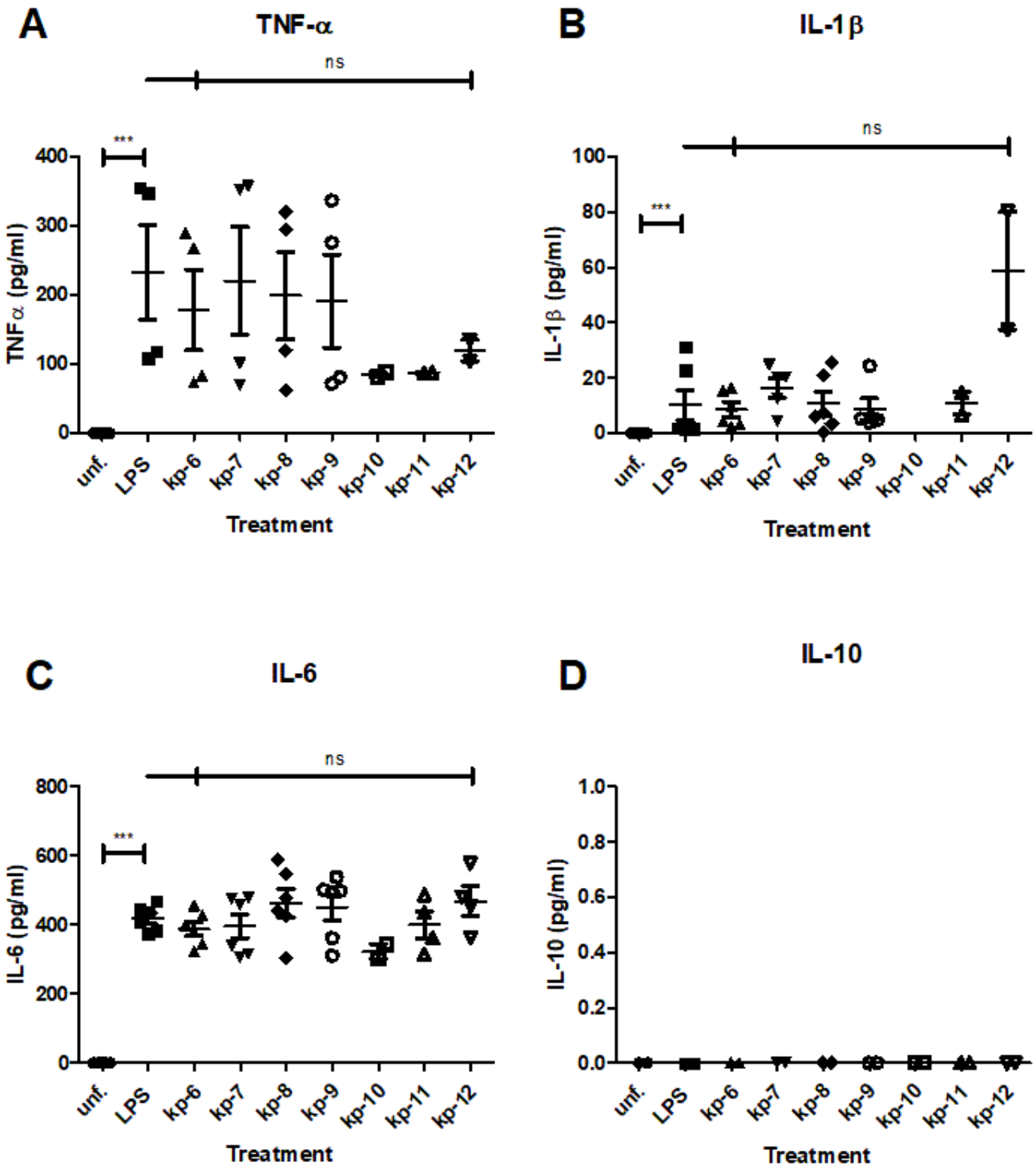

Figure 3.11: Effect of Kp-10 on selected cytokines on protein expression following a LPS-induced inflammatory response. Whole blood was treated with 10 ng/ml of LPS for 4 hours following a 8 hour pre-incubation with treatment (Kp-10 1µM [-6] to 1 pM [-12]). Stimulation was done in the presence of fresh treatment. Cell culture Supernatants were harvested and subjected to protein expression analysis via sandwich ELISA to detect TNF-α expression. Graphs display pooled data, representative of 4 independent samples (±SEM). IL-10 had no response detected. One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the difference between treatments. $p < 0.05$ defines statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively. The symbol "ns" depicts no significance.

3.2.3 Comparison of EDTA and Sodium Heparin (NaHep) blood collection tubes for assay design

Previously, using a 8 hour pre-incubation time did not yield significant data. It was decided that one may achieve a greater response utilising a longer pre-incubation time with Kp-10. Since there was no response/induction at 24 hours for whole blood collected in K3 EDTA tubes (Figure 3.8B), it was decided to use NaHep tubes as an alternative based on the results of previous studies, reporting a better use for cytokine expression analysis [144]. Following the usual model set-up as described in Figure 3.10, observations illustrated that all concentrations of Kp-10 had no effect on TNF-α production (Figure 3.12) although induction at 10 ng/ml was much higher (± 4 fold) at 1007 pg/ml ± 3.13 (Figure 3.12) compared to at 10 ng/ml 233.00 pg/ml ± 68.90 for blood collected in EDTA tubes (Figure 3.11A). The effect of Kp-10 at varying concentrations on TNF-α induction remained consistent throughout (ranged from lowest being Kp-10 at 1 nM = 971 pg/ml ± 13.80 to the highest being Kp-10 at 10^{-7} M = 990.60 ± 7.70) and in general Kp-10 had no effect on TNF-α induction. It was observed that cells did respond after 24 hour pre-incubation (LPS 10 ng/ml = 866.10 pg/ml ± 114.30) (Figure 3.13) unlike previous experiments whereby cells would not respond after 24 hours of pre-incubation and then stimulated (Figure 3.8B).

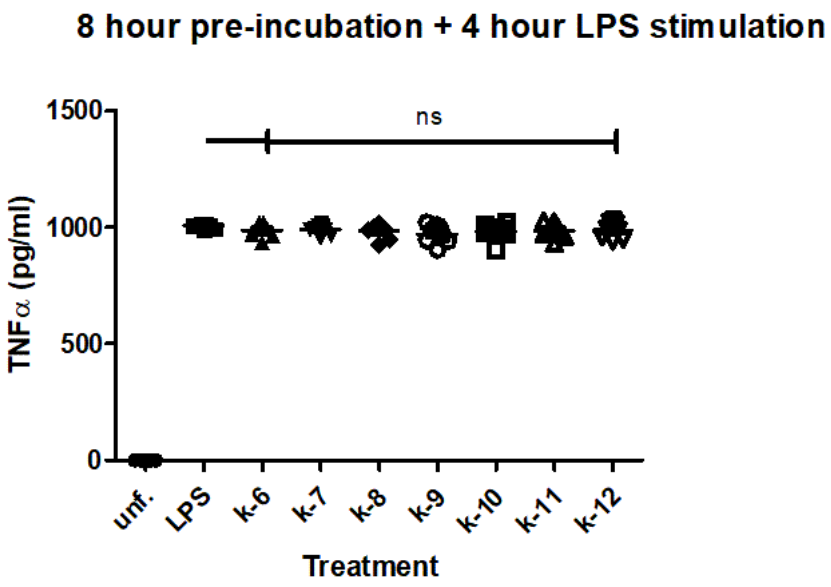

Figure 3.12: Effect of Kp-10 on TNF-α protein expression following a LPS-induced inflammatory response using NaHep blood collection tubes. Whole blood was treated with 10 ng/ml of LPS for 4 hours following a 8 hour pre-incubation with treatment (Kp-10 1µM [-6] to 1 pM [-12]). Stimulation was done in the presence of fresh treatment. Cell culture Supernatants were harvested and subjected to protein expression analysis via sandwich ELISA to detect TNF-α expression. Graphs display pooled data, representative of 4 independent samples for TNF-α (±SEM). One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the difference between treatments. $p < 0.05$ defines statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively. The symbol "ns" depicts no significance.

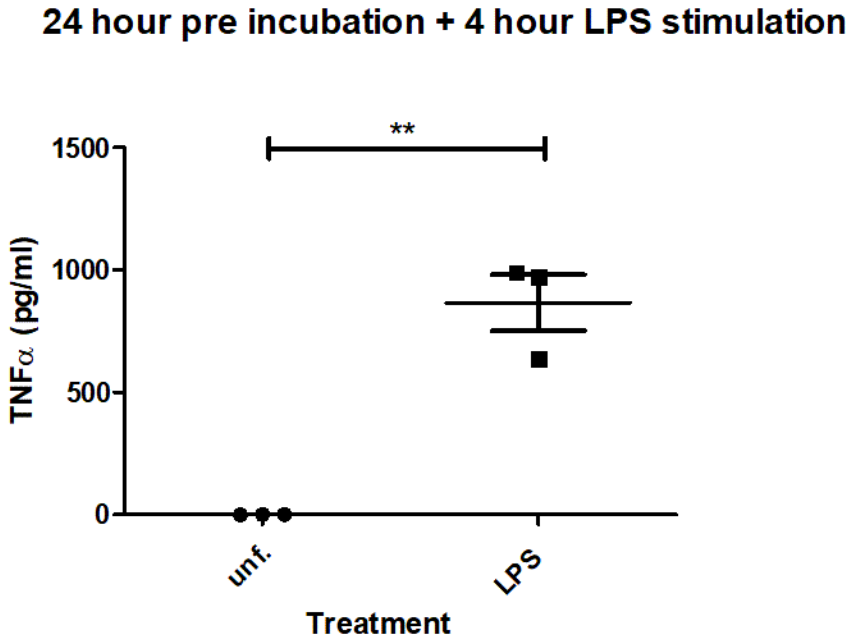

Figure 3.13: Effect of Kp-10 on TNF-α protein expression following a LPS-induced inflammatory response using NaHep blood collection tubes. Whole blood was treated with 10 ng/ml of LPS for 4 hours following 24 hours of incubation without treatment. Cell culture Supernatants were harvested and subjected to protein expression analysis via sandwich ELISA to detect TNF-α expression. Graphs display pooled data, representative of 3 independent samples for TNF-α (±SEM). One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test were performed to compare the difference between treatments. $p < 0.05$ defines statistical significance, where *, **, *** denotes $p < 0.05$; $p < 0.001$ and $p < 0.0001$ respectively. The symbol "ns" depicts no significance.

Due to the lack of Kp-10 induced effects at a pre-incubation time of 8 hours with 4 hours of LPS stimulation using NaHep blood collection tubes, paired with the ability to culture whole blood for a longer time point, it was decided to test the effects of Kp-10 usingr a longer pre-incubation time. We thus treated whole blood for 24 hours, 48 hours and 72 hours (Figure 3.14, Figure 3.15 and Figure 3.16) prior to being stimulated with LPS at 10 ng/ml in the presence of treatment for 4 hours, replenishing the Kp treatment every 24 hours for the 48 and 72 hour time points. It must be noted that only two Kp-10 concentrations (1 μM and 10 -8 M) based on performance in previous experiments) could be tested at 48 hours (Figure 3.15) due to reagent and patient recruitment (sample) limitations

When testing the effect of Kp-10 on LPS -induced IL-6, IL-1β, IL-10 and TNF-α expression (Figure 3.14, Figure 3.15 and Figure 3.16) over 24, 48 and 72 hours, the following effects were seen:

For TNF-α LPS-induced expression at 24 hours (Figure 3.14A), the results remained fairly consistent as to what was seen in Figure 3.12. At 24 hours LPS induction at 10 ng/ml for TNF-α expression was relatively the same as those samples treated with Kp-10 at all concentrations (24 hours = 1006 pg/ml ± 3.36). For IL-6, at 24 hours (Figure 3.14B), LPS at 10 ng/ml induced IL-6 expression (792.0 pg/ml ± 0.90). Only a slight inhibitory effect was seen by Kp-10 and that was at 10^{-7} M (757.50 pg/ml ± 34.24). At 24 hour, in general, no Kp-10-mediated effects were observed.

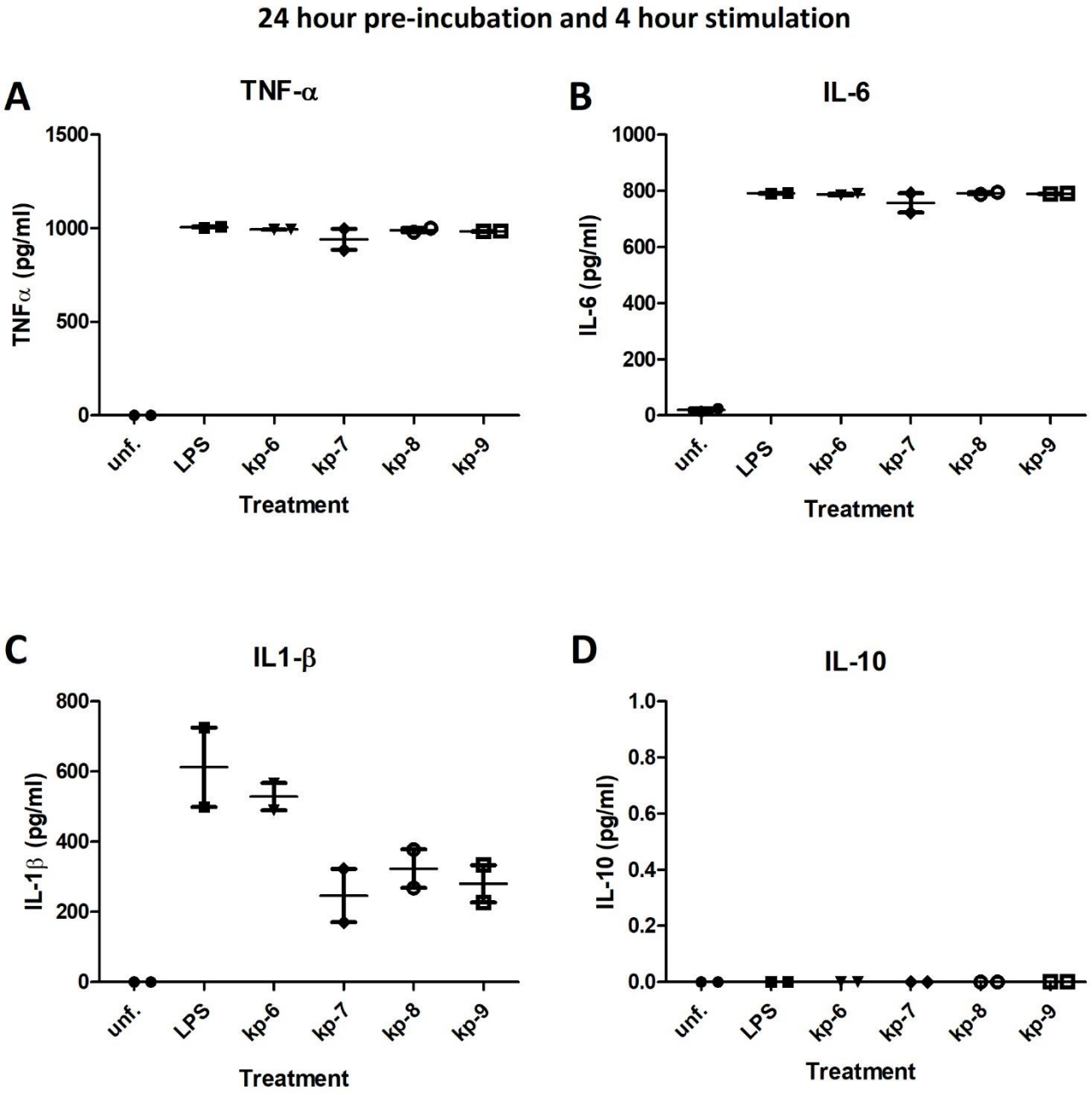

Figure 3.14: Effect of Kp-10 on selected cytokines on protein expression following a LPS-induced inflammatory response using NaHep blood collection tubes. Whole blood was treated with 10 ng/ml and 1 µg/ml of LPS for 4 hours following a 24, 48 and 72 hour pre-incubation with treatment (Kp-10 1µM [-6] to 1 nM [-9] and 1 µM + 10-8 M for 48 hours). Treatments were replenished every day and Stimulation was done in the presence of fresh treatment. Cell culture Supernatants were harvested and subjected to protein expression analysis via sandwich ELISA to detect TNF-α expression. Graphs display pooled data, representative of 2 independent samples for the selected cytokines (± mean/range). Statistical analysis could not be conducted as the viable sample size was too small.

A varied display of results was observed for IL-1β (Figure 3.14C). At 24 hours, LPS at 10 ng/ml induced expression of IL-1β (612.30 pg/ml ± 113.2). This induction was inhibited by Kp-10 at all concentrations with Kp-10 at 10^{-7} M (246.50 pg/ml ± 75.97 (± 3 fold less)) having the greatest inhibitory effect and 1 µM being the least effective at 528.30 pg/ml ± 38.74 (slight inhibition) which proved to be unexpected. The remaining two concentrations inhibited LPS-induced expression in a similar manner to that of Kp-10 at 10^{-7} M (10^{-8} M = 322.90 pg/ml ± 54.49 and 1 nM = 280.80 pg/ml ± 53.04). For IL-10 (Figure 3.14D) no effect from Kp-10 was seen and once again, as previously seen, there was no induction by LPS at 10 ng/ml at 24 hours.

At 48 hours LPS induced TNF-α expression at 10 ng/ml for (1014 pg/ml ± 0.43) (Figure 3.15A). An inhibitory effect of Kp-10 on TNF-α induced expression was only seen at 48 hours for 10^{-8-} M (755.8 pg/ml ± 253.60) (Figure 3.15A). The remaining effects were consistent with that seen for normal LPS-induction at 10 ng/ml. At 48 hours LPS at 10 ng/ml induced a response in IL-6 induction (784.10 pg/ml ± 1.07) (Figure 3.15B). An inhibitory effect was seen for Kp-10 at 10^{-8} M (10^{-8} M = 580.70 pg/ml ± 192.60), however due to the spread in error range, this can be ruled out and declared negligible. At 48 hours for IL-1β (Figure 3.15C), Kp-10 only inhibited LPS induction at a concentration of 10^{-8} M (from LPS 10 ng/ml = 50.60 pg/ml ± 14.05 to 10^{-8} M = 32.23 pg/ml ± 6.34). There was induction by 10 ng/ml LPS for IL-10 at 48 hours of 133.60 pg/ml ± 49.65 (Figure 3.15D). Kp-10 inhibited the effects of LPS-induced IL-10 expression, having a greater effect at a concentration of 10^{-8} M (1 µM = 88.34 pg/ml ± 20.22 and 10^{-8} M = 74.97pg/ml ± 20.70).

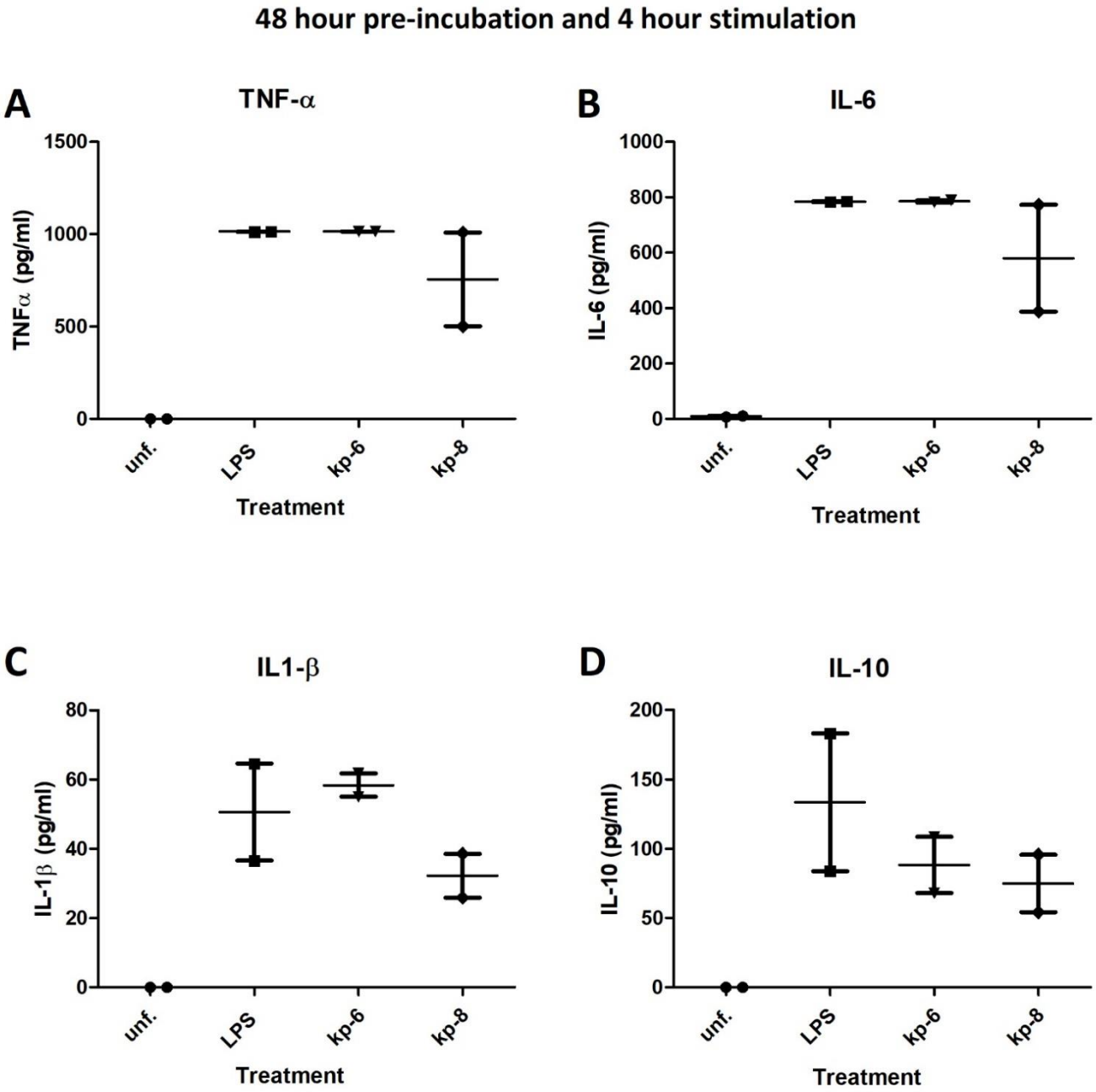

Figure 3.15: Effect of Kp-10 on selected cytokines on protein expression following a LPS-induced inflammatory response using NaHep blood collection tubes. Whole blood was treated with 10 ng/ml and 1 µg/ml of LPS for 4 hours following a 24, 48 and 72 hour pre-incubation with treatment (Kp-10 1µM [-6] to 1 nM [-9] and 1 µM + 10-8 M for 48 hours). Treatments were replenished every day and stimulation was done in the presence of fresh treatment. Cell culture Supernatants were harvested and subjected to protein expression analysis via sandwich ELISA to detect TNF-α expression. Graphs display pooled data, representative of 2 independent samples for the selected cytokines (± meain/range). Statistical analysis could not be conducted as the viable sample size was too small.

For TNF-α at 72 hours there was successful induction for LPS alone which was 999.90 pg/ml ± 10.85 (Figure 3.16A). Kp-10, at all concentrations, had no effect on TNF-α induction at 72 hours. For IL-6 at 72 hours (Figure 3.16B), slight inhibition was seen at Kp-10 = 10^{-7} M (487.50 pg/ml ± 24.34) when compared to LPS alone at 10 ng/ml (612.10 pg/ml ± 160.0). The rest of the Kp-10 concentrations displayed no effect on IL-6 induction at 72 hours. The error range for LPS alone is wide, therefore although we do see inhibition at 10^{-7}M this is not significant. For IL-1β at 72 hours (Figure 3.16C) no effects were seen besides an enhancement of induction at Kp-10 10^{-8} M (from 1.32 pg/ml ± 0.72 for LPS alone to 4.13 pg/ml ± 1.19). In general induction at 72 hours was negligible, so the results cannot be deemed conclusive in any way. For IL-10 at 72 hours there was induction by LPS of 3.99 pg/ml ± 3.10 (Figure 3.16D). Kp-10 enhanced IL-10 expression at all concentrations except for 1 μM Kp-10 which showed inhibition (0.30 pg/ml ± 0.30). Again the greatest effect was shown at 10^{-8} M (13.42pg/ml ± 3.33). The other two concentrations displayed similar enhancement of IL-10 expression: 10^{-7} M = 5.48 pg/ml ± 1.03 and 1 nM = 5.52 pg/ml ± 5.52.

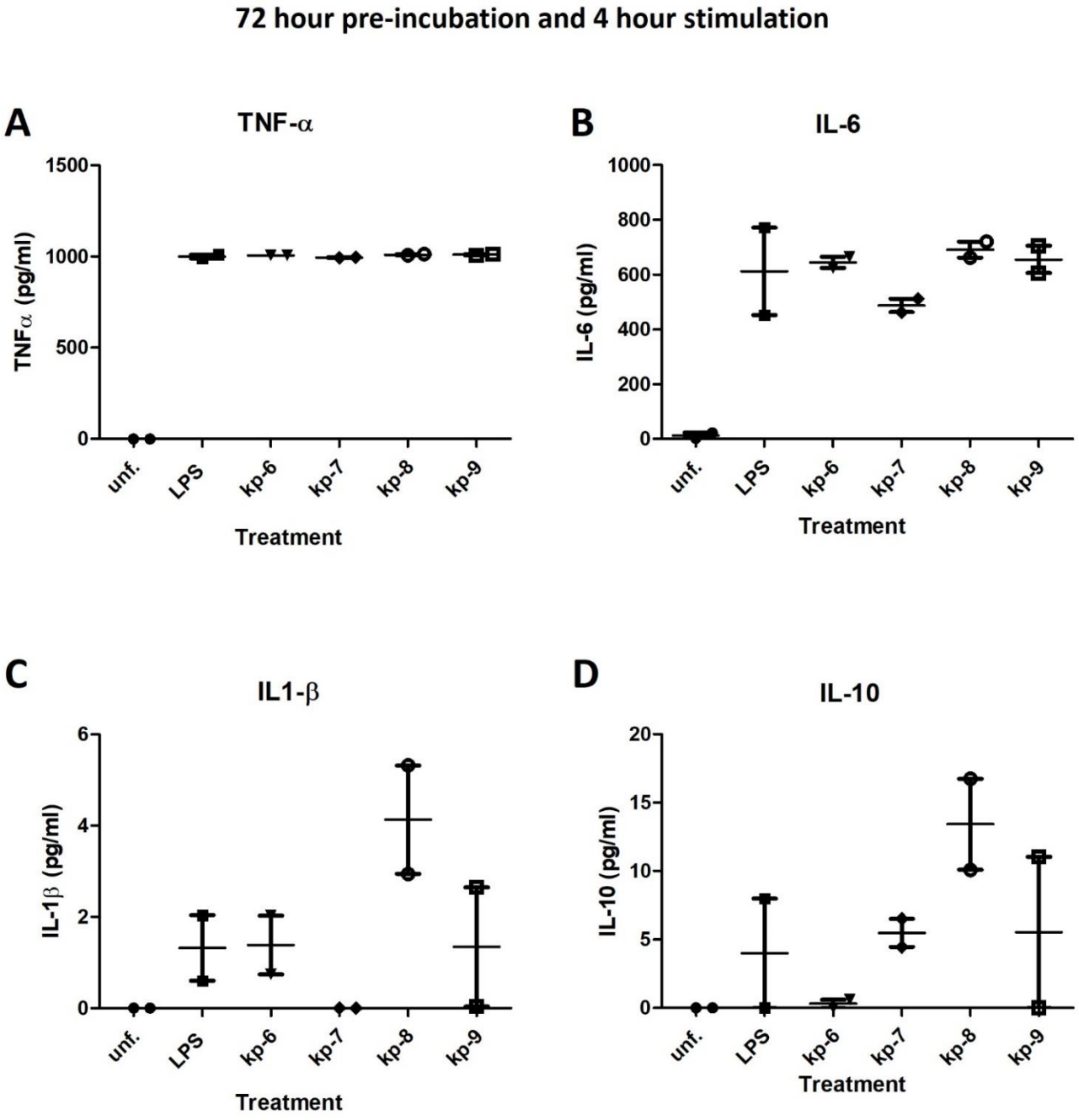

Figure 3.16: Effect of Kp-10 on selected cytokines on protein expression following a LPS-induced inflammatory response using NaHep blood collection tubes. Whole blood was treated with 10 ng/ml and 1 µg/ml of LPS 4 hours following a 24, 48 and 72 hour pre-incubation with treatment (Kp-10 1µM [-6] to 1 nM [-9] and 1 µM + 10-8 M for 48 hours). Treatments were replenished every day and Stimulation was done in the presence of fresh treatment. Cell culture Supernatants were harvested and subjected to protein expression analysis via sandwich ELISA to detect TNF-α expression. Graphs display pooled data, representative of 2 independent samples for the selected cytokines (± meain/range). Statistical analysis could not be conducted as the viable sample size was too small.

Chapter 4 Discussion

Successful pregnancy is dependent on the development of physiological immune tolerance of the maternal immune system towards the genetically foreign fetus. It is known that the mother's body undergoes significant changes during pregnancy whereby hormonal regulation within the periphery and within circulation dictates, with specific reference to immune reactivity and metabolism, these changes [7]. Hormones therefore are important regulators of the functional activity of the immune system and immune cells comprising this system [5,6,7]. At the site of placentation, hormones are secreted by the placenta which, in part, function to protect the fetus from the aggressive immune response of the mother [16]. Previously, kisspeptin has been positively implicated in regulating the functional activity of immune cells [7].

Development of maternal immune tolerance towards the genetically foreign fetus is dependent on the several factors, of which the most important factors include the interactions between a range of cytokines secreted at the site of implantation by maternal and fetal cells in addition to a shift in the ratios of Th1/Th2 cytokine subsets [10]. Gestational hormones and stimulation of trophoblast cells is essential for the development of the decidua from the endometrium. This development is dependent on the communication between the developed decidua and trophoblast cells which is mediated by cytokines and cell surface receptors [10,104]. In addition to placentation, cellular immunity is mediated by specific effector cells as well as the cytokines they produce. It has been shown that the effects of cell-mediated immunity have deleterious consequences on the fetus during pregnancy.

The two functional subsets of Th cells responsible for inducing different effector responses include: Th1 cells which induce several responses including cell-mediated cytotoxic and inflammatory reactions through the release of IFN-γ, TNF-α and IL-2 [8,17,18] and contrasting to this, Th2 cells, which have been associated with full term and successful pregnancy, enhance the production of B cell antibodies through the release of cytokines: IL-4, IL-5, IL-6 and IL-10 [17,18]. Maintaining certain levels of cytokines and chemokines is imperative for the regulation of the immune system during pregnancy. More so, maintenance of an appropriate cytokine balance at the maternal–fetal interface and in circulation is crucial for successful pregnancy.

The effects of kisspeptin have been studied on the cytokines IL-10 and IL-17A as well as aTreg and Th17 cells, which have been shown to be significant role players in the immune response during pregnancy [5,7]. The experiments done for this study were established to investigate the effects of kisspeptin on all cytokines (pro- and anti-inflammatory) which, based on the results, may elucidate certain mechanisms which are implicated in the determination of pregnancy outcome. In this study we tried

to establish the role of kisspeptin in regulating cytokine and chemokine expression, which would provide further elucidation into how the hormone plays a role in facilitating placentation and ultimately full term pregnancy.

4.1 Establishment of the immunomodulatory effects of kisspeptin using an *in vitro* PBMC infection assay

In this study, we used a previously established *in vitro* PBMC infection assay model to determine the immunomodulatory effects of kisspeptin treatment on *Mycobacterium tuberculosis* (Mtb) induced immune response in PBMCs. The experimental work conducted in this thesis is a pilot study, aiming to establish a foundation i.e. an *in vitro* proof-of-concept required for further investigation and experimentation. The PBMC infection model was not adequate to establish the effect of Kp-10 on Mtb-induced cytokine expression.

4.2 Investigating the role of kisspeptin in regulating the induction of cytokines

In this investigation the pro-inflammatory cytokines IFN-γ, IL-17A, IL-1β, IL-1α, IL-6, TNF-α, and GM-CSF , chemokines MCP-1, MIP-1α and MIP-1β, and anti-inflammatory cytokine IL-10 were selected for protein expression analysis. The chemokines and pro-inflammatory cytokines have previously been shown to be upregulated during infection whilst IL-10 can function as either an anti-inflammatory or immunosuppressive cytokine during infection [141,142]. Literature highlights that IL-1β is the most stable (longest half life)cytokine followed by IFN-α, IL-1α, IFN-γ, IL-6, and TNF-α [27,31] which is important to note in the context of this study. A Luminex multiplex assay was conducted in order to assay multiple cytokines at once and gain a broader determination of the immunomodulatory effects of kisspeptin on cytokine expression when stimulated with a pathogen. It must be noted that the data presented was limited in terms of patient number. 5 patients were used in the study investigating Mtb-induced cytokine repsponses in PBMCs, in order to outline the effects of kisspeptin on cytokine expression. The initial study was low-powered with the goal of establishing the hypothesis presented in this study and therefore justifying future investigations and experimentation. Of the 5 patients recruited for this initial investigation, only data for 2 patients was viable, meaning that of the analytes analysed, 2 patients had responses that fell within the observable range of the assay i.e. the standard

curve, deeming the data accurate for quantification. For the remaining 3 patients there was insufficient induction by Mtb and the readings all fell below the observable range therefore the repsonse was not sufficient in order to be measured. Of the 12 analytes investigated using the luminex multiplex assay, only 5 analytes (GM-CSF, TNF-α, MIP-1α, MIP-1β and IL-10) had responses that were within the observable range of the standard curve (Figure 3.1, Figure 3.2 and Figure 3.3). Therefore, data from the initial experiments were suggestive and not reliable based on the number of repeats included in the analysis. Statistics could not be conducted on luminex data based on the fact that only 2 out of the 5 patient samples produced observable/viable data, therefore the data collect is only suggestive of a pattern and more samples would be needed to make more accurate conclusions.

Major findings from the protein expression analysis of this initial investigation include the effect of kisspeptin-10 on pathogen (Mtb) induced pro- and anti- inflammatory cytokine and chemokine expression. In addition, the efficiency of AIM-V medium in conjunction with prolonged Mtb exposure for the culturing PBMCs over a certain duration was investigated.

At the 2 hour time point, cytokine responses were induced and measured for MIP-1α, MIP-1β and TNF-α (Figure 3.1, Figure 3.2 and Figure 3.3). However, in general, Kp-10 at both concentrations (1 μM and 10 nM) had no effect on cytokine expression for TNF-α, MIP-1β and MIP-1α at 2 hours. Protein expression analysis data showed, in general, inhibition of MIP-1α, MIP-1β, TNF-α, IL-10 and GM-CSF by Kp-10 relative to the control (100%) following 24 hours. Following analysis of Figures 3.1, Figure 3.2 and Figure 3.,3, it was expected to see the inhibition of pro-inflammatory cytokines, however the inhibition of IL-10 was not expected, as literature has shown kisspeptin to have the opposite effect on IL-10 i.e. induction of anti-inflammatory immunosuppressive properties and thus induction of IL-10 expression [5,16]. It has been shown that elevated IL-4 and IL-10 and reduced IL-2 and IFN-γ production by peripheral blood mononuclear cells (PBMCs) are associated with successful pregnancy outcomes [37,141]. Unfortunately, based on the fact that an inhibition of IL-10 by Kp-10 was observed as well as the inhibition of pro-inflammatory cytokines, it is difficult to correlate Kp-10-mediated effects with literature which suggests the inhibition of pro-inflammatory cytokines and induction of anti-inflammatory cytokines being associated with successful pregnancy outcomes. However, the inhibition of MIP-1α, MIP-1β and TNFα and GM-CSF by Kp-10 may suggest a potential inhibitory role of Kp-10 of Th1 type/pro-inflammatory cytokines and chemokines whereby the suppression of Th1 type cytokines has been associated with fetal uptake and successful pregnancy outcomes [17]. No accurate statement can be made based on the large inter-patient variability within the results, lack of statistical analysis due to low samples size and in general low sample size. The differences between the findings of this study and others, like that of Gorbunova, et al. and Shirshev, et al., may be

attributed to the same reason, as these other studies had larger patient cohort/sample size and therefore could infer more from their data.

For the kisspeptin antagonist, p356, a suggestive partial agonistic effect was observed when used alone and in combination with Kp-10 (Figure 3.1 , Figure 3.2 and Figure 3.3). This effect has been previously supported by an investigation using the blood of canines in literature [145]. This finding suggests that p356 may act as an agonist depending on the induction/stimulation model used in conjunction with the kisspeptin-induced system measured. The observed unexpected inhibitory effect of p356 (kisspeptin antagonist) on cytokine responses, which was similar to the effect of Kp-10, indicates that p356 in this signalling system has partial agonistic activity. Therefore, in agreement with literature, a synthetic ligand that is an antagonist in one signalling system may be found to be agonistic in a different signalling system [145]. In future, it would be better to use other known kisspeptin antagonists (p234, p271, p354) [145], which may elicit improved responses and further validate that the effects seen are specifically via the kisspeptin receptor GPR54.

Although there was successful induction for certain cytokines and chemokines by Mtb for the 2 hour and 24 hour timepoint, at the day 6 time point no cytokines were measured and the response for all 5 patients was negligible and fell outside of the observable range thus could not be accurately quantified for all analytes. In order to establish why there was no response at the day 6 time point, the viability of the isolated PBMCs over the allotted time points i.e. 2 hours, 24 hours and day 6, was investigated (Figure 3.4). Uninfected cells had a decrease in viability at day 6 based on a reduction in absorbance readings (2 hours (0,251), day 1 (0,442) and day 6 (0,052)).A possible explanation for this could be that the use of AIM-V was not sufficient for culturing PBMCs up to a day 6 time point. In addition, cell viability for infected samples and samples with treatment saw a reduction of greater then 50% in cell viability at day 6 when compared to both 2 hours and 24 hours. A reason for this reduction could be that the MOI was possibly too high and therefore prolonged Mtb infection over 6 days may have caused cell death which would have had an impact on cell viability. A possible suggestion to alleviate this problem would be to either lower the MOI, increase cell seeding number or do an experiment over lesser duration of time.

For future work, making use of Mtb infection at a lower and more appropriate MOI could yield more consistent results. The extreme exposure of PBMCs to Mtb infection could have resulted in cell death therefore leading to an response reduction in cell viability over 6 days. Mtb infection of PBMCs (macrophages included), is representative of acute immune response and not necessarily chronic inflammation which may be a more consistent and representative obstacle to overcome during the early stages of pregnancy. In this study only the acute immune response was tested. Increasing cell

number when seeding could yield cytokine responses sufficient enough to be quantified via Luminex as the amount of cells expression cytokines would be greater and this would lead to a more concentrated cytokine response. It was concluded, from the data, that PBMCs could not be cultured for the duration of a full experiment as less than 50% of total cells were viable by day 6 (Figure 3.4C) when compared to 2 hours and 24 hours.

Further optimisation of the existing *in vitro* infection model needs to be done whereby a titration of kisspeptin could be included along with an MTT analysis as to establish the relative IC_{50} of the hormone and completely characterise the hormone. However, the major setback of this model system would be that the requirements for experimentation proved to arduous and the use of Mtb in the biosafety level 3 was deemed inefficient and uneccessary for what was being tested. It was thus better to move to an alternative model system. Based on the demands and limitations of the assay, it was decided to move on to a more established means of inflammatory stimulation using LPS. LPS is a more controlled means of stimulation based on the ability to use set concentrations which may be deemed more consistent than using Mtb. However, LPS is still only representative of acute inflammation. An improvement and possible extension in order to investigate modulation of a chronic response, especially when this might be better in representing the physiology of pregnancy, would be to conduct stimulation/infection assays using pregnant mice models over a gestational period. In addition, it was intended that through the use of a time course in the stimulation assays, chronic inflammation could be replicated, however this did not prove to be completely successful.

Immune response is the product of multiple events and relies on several avenues of communication between many different cell types (specialised adaptive immune cells, fibroblasts, epithelial cells and specialised innate immune cells) within their environment [88,142,143]. The use of PBMCs, although beneficial for culturing and well established for protein expression analysis, makes use of monoculture taken out of the usual context of the tissue microenvironment. This justified an additional need for an alternative, and that was making use of whole blood. In saying this, although the PBMC infection model was previously established and tested, further optimisations need to be done to ensure that it is robust and that the results obtained are reproducible. In addition, the concentrations of kisspeptin used in this model were not biologically relevant to *in vivo* experiments where the concentrations of kisspeptin ranged from 4.60 pmol/L to 9.60 pmol/L [5,6,16].

In summary, the findings from this investigation were suggestive and non-significant, and kisspeptin may regulate the immune response based on the effects it elicits on pro-inflammatory and anti-inflammatory cytokines.

4.2.1 Kisspeptin showed little to no effect on Mtb uptake/infection

It was important to investigate the effect of Kp-10 on Mtb uptake in order to understand whether cells displayed different cytokine responses based on the severity of Mtb uptake/infection. Overall the results from the CFU analysis (Figure 3.5) showed no significance difference between the treatments and the infected untreated control for both time points i.e. at 2 hours and 24 hours. Therefore, Kp-10 had no effect on Mtb uptake so the state of infection was relatively consistent throughout all samples meaning that this would have no effect on cytokine expression. Due to the large inter-patient variability and low sample size, it is difficult to affirm anything except that Mtb uptake was not effected by Kp-10 and p356 treatment.

4.3 Establishing a *in vitro* whole blood immune response model

For this section of the study, an *in vitro* immune response model was established in order to determine the effects of Kp-10 on LPS-induced cytokine expression using whole blood. It was decided to use a model that does not involve a pathogen-induced means of inducing cytokines nor would it require the use of the P3 faclitity. In addition, the use of PBMCs requires a large quantity of blood from patients thus it was decided to move to the use of whole blood which requires less blood and which would also beneficial for future work when testing pregnant women. Due to ethical limitations, one cannot take a large quantity of blood from patients, especially in the case of pregnant women (future studies), as not to effect a patient's health and well-being. Whole blood, based on previous studies, proves to be more physiologically relevant than PBMCs and therefore would be a better representation of kisspeptin effects in response to inflammation based on the fact that the immune response is the product of multiple events and relies on several avenues of communication between many different cell types (specialised adaptive immune cells, fibroblasts, epithelial cells and specialised innate immune cells) [143,146]. It is thus preferred to make use of whole blood for studying cytokine and chemokine induction by LPS as it allows optimal preservation of the physiological milieu in which cell activation is as a results of the interaction between LPS and plasma molecules [143,146]. In addition, there was a greater response to kisspeptin treatment when using LPS as a stimulating agent as opposed to the previously used Mtb.

Based off preliminary tests using varying concentrations of LPS at 4, 6, 12 and 24 stimulation time, 4 hours of stimulation was concluded to be the optimum time point for LPS stimulation. Following this,

the optimum pre-incubation time point with kisspeptin was tested. Literature suggests that 72 hours is optimum for kisspeptin reactivity using a LPS stimulation assay of monocytes [5], however using K3 Ethylenediaminetetraacetic acid (EDTA) tubes for blood collection did not allow for cell culturing up to 24 hours prior to LPS stimulation. The cytokines present within the supernatant are a product of the initial stimulation as TNF-α is expressed early upon infection [136], however cytokines may also degrade over time which may be a reason as to why there was no response following 24 hours. As expected, the peak in TNF-α protein expression was seen at a shorter pre-incubation time with Kp-10 (4 hours) followed by a 4 hour LPS stimulation. This may be explained by optimum cell viability and/or initial immune response and accumulation of cytokine at this timepoint which can be further validated by the complete lack of TNF-α expression at 24 hour of pre-incubation. To prove that the lack of induction in due to cell death an MTT should have had to been carried out. Unfortunately, this was not done and remains for future work. In addition, a dose response can be conducted to obtain an IC_{50} value for kisspeptin in order to characterise the hormone.

A LPS concentration of 10 ng/ml at a 4 hour stimulation time proved sufficient in terms of TNF-α induction. The LPS concentration of 10 ng/ml was also chosen due to the sensitivity of the ELISA. It was found that the response/induction was concentration dependent and not time dependent as higher concentrations of cytokine expression were detected due to higher concentrations of LPS which lead to the reading falling out of the standard curve range and therefore allowing for inaccurate quantification (Figure 3.6). Therefore, there would be a need for further dilutions of supernatant to allow for accurate cytokine quantification which was not feasible in this present study. The lower concentration of LPS was ultimately chosen as it was expected that kisspeptin would present with only minor immunomodulatory effects. It was also chosen as it was the lowest concentration that would provide measurable results and provide Kp-10 a better chance for inhibition. As kisspeptin showed better inhibition of LPS induction for TNF-α following 8 hour pre-incubation it was decided to proceed with a final assay design of 8 hour pre-incubation with kisspeptin followed by a 4 hour LPS stimulation (Figure 3.9). The pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Th1 subset) were investigated, as well as anti-inflammatory cytokine, IL-10 (Th2 subset). These cytokines were chosen based on their ability to induce and reduce inflammation respectively and based on their role in maternal immune tolerance formation during pregnancy.

Previously it has been stated that the use of EDTA blood collection tubes is for specific use in gene expression analysis whilst NaHep blood collection tubes are optimum for protein expression analysis [146]. For this study we used K3 EDTA blood collection tubes as it was initially planned to compare gene expression to protein expression and in order to be consistent, the same blood collection tube was to be used. However, it was determined that EDTA tubes were not sufficient for culturing whole

blood as cells were unresponsive after 24 hours (Figure 3.7B). It can be suggested that the function of EDTA i.e. acting as a clotting and chelating agent may have an effect on protein expression following induction.

This type of study was a pilot study aimed at establishing an optimised and robust *in vitro* immune response model using whole blood which would provide the basis for extensive research into the immunomodulatory effects of kisspeptin within pregnant and non-pregnant women with and without an history of idiopathic recurrent miscarriage and/or preeclampsia. In addition, this model would allow for larger studies as less blood would be needed from patients to conduct experiments. The model presented was designed to best represent the physiological response to inflammation within the periphery and circulation.

4.3.1 Kisspeptin-10 had varied effects on cytokine production

The results from analysis of protein expression data indicated that LPS-induced expression of TNF-α (Figure 3.10A), IL-1β (Figure 3.10B) and IL-6 (Figure 3.10C) whereas there was no induction by LPS on IL-10 expression (Figure 3.10D). In general, Kp-10 treatment inhibited LPS induction on all cytokines with the exception of IL-10. In saying this the results were highly variable as kisspeptin also enhanced expression at some concentrations. This is with specific reference to IL-6 production whereby expression was slightly enhanced at concentrations of 10^{-8} M, 1 nM and 1 pM. The same can be seen for IL-1β as Kp-10 induced expression at 1 pM and 10^{-7} M. However, for IL-1β, induction was not sufficient, meaning that induction across all conditions was not great enough to achieve a measurable response so these results cannot be deemed reliable.

TNF-α production (Figure 3.10A) was inhibited by kisspeptin at all concentrations, having the strongest inhibition from concentrations 10^{-10} M, 10^{-11} M and 1 pM. It was interesting to see that kisspeptin elicited stronger inhibitory effects at lower concentrations (10^{-10} M) as supposed to the higher concentrations. This could be as a results of the lower concentrations being more physiologically relevant [5,7,16]. An MTT assay would need to be conducted to establish the IC_{50}/EC_{50} value of kisspeptin. This would provide the optimum concentration of kisspeptin to treat cells in order to illicit a response. Literature has indicated the usage of kisspeptin concentrations ranging from 4.6 pm/L to 9.6 pmol/L [16]. In this study it was possible that the concentration of kisspeptin was too high and could have had the opposing effects to what has previously been seen. Positively, it was encouraging to observe kisspeptin's inhibition of TNF-α using both the whole blood model and PBMC model as pro-inflammatory cytokines, like IFN-γ and TNF-α, can either have a direct damaging effect on the placenta and developing fetus or indirect effect by activating cytotoxic cells, NK cells or T cells [26] Previously, TNF-α has been shown to induce apoptosis in human primary villous trophoblast cells whilst IFN-γ

enhances TNF-mediated cytotoxicity of these cells [17,35]. In addition, both the cytokines were shown to inhibit human trophoblast outgrowth *in vitro* [17,36]. As stated before, an immunological shift away from pro-inflammatory responses is necessary for a successful pregnancy and maternal immune tolerance formation.

It was disappointing to observe no induction of IL-10 by LPS and to observe no kisspeptin-mediated effects on IL-10 expression. This would have provided great insight into immunomodulation by kisspeptin based on IL-10 having immunosuppressive capabilities [140]. As literature supports, the importance of IL-10 for pregnancy maintenance is essential to prevent inflammation-induced abortion, which was shown to occur in mice with an IL-10 knockout mutation [51]. It has been shown in mice that elevated concentrations of anti-inflammatory factors, such as IL-10, can prevent spontaneous abortions [39]. An increased production of interleukin-2 (IL-2) and IFN-γ by PBMCs along with a reduction in IL-10 have been associated with spontaneous miscarriage in humans [17,20]. The role of kisspeptin has been shown to enhance the secretion IL-10 by CD4+ T lymphocytes primed with transforming growth factor (TGF)-β1 which contributes to the decrease of IL-17A secretion by CD4+ T lymphocytes primed with IL-1β/IL-6 [16]. However, the results from this study could not validate this role based on the lack of IL-10 induction and Kp-10 mediated effects on IL-10 expression. It must be noted that in one study, it was noticed that there was much variation in results when using a whole blood assay, however, production of IL-10 seems to better reflect an innate pro- or anti-inflammatory response whereas production of TNF-α was more reflective of immediate immunological challenges such as LPS stimulation [143].

IL-6 induces the development of Th17 cells from undifferentiated T cells together with TGF-β and inhibits TGF-β-induced aTreg differentiation promoting inflammation during pregnancy [47]. Varied results were observed regarding kisspeptin mediated effects on IL-6 expression, however it was promising to see inhibition of IL-6 expression at lower concentrations of kisspeptin. The cytokine, IL-6, is multifunctional and plays a significant role in the context of this study i.e. playing a role in immune adaptations which are necessary for the development of immune tolerance. In short, Kp-10 inhibited the expression of IL-1β and IL-6 at certain concentrations. The suggestive nature of the results highlights the need to further optimise and repeat these experiments in future as this would have been a major finding if significant. As Kp-10 slightly inhibited IL-6, TNF-α and IL-1β we may suggest an immunomodulatory role on group 1 and group 2 type cytokines in this context.

In future, it would be of great benefit to relate protein expression to gene expression by comparing protein expression analysis data to data acquired from a reverse transcription polymerase chain reaction (RT-PCR). This would provide a clearer indication of the immunomodulatory effects of

kisspeptin as well as further validation of the reproducibility of the assay. In addition, it would be beneficial to expand the scope in terms of cytokine profiles being investigated to those playing an essential role in maternal immune tolerance for example: IL-17A [47, 48] and TGF-β [47]. One would also need to investigate a full time course as it is known that cytokines and chemokines are expressed differently over a certain time duration when stimulated [136].

Much like the earlier model test, this model does present limitations. Following further testing and optimisation, it may provide the necessary proof-of-concept and foundation required for further study into the immunomodulatory effects of kisspeptin *in vitro* and for an ethics application for *in vivo* studies. These experiments were to be done to establish a working *in vitro* infection model to be used, in future, to further investigate the immunomodulatory properties of Kp-10 using the blood from women suffering from idiopathic recurrent miscarriage (IRM) or preeclampsia outside or within pregnancy. One way to establish the basis for further development of the model would be to identify and quantify kisspeptin receptor levels via immunocytochemistry within whole blood and PBMCs as this could be a factor negatively affecting results due to low expression levels. In addition, one could expand the study and investigate primary immune cells isolated from the placenta under the same conditions to see the effect of Kp-10 on immune stimulation.

4.4 Sodium Heparin (NaHep) blood collection tubes are more suitable for protein expression analysis than K3 EDTA tubes

It has been shown that heparin enhances monocyte production of TNF following LPS-induction, whilst EDTA inhibits the production of LPS-induced cytokines [142,147]. The reason for this is based on the effect of the two anticoagulants on neutrophil-derived protein cationic antimicrobial protein of 18 kDa (CAP18) and heparin-binding-protein (HBP/CAP37) which inhibit and enhance LPS-induced cellular activation, respectively [146]. CAP18's inhibitory effect is increased by EDTA and removed by heparin. In addition, the LPS-induced TNF-α response is calcium dependent and is inhibited by EDTA, confirming the hypothesis that EDTA is unsuitable for measuring TNF-α [144,146]. Therefore, it is important to consider these effects following review of the previous section and further justifies the reason for using NaHep blood collection tubes for testing the hypothesis. The main reason validating the use of NaHep tubes would be the ability to culture whole blood over an extended period of time i.e. greater than 24 hours and still achieve measurable induction following LPS stimulation.

The results from NaHep tubes depicted similar Kp-10 mediated effects to that seen in previous experiments using EDTA tubes. Since viability was low at 24 hours using EDTA tubes and literature

suggesting that 72 hours is optimum for kisspeptin reactivity [5], it was decided to test sodium heparin blood collection tubes under the same conditions. Therefore, we compared the EDTA tubes used under this section to that of the NaHep tubes used for the PBMC infection assay. NaHep tubes have previously been used for protein expression analysis [146]. Kp-10 induced IL-1β production at 24 and 48 hours with notable efficacious concentrations being 10^{-8} M. At 72 hours IL-1β expression was enhanced but these results were negligible based on the fact that IL-1β induction by LPS was too low for all treatments and controls. There was induction for IL-10 following 48 and 72 hours which was expected [136]. Kp-10 enhanced IL-10 production at 72 hours for 10^{-8} M but inhibited expression at 48 hours at the same concentration. This was positive to see and further justified the use of a 72 hour pre-incubation time. For the remaining cytokines the results were negligible in that there were not Kp-10 mediated effects.

4.5 Patient variability is a major contributor to varied results

Patient variability was a major contributor to the negative results seen in this study. Each patient presented, in most cases, a completely different response to the next. One possible factor contributing to this could be that having patients with normal functioning immune responses, much like the patients in this study, may contribute to varied immune response whereas patients with compromised immune states may present with a more uniform or consistent response when stimulated. It is known that using human samples presents a large discrepancy between patients, but with a large cohort, it would be expected to see a trend/pattern for the majority of patients. This remains to be investigated as this was a low-powered study. One would need to increase the study cohort in order for the elucidation of reliable cytokine and chemokine expression profiles as well as the effect of Kp-10 on these profiles. There was a lot less inter-sample variability observed in NaHep tubes when compared to EDTA tubes. This observation could be attributed to the stability of cytokines within NaHep tubes, as previously discussed. This in conjunction with possible inconsistent sample handling could contribute to descrepancies observed.

Conclusion

In this study we employed an *in vitro* PBMC infection assay model as well as an *in vitro* whole blood infection model in order to determine the effects of kisspeptin on Mtb and LPS-induced cytokine and chemokine expression. Results from the PBMC infection assays showed varied yet no effect of

kisspeptin-10 on selected cytokine expression at 2 hours post-infection, however suggested an inhibitory effect of kisspeptin-10 on selected cytokine expression after 24 hours. These effects were not observed 6 days post-infection. Results from the whole blood stimulation assay suggested an inhibitory effect of kisspeptin-10 on LPS-induced pro-inflammatory cytokines whilst generally not having an effect on anti-inflammatory cytokines. Overall we could only suggest an immunomodulatory role of kisspeptin-10 based on the observed inhibition of pro-inflammatory cytokines at the protein level. Again it must be stated that the data was not significant so all claims within this study are only indicative of potential patterns or effects, which remain to be investigated and/or seen.

This study aimed to provide further insight into the role of kisspeptin in the development of maternal immune tolerance, based on its effect on cytokine and chemokine expression. It was a low-powered study aimed at establishing a model system to examine the hypothesis presented in order to justify further investigation and experimentation. Understanding of key regulators and mechanisms of maternal immune tolerance is essential to understanding the pathophysiological mechanisms underlying certain pregnancy-related disorders. This is a pilot study aimed at characterising kisspeptin along with its role in establishing maternal immune tolerance based on its effects on several cytokines and chemokines. Manipulation of regulatory hormones such as kisspeptin could represent a potentially attractive approach in the treatment of various pregnancy-related disorders including preeclampsia and unexplained recurrent miscarriage, however this remains a goal for the future as the effects of kisspeptin and its role in such disorders as well as during pregnancy needs to be fully elucidated and established.

It is important to further investigate kisspeptin mediated regulation of cytokoines and chemokines as it may be possible to develop strategies to regulate the immune response toward fetal antigens via cytokine and chemokine manipulation through kisspeptin administration, which could induce maternal immune tolerance against fetal antigens throughout immune-mediated recurrent and unexplained spontaneous abortion. It is imperative to establish a foundation for further investigations as one cannot get too excited about potential applcations until the underlying mechanisms have been elucidated and therefore better understood. If kisspeptin does indeed play a role in immune tolerance formation during pregnancy, then we can move forward to investigating therapeutic applications.Further studies would need to be done to elucidate the role played by kisspeptin in maternal immune tolerance formation.

Despite the non-conclusive/-significant nature of the results presented in this study, hopefully this study has outlined the potential for further research into the potential immunomodulatory roles of kisspeptin in the development of maternal immune tolerance and its implications for pregnancy.

References:

1. Matjila, M.J. (2014) *The role of kisspeptin and its cognate receptor GPR-54 in normal and abnormal placentation*. PhD thesis, University of Cape Town, Cape Town South Africa.

2. Moodley, J. (2011) Maternal deaths associated with hypertension in South Africa: lessons to learn from the Saving Mothers report, 2005-2007. *Cardiovascular Journal Africa.* **22,** 31-35.

3. Sanders, F. N. (1999) Saving mothers--report on maternal deaths in South Africa. *South African Medical Journal.* **89,** 1245-1246.

4. Moodley, J. (2014-2016) Saving mothers--report on maternal deaths in South Africa short report. *South African Medical Journal.* **89,** 1-90.

5. Gorbunova, O.L., Shirshev, S.V. (2014) The role of kisspeptin in immune tolerance formation during pregnancy. *Doklady Biological Sciences*. **457**, 259-260.

6. Shirshev, S.V. (2009) The immunology of mother-fetus interations. *Yekaterinburg: UrO RAS.*

7. Gorbunova, O.L., Shirshev, S.V. (2016) Molecular mechanisms of the regulation by kisspeptin of the formation and functional activity of Treg and Th17. *Biochemistry (Moscow), Supplement Series A: Membrane and Cell Biology*. **10**, 180-187.

8. Jamieson, D.J., Theiler, R.N., Rasmussen, S.A. (2006) Emerging infections and pregnancy. *Emerging infectious Diseases*. **12**, 1638-1643.

9. Matjila, M.J, Hoffman, A., van der Spuy, Z.M. (2017) Medical conditions associated with recurrent miscarriage – Is BMI the tip of the iceberg? *European Journal of Obstetrics & Gynecology and Reproductive Biology*. **214**,91-96.

10. Saini, V., Arora, S., Yadav, A., Bhattacharjee, J. (2011) Cytokines in recurrent pregnancy loss. 2011. *Clinica Chimica Acta*. **412**, 702-708

11. Quenby, S., Vince, G., Farquharson R., Aplin, J. (2002) Recurrent miscarriage: a defect in nature's quality control? *Human Reproduction.* **17**, 1959–1963.

12. van Mourik, M.S., Macklon, N.S., Heijnen, C.J. (2009) Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. *Journal of Leukocyte Biology*. **85**, 4-19.

13. Babbage, S.J., Arkwright, P.D., Vince, G.S., et al. (2001) Cytokine promoter gene polymorphisms and idiopathic recurrent pregnancy loss. *Journal of Reproductive Immunology*. **51**, 21–27.

14. Liu, Y.S., Wu, L., Tong, X.H., et al. (2011) Study on the relationship between Th17 cells and unexplained recurrent spontaneous abortion. *American Journal of Reproductive Immunology*. **411**, 503-506.

15. Mellor, A.L., Munn, D.H. (2001) Tryptophan catabolism prevents maternal T cells from activating lethal anti-fetal immune responses. *Journal of Reproductive Immunology*. **52**, 5-13.

16. Shirshev, S.V., Gorbunova, O.L., Orlova E.G. (2017) Role of kisspeptin and leptin in the development of immune reactivity during pregnancy. *Human Physiology*. **43**, 705-710.

17. Raghupathy, R., Makhseed, M., Azizieh, F., Omu, A., Gupta, M., Farhat, R. (2000) Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. *Human Reproduction*. **15**, 713-718.

18. Mosmann, T.R., Sad, S. (1996) The expanding universe of T-cell subsets. *Immunology Today*. **17**, 138-146.

19. Erdmann, A.A., Jung, U., Foley, J.E., Toda, Y., Fowler, D.H. (2004) Co-stimulated/Tc2 cells abrogate murine marrow graft rejection. *Biology of Blood and Marrow Transplant*. **10**, 604–613.

20. Burns, W.R., Wang, Y., Tang, P.C.Y., et al. (2005) Recruitment of CXCR3+ and CCR5+ T cells and production of interferon-gamma-inducible chemokines in rejecting human arteries. *American Journal of Transplantation.* **5**, 1226–36.

21. Nickerson, P., Steurer, W., Steiger, J., Zheng, X., Steele, A.W., Strom, T.B. (1994) Cytokines and the Th1/Th2 paradigm in transplantation. *Current Opinions in Immunology*. **6**, 757–764.

22. Strom, T.B., Chaudury, P.R., Manfro, R., et al. (1996) The Th1/Th2 paradigm and allograft response. *Current Opinion in Immunology*. **8**, 688–693.

23. Li, X.C., Zand, M.S., Li, Y., Zheng, X.X., Strom, T.B. (1998) On histocompatibility barriers, Th1 to Th2 immune deviation, and the nature of the allograft responses. Journal of Immunology. **161**, 2241–2247.

24. Dealtry, G.B., O'Farrel, M.K., Fernandez, N. (2000) The Th2 cytokine environment of the placenta. *International Archives of Allergy and Immunology*. **123**, 107–119.

25. Piccinni, M.P., Beloni, L., Livi, C., Maggi, E., Scarselli, G., Romagnani, S. (1998) Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. *Nature Medicine*. 4, 1020–1024.

26. Raghupathy, R. (1997) Th1-type immunity is incompatible with successful pregnancy. *Immunology Today*. **18**, 478–482.

27. Pavlov, O.V., Selutin, A.V., Pavlova, O.M., Selkov, S.A. (2020) Two patterns of cytokine production by placental macrophages. *Placenta*. **91**, 1-10.

28. Hayashi, M., Zhu, K., Sagesaka, T., Fukasawa, I. (2008) Amniotic fluid levels of tumor necrosis factor alpha and soluble tumor necrosis factor receptor 1 before and after the onset of labor in normal pregnancies. *Hormone and Metabolic Research*. **40,** 251-256.

29. Saito, S., Kasahara, T., Ishihara, Y., Ichijo, M. (1993) Elevation of amniotic fluid interleukin 6 (IL-6), IL-8 and granulocyte colony stimulating factor (G-CSF) in term and preterm parturition. *Cytokine.* **5**, 81-88.

30. Grenache, D.G., Hankins, K., Parvin, C.A., Gronowski, A.M. (2004) Cervicovaginal interleukin-6, tumor necrosis factor-alpha, and interleukin-2 receptor as markers of preterm delivery. *Clinical Chemistry*. **50**, (2004) 1839-1842.

31. Steinborn, A., Kuhnert, M., Halberstadt, E. (1996) Immunomodulating cytokines induce term and preterm parturition. *Journal of Perinatology*. **24**, 381-390.

32. van Mourik, M.S.K., Macklon, N.S., Heijnen, C.J. (2009) Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. *Journal of Leukocyte Biology*. **85**, 4–19.

33. Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology*. **136**, 2348–2357.

34. Yui, J., Garcia-Lloret, M., Wegmann, T.G., Guilbert, L.J. (1994) Cytotoxicity of tumor necrosis factor-α (TNF-α) and gamma-interferon (IFN-γ) against primary human placental trophoblasts. *Placenta*. **15**, 819-826.

35. Berkowitz, R., Hill, J.A., Kurtz, C.B., Anderson, D.J. (1988) Effects of products of activated leukocytes (lymphokines and monokines) on the growth of malignant trophoblast cells *in vitro*. *American Journal of Obstetrics and Gynaecology*. **151**, 199-203.

36. Marzi, M., Vigano, A., Trabattoni, D., et al. (1996) Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clinical Experimental Immunology*. **106**, 127-133.

37. Hill, J.A. (1995) Immunologic factors in recurrent spontaneous abortions. In Kurpisz, M. and Fernandez, N. (eds), *Immunology of Human Reproduction.* BIOS, Oxford, 401-424.

38. Chaouat, G., Meliani, A.A., Raghupathy, M.R., et al. (1995) IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. *The Journal of Immunology*. **154**, 4261-4268.

39. Saito, S., Sakai, M., Sasaki, Y., Tanebe, K., Tsuda, H., Michimata, T. (1999) Quantitative analysis of peripheral blood Th0, Th1, Th2 and the Th1:Th2 cell ratio during normal human pregnancy and preeclampsia. *Clinical Experimental Immunology*. **117**, 550–555.

40. Makhseed, M., Raghupathy, R., Azizieh, F., Omu, A., Al-Shamali, E., Ashkanani, L. (2001) Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions. *Human Reproduction*. **16**, 2219–2226.

41. Zhu, X.Y., Zhou, Y.H., Wang, M.Y., Jin, L.P., Yuan, M.M., Li, D.J. (2005) Blockade of CD86 signaling facilitates a Th2 bias at the maternal–fetal interface and expands peripheral CD4CCD25C regulatory T cells to rescue abortion-prone fetuses. *Biology of Reproduction*. **72**, 338–345.

42. Wegmann, T.G., Lin, H., Guilbert, L., Mosmann, T.R. (1993) Bidirectional cytokine interactions in the maternal–fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunology Today*. **14**, 353–356.

43. Bates, M.D., Quenby, S., Takakuwa, K., Johnson, P.M., Vince, G.S. (2002) Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss? *Human Reproduction*. **17**, 2439–2444.

44. Chaouat, G., Lédée-Bataille, N., Zourbas, S., et al. (2003) Cytokines, implantation and early abortion: re-examining the Th1/Th2 paradigm leads to question the single pathway, single therapy concept. *American Journal of Reproductive Immunology*. **50**, 177–186.

45. Babbage, S.J., Arkwright, P.D., Vince, G.S., et al. (2001) Cytokine promoter gene polymorphisms and idiopathic recurrent pregnancy loss. *Journal of Reproductive Immunology*. **51**, 21–27.

46. La Rocca, C., Carbone, F., Longobardi, S., Matarese, M. (2014) The immunology of pregnancy: Regulatory T cells control maternal immune tolerance toward the fetus. *Immunology Letters*. **162**, 41-48.

47. Wang, W.J., Hao, C.F., Yi-Lin Yin, G.J., Bao, S.H., Qiu, L.H., et al. (2010) Increased prevalence of T helper 17 (Th17) cells in peripheral blood and decidua in unexplained recurrent spontaneous abortion patients. *Journal of Reproductive Immunology*. **84**,164–170.

48. Sakaguchi, S. (2005) Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nature Immunology*. **6**, 345-352.

49. Akbar, A.N., Vukmanovic-Stejic, M., Taams, L.S., Macallan, D.C. (2007) The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. *Nature Reviews Immunology*. **7**, 231–237.

50. Robertson, S.A., Care, A.S., Skinner, R.J. (2007) Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice. *Biology of Reproduction*. **76**, 738–748.

51. Saito, S., Nakashima A, Shima T, Ito M. (2010) Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *American Journal of Reproductive Immunology*. **63**, 601–610.

52. Dhama, K., Saminathan, M., Jacob, S.S., Singh, M., et al. (2015) Effect of immunomodulation and immunomodulatory agents on health with some bioactive principles, modes of action and potent biomedical applications. *International Journal of Pharmacology.* **11**, 253-290.

53. Shrestha, S., Sharma, P., Kumar, P., Saxena, S.P., Sharma, R. (2018) Natural killer cells: an insight into its role in pregnancy. *Journal of Clinical and Diagnostic Research*. **12**, BE01-BE07.

54. Xiong, S., Sharkey, A.M., Kennedy, P.R., Gardner, L., Farrell, L.E., Chazara, O., et al. (2013) Maternal uterine NKcell-activating receptor KIR2DS1 enhances placentation. *Journal of Clinical Investigation*. **123**, 4264-4272.

55. Arcuri, F., Cintorino, M., Carducci, A. (2006) Human decidual natural killer cells are a source and target of macrophage migration inhibitory factor. *Reproduction*. **131**, 175-182.

56. Bouman, A., Moes, H., Meineman, M.J., De Leij, L., Faas, M.M. (2001) Cytokine production by NK-lymphocytes in follicular and luteal phase of the ovarian cycle in humans. *American Journal of Reproductive Immunology*. **45**, 130-134.

57. Matjila, M.J., Millar, R.P., van der Spuy, Z., Katz, A.A. (2016) Elevated placental expression at the maternal–fetal interface but diminished maternal circulatory kisspeptin in preeclamptic pregnancies. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health.* **6**, 79-87.

58. Zahumensky, J. (2009) Doppler flowmetry in preeclampsia. *Bratisl Lek Listy*. **110**, 432-435.

59. Davey, D.A., Macgillivray, I. (1986) The classification and definition of the hypertensive disorders of pregnancy: proposals submitted to the international society for the study of hypertension in pregnancy. *Hypertension in Pregnancy*. **b5**, 97–133.

60. Duley, L. (2009) The global impact of pre-eclampsia and eclampsia. *Seminars in Perinatology* **33**, 130–137.

61. Roberts, C.L., Ford, J.B., Algert, C.S., Antonsen, S., Chalmers, J., Cnattingius, S. et al. (2011) Population-based trends in pregnancy hypertension and pre-eclampsia: an international comparative study. *BMJ Open.* **1**, e000101.

62. Martinez-Varea, A., Pellicer, B., Perales-Marin, A., Pellicer, A. (2014) Relationship between Maternal Immunological Response during Pregnancy and Onset of Preeclampsia. *Journal of Immunology Research.* **210241,** 1-68.

63. Huang, S. J., Zenclussen, A. C., Chen, C.-P., et al. (2010) The implication of aberrant GM-CSF expression in decidual cells in the pathogenesis of preeclampsia. *The American Journal of Pathology*. **177,** 2472–2482.

64. Hayashi, M., Hamada, Y., and Ohkura, T. (2004) Elevation of granulocyte-macrophage colony-stimulating factor in the placenta and blood in preeclampsia. *The American Journal of Obstetrics and Gynecology*. **190**, 456–461.

65. Sierra, S., Stephenson, M. (2006) Genetics of recurrent pregnancy loss. Seminars in Reproductive Medicine. **24**, 17–24.

66. Quenby, S., Vince, G., Farquharson, R., Aplin, J. (2002) Recurrent miscarriage: a defect in nature's quality control? *Human Reproduction*. **17**, 1959–1963.

67. Shaarawy, M., Nagui, A.R. (1997) Enhanced expression of cytokines may play a fundamental role in the mechanisms of immunologically mediated recurrent spontaneous abortion. A*cta Obstetricia et Gynecologica Scandinavica*. **76**, 205-211.

68. Vince, G.S., Johnson, P.M. (1996) Is there a Th2 bias in human pregnancy? *Journal of Reproductive Immunology*. **32**, 101-104.

69. Vives, A., Balasch, J., Yague, J., Quinto, L., Ordi, J., Vanrell, J.A. (1999) Type-1 and type-2 cytokines in human decidual tissue and trophoblasts from normal and abnormal pregnancies detected by reverse transcriptase polymerase chain reaction (RT-PCR). *American Journal of Reproductive Immunology*. **42**, 361–368.

70. Lee, J. H., Miele, M. E., Hicks, D. J., Phillips, K. K., Trent, J. M., Weissman, B. E. & Welch, D. R. (1996) KiSS-1, a novel human malignant melanoma metastasis suppressor gene. *Journal of the National Cancer Institute.* **88,** 1731-1737.

71. Jayasena, C.N., Abbara, A., Comninos, A.N., Nijher, G.M.K., et al. (2014) Kisspeptin-54 triggers egg maturation in women undergoing in vitro fertilization. *The Journal of Clinical* Investigation. **124**, 3667–3677.

72. Hu, K., Zhao, H., Chang, H., Yu, Y., Qiao, J. (2018) Kisspeptin/Kisspeptin Receptor System in the Ovary. *Frontiers in Endocrinolog*. **8**, Article 365.

73. Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashil, K., Terao, Y., Kumano, S., Takatsu, Y., et al. (2001) Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature.* **411,** 613-617.

74. Hiden, U., Bilban, M., KnÖfler, M., Desoye, G. (2007) Kisspeptins and the placenta: Regulation of trophoblast invasion. Reviews in *Endocrine and Metabolic Disorders*. **8**, 31-39.

75. Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwinden, J.M., Le Poul, E., et al. (2001) The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *The Journal of Biological Chemistry*. **276**, 34631-34636.

76. Stafford, L.J., Xia, C., Ma, W., Cai, Y., Liu, M. (2002) Identification and characterization of mouse metastasis-suppressor KiSS1 and its G-protein-coupled receptor. *Cancer Research*. **62**, 5399-5404.

77. Muir, A. I., Chamberlain, L., Elshourbagy, N. A., Michalovich, D., Moore, D. J., Calamari, A., Szekeres, P. G., et al. (2001) AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *The Journal of biological chemistry.* **276,** 28969-28975.

78. Tomita, K., Niida, A., Oishi, S., Ohno, H., Cluzeau, J., Navenot, JM., et al. (2006) Structure activity relationship study on small peptidic GPR54 agonists. *Bioorganic Medicinal Chemistry*. **14**, 7595-7603.

79. Nijher, G., Dhillo, W., Chaudhri, O., Murphy, K, Ramachandran R, Fowler A, et al. (2009) Plasma kisspeptin levels in hypertensive diseases of pregnancy and the identification of kisspeptin in amniotic fluid. *Endocrine*. **19**, 300.

80. Muir, A.I., Chamberlain, L., Elshourbagy, N.A., Michalovich, D., Moore, D.J., Calamari, A., et al. (2001) AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *The Journal of Biological Chemistry*. **276:289**, 69-75.

81. Nijher, G., Dhillo, W., Chaudhri, O., Murphy, K., Ramachandran, R., Fowler, A., et al. (2009) Plasma kisspeptin levels in hypertensive diseases of pregnancy and the identification of kisspeptin in amniotic fluid. *Endocrine*. **19**, 300.

82. Dhillo, W.S. (2008) Kisspeptin: A novel regulator of reproductive function. *Journal of Neuroendocrinology*. **20**, 963-970.

83. 84 >> Kuklina, E.M., Shirshev, S.V. (2000) cAMP-dependent signal transduction in control of T-lymphocyte activation. *Biokhimia (Russia)*. **65**, 741-752.

84. Schwartz, J.H. (2001) The many dimensions of cAMP signalling. *PNAS.* **98**, 13482-13484.

85. Bilban, M., Ghaffari-Tabrizi, N., Hintermann, E., Bauer, S., Molzer, S., Zoratti, C., et al. (2004) Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. *Journal of Cell Science*. **117**, 1319–1328.

86. Roa, J., Vigo, E., Castellano, J.M., Navarro, V.M., Fernandez-Fernandez, R., Casanueva, F.F., et al. (2006) Hypothalamic expression of KiSS-1 system and gonadotropin-releasing effects of kisspeptin in different reproductive states of the female Rat. *Endocrinology*. **147**, 2864-2878.

87. Seminara, S.B., Kaiser, U.B. (2005) New gatekeepers of reproduction: GPR54 and its cognate ligand, KiSS-1. *Endocrinology*. **146**, 1686-1688.

88. Irwig, M.S., et al. (2004) Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology*. **80**, 264–272.

89. Navarro, V.M., et al. (2004) Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinising hormone-releasing activity of KiSS-1 peptide. *Endocrinology*. **145**, 4565–4574.

90. Gottsch, M.L., et al. (2004) A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology*. **145**, 4073–4077.

91. Messager, S., et al. (2005) Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proceedings of the National Academy Sciences of the U.S.A*. **102**, 1761–1766.

92. Shahab, M., Mastronardi, C., Seminara, S.B., Crowley, W.F., Ojeda, S.R., Plant, T.M. (2005) Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. *Proceedings of the National Academy Sciences of the U.S.A*. **102**, 2129–2134.

93. Caraty, A., et al. (2007) Kisspeptin synchronizes preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. *Endocrinology*. **148**, 5258–5267.

94. Dhillo, W.S., et al. (2005) Kisspeptin-54 stimulates the hypothalamic-pituitary-gonadal axis in human males. *Journal of Clinical Endocrinology and Metabolism*. **90**, 6609–6615.

95. Dhillo, W.S., et al. (2007) Kisspeptin-54 stimulates gonadotropin release most potently during the preovulatory phase of the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*. **92**, 3958–3966.

96. Chan, Y.M., et al. (2011) Kisspeptin resets the hypothalamic GnRH clock in men *Journal of Clinical Endocrinology and Metabolism*. **96**, 908–915

97. George, J.T., et al. (2011) Kisspeptin is a potent stimulator of LH and increases pulse frequency in men. *Journal of Clinical Endocrinology and Metabolism*. **96**, 1228–1236.

98. Chan, Y.M., Butler, J.P., Sidhoum, V.F., Pinnell, N.E., Seminara, S.B. (2012) Kisspeptin administration to women: a window into endogenous kisspeptin secretion and GnRH responsiveness across the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*. **97**, 1458–1467.

99. Young, J., et al. (2013) Kisspeptin restores pulsatile LH secretion in patients with neurokinin B signalling deficiencies: physiological, pathophysiological and therapeutic implications. *Neuroendocrinology*. **97**, 193–202.

100. Jayasena, C.N., et al. (2009) Subcutaneous injection of kisspeptin-54 acutely stimulates gonadotropin secretion in women with hypothalamic amenorrhea but chronic administration causes tachyphylaxis. *Journal of Clinical Endocrinology and Metabolism*. **94**, 4315–4323.

101. Jayasena, C.N., et al. (2013) Twice-daily subcutaneous injection of kisspeptin-54 does not abolish menstrual cyclicity in healthy female volunteers. *Journal of Clinical Endocrinology and Metabolism*. **98**, 4464–4474.

102. Loon, E. (2015) Kisspeptin signalling and its roles in humans. *Singapore Medical Journal*. **56**, 649-656.

103. Matjila, M.J., Millar, R., van der Spuy, Z., Katz, A. (2013) The differential expression of Kiss1, MMP9 and angiogenic regulators across the feto-maternal interface of healthy human pregnancies: Implications for trophoblast invasion and vessel development. *PLoS ONE*. **8**, 63574.

104. Aparicio, S.A.J.R. (2005) Kisspeptins and GPR54 – The new biology of the mammalian GnRH axis. *Cell Metabolism*. **1**, 293-296.

105. Goodman, R.L., et al. (2007) Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology.* **148**, 5752–5760.

106. Clarkson, J., Herbison, A.E. (2006) Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology*. **147**, 5817–5825.

107. Seminara, S. B., Messager, S., Chatzidaki, E. E., Thresher, R. R., Acierno, J. S., Shagoury, J. K., Bo-Abbas, Y., Kuohung, W., Schwinof, K. M., Hendrick, A. G., Zahn, D., Dixon, J., Kaiser, U. B., Slaugenhaupt, S. A., Gusella, J. F., o'Rahilly, S., Carlton, M. B. L., Crowley, W. F., Aparicio, S. A. J. R., Colledge, W. H. (2003) The GPR54 Gene as a Regulator of Puberty. *New England Journal of Medicine.* **349**, 1614-1627.

108. de Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., Milgrom, E. (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proceedings of the National Academy Sciences of the U.S.A*. **19**, 10972–10976.

109. Topaloglu, A.K., et al. Inactivating KISS1 mutation and hypogonadotropic hypogonadism. (2012) *The New England Journal of Medicine*. **366**,629–635.

110. Teles, M.G., et al. A GPR54-activating mutation in a patient with central precocious puberty. (2008) *The New England Journal of Medicine*. **358**, 709–715.

111. Cetkovic, A., Miljic, D., Ljubic, A., Patterson, M., Ghatei, M., Stamenkovic, J., et al. (2012) Plasma kisspeptin levels in pregnancies with diabetes and hypertensive disease as a potential marker of placental dysfunction and adverse perinatal outcome. *Endocrine Research.* **37**, 78-88.

112. Horikoshi, Y. (2003) Dramatic elevation of plasma metastin concentrations in human pregnancy: metastin as a novel placenta-derived hormone in humans. *The Journal of Clinical Endocrinology and Metabolism*. **88**, 914–919.

113. Gude, N. M., Roberts, C. T., Kalionis, B. and King, R. G. (2004) Growth and function of the normal human placenta. *Thromb Research.* **114,** 397-407.

114. Burton, G. J., Hempstock, J. and Jauniaux, E. (2001) Nutrition of the human fetus during the first trimester-- a review. *Placenta.* **22,** S70-77.

115. Miljic, D., Cetkovic, A., Ljubic, A., Patterson, M., Ghatei, M., Bloom, S., et al. (2010) Kisspeptin levels in high risk pregnancies related to chronic diseases: insulin dependent diabetes mellitus (IDDM) and arterial hypertension (H). *Endocrine Research*. **22**, 464.

116. Park, D.W., Lee, S.K., Hong, S.R., Han, A.R., Kwak-Kim, J., Yang, K.M. (2012) Expression of Kisspeptin and its receptor GPR54 in the first trimester trophoblast of women with recurrent pregnancy loss. *The American Journal of Reproductive Immunology*. **67**, 132-139.

117. Cetkovic, A., Miljic, D., Ljubic, A., Patterson, M., Ghatei, M., Stamenkovic, J., Nikolic-Djurovic, M., Pekic, S., Doknic, M., Glisic, A., Bloom, S., Popovic, V. (2012) Plasma kisspeptin levels in pregnancies with diabetes and hypertensive disease as a potential marker of placental dysfunction and adverse perinatal outcome. *Endocrine Research*. **37**, 78-88.

118. Armstrong, R.A., Reynolds, R.M., Leask, R., et al. (2009) Decreased serum levels of kisspeptin in early pregnancy are associated with intra-uterine growth restriction and pre-eclampsia. *Prenatal diagnosis.* **29**, 982-985.

119. Brosens, I., Robertson, W.B., Dixon, H.G. (1967) The physiological response of the vessels of the placental bed to normal pregnancy. *The Journal of Pathology and Bacteriology.* **93**, 569-579.

120. Robertson, W. B., Brosens, I., Dixon, H. G. (1967) The pathological response of the vessels of the placental bed to hypertensive pregnancy. *The Journal of Pathology and Bacteriology.* **93,** 581-592.

121. Janneau, J. L. (2002) Transcriptional Expression of Genes Involved in Cell Invasion and Migration by Normal and Tumoral Trophoblast Cells. *Journal of Clinical Endocrinology & Metabolism.* **87,** 5336-5339.

122. Ramaesh, T., Logie, J. J., Roseweir, A. K., Millar, R. P., Walker, B. R., Hadoke, P. W., Reynolds, R. M. (2010) Kisspeptin-10 inhibits angiogenesis in human placental vessels ex vivo and endothelial cells in vitro. *Endocrinology*. **151**, 5927-34.

123. Francis, V.A., Abera, A.B., Matjila, M.J., Millar, R.P., Katz, A.A. (2014) Kisspeptin Regulation of Genes Involved in Cell Invasion and Angiogenesis in First Trimester Human Trophoblast Cells. *PLoS ONE*. **9**, e99680.

124. Moffett, A., Colucci, F. (2014) Uterine NK cells: active regulators at the maternal-foetal interface. *Journal of Clinical Investigation*. **124**, 1872-1879.

125. Ashkar, A.A., Santo, J.P., Croy, B.A. (2000) Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *Journal of Experimental Medicine*. **192**, 259-270.

126. Sharma, S. (2014) Natural killer cells and regulatory T cells in early pregnancy loss. *International Journal of Developmental Biology*. **58**, 219-229.

127. Roseweir, A.K., Katz, A.A., Millar, R.P. (2012) Kisspeptin-10 inhibits cell migration *in vitro* via a receptor-GSK3 beta-FAK feedback loop in HTR8SV neo cells. *Placenta.* **33**, 408–415.

128. Iwasa, T., Matsuzaki, T., Tungalagsuvd, A., Munkhzaya, M., Kawami, T., Niki, H., et al. (2014) Hypothalamic kiss1 and RFRP gene expressions are changed by a high dose of lipopolysaccharide in female rats. *Hormones and Behaviour*. **66**, 309-316.

129. Prabhu, V.V., Sakthivel, K.M., Guruvayoorappan, C. (2013) Kisspeptins (KiSS-1): essential players in suppressing tumor metastasis*. Asian Pacific Journal of Cancer Prevention*. **14**, 6215-6220.

130. Park, D.W., Lee, S.K., Hong, S.R., Han, A.R., Kwak-Kim, J., Yang, K.M. (2012) Expression of kisspeptin and its receptor GPR54 in the first trimester trophoblast of women with recurrent pregnancy loss. *The American Journal Reproductive Immunology*. **67**, 132-139.

131. Murugaiyan, G., Mittal, A., Lopez-Diego, R., Maier, L.M., Anderson, D.E., Weiner, H.L. (2009) IL-27 is a key regulator of IL-10 and IL-17 production by human CD4+ T cells. *Journal of Immunology*. **183**, 2435–2443.

132. Schumacher, A., Brachwitz, N., Sohr, S., Engeland, K., Langwisch, S., et al. (2009) Human Chorionic Gonadotropin Attracts Regulatory T Cells into the Fetal-Maternal Interface during Early Human Pregnancy. *The American Journal of Immunology.* **182**, 5488-5497.

133. Gorbunova, O.L., Shirshev, S.V., Zamorina, S. (2013) Kisspeptin effect on monocyte functional activity. *Immunologia (Moscow).* **34**, 247-251.

134. Kumar, H., Kawai, T. & Akira, S. (2011) Pathogen recognition by the innate immune system. *International Reviews Immunology*. **30**, 16–34.

135. Schildberger, A., Rossmanith, E., Eichhorn., T., et al. (2013) Monocytes, Peripheral Blood Mononuclear Cells, and THP-1 Cells Exhibit Different Cytokine Expression Patterns following Stimulation with Lipopolysaccharide. *Mediators of Inflammation*. **697972**

136. Mukherjee, S. et al. (2009) LPS-driven Th2 cytokine production in macrophages is regulated by both MyD88 and TRAM. *Journal of Biological Chemistry*. **284**, 29391–29398.

137. Buettner, R. et al. (2012) Alkylation of cysteine 468 in Stat3 defines a novel site for therapeutic development. *American Chemical Society of Chemical Biology*. **6**, 432–443.

138. Liu, D., Yang, P. S. (2012) Minocycline hydrochloride nanoliposomes inhibit the production of TNF-α in LPS-stimulated macrophages. *International Journal of Nanomedicine*. **7**, 4769–4775.

139. Baniyash, M. (2006) Chronic inflammation, immunosuppression and cancer: New insights and outlook. *Seminars in Cancer Biology*. **16**, 80–88.

140. Robinson, D.P., Klein, S.L. (2012) Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis. *Hormonal Behaviour.* **62**, 263-271.

141. Schultz, G. S., Davidson, J. M., Kirsner, R. S., Bornstein, P., Herman, I. M. (2011) Dynamic reciprocity in the wound microenvironment. *Wound Repair and Regeneration*. **19**, 134–148

142. Deenadayalan, A., Maddineni, P., Raja, A., (2013) Comparison of whole blood and PBMC assays for T-cell functional analysis. *BMC Research Notes*. **6**, 120-125.

143. Brunialti, M.K., Kallas, E.G., Freudenberg, M., Galanos, C., Salomao, R. (2002) Influence of EDTA and heparin on lipopolysaccharide binding and cell activation, evaluated at single-cell level in whole blood. *Cytometry*. **50**, 14–8.

144. Albers-Wolthers, C.H.J., de Gier, J., Walen, M., van Kooten, P.J.S., Lambalk, C.B., Leegwater, P.A.J., et al. (2017) *In vitro* and *in vivo* effects of kisspeptin antagonists p234, p271, p354, and p356 on GPR54 activation. *PLoS ONE*. **12**, 1-16.

145. Banfi, G., Luca Salvagno, G., Lippi, G. (2007) The role of ethylenediamine tetraacetic acid (EDTA) as *in vitro* anticoagulant for diagnostic purposes. *Clinical Chemistry and Laboratory Medicine.* **45**, 565-575.

146. Engstad, C.S., Guttenberg, T.J., Osterud, B. (1997) Modulation of blood cell activation by four commonly used anticoagulants. *Journal of Thrombosis and Haemostasis*. **77**, 690–696.

Appendix A Reagent Recipes

10X Ammonium, Calcium and Potassium (ACK) lysis buffer	82.9 g NH_4Cl; 10 g $KHCO_3$; 0.372 g EDTA – fill up to 1 000 ml with dH_2O, pH 7.2
10X Phosphate Buffer Saline (PBS)	1.37 M NaCl; 14 mM KH_2PO_4; 27 mM KCl; 43 mM Na_2HPO_4
7H9 media	2.4 g Agar; 450 ml dH_2O and 1 ml Glycerol
7H11 media	21 g Agar; 900 ml dH_2O and 5 ml Glycerol
MTT Reagent	5 mg/ml MTT reagent, Cat no. M2128 (Sigma Aldrich) in 1X PBS, pH 7.4
0.05% Sodium dodecyl sulphate (SDS)	Prepare 10% master stock – 50 g SDS in 500 ml dH_2O then make 1:200 dilution by adding 1 ml of 10% SDS to 199 ml of dH_2O
ELISA coating buffer	0.1 M Sodium Carbonate, pH 9.5; 7.13 g $NaHCO_3$; 1.59 g Na_2CO_3; make up to 1 L; pH to 9.5 with 10N NaOH
ELISA wash buffer	1X PBS with 0.05% Tween-20
ELISA assay diluent	1X PBS with 10% fetal bovine serum (FBS), pH 7.0
ELISA stop solution	1 M H_3PO_4 or 2 N H_2SO_4

Appendix B Luminex assay protocol

Summarised version of protocol taken from – **MILLIPLEX® MAP HUMAN CYTOKINE/CHEMOKINE MAGNETIC BEAD PANEL KIT 96 Well Plate Assay Cat. # HCYTOMAG-60K. Instruction Manual**

NOTE: All reagents provided in kit

Allow all reagents to warm to room temperature (20-25°C) before use in the assay.

1. Add 200 µL of Wash Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).

2. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.

3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 pg/mL standard (Background).

4. Add 25 µL of Assay Buffer to the sample wells.

5. Add 25 µL of appropriate matrix solution to the background, standards, and control wells. When assaying serum or plasma, use the Serum Matrix provided in the kit. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.

6. Add 25 µL of serum/plasma Sample (1:100 dilution for RANTES, PDGF-AA, and PDGF-BB, Neat for all other 38 cytokines) or 25 µl cell culture sample into the appropriate wells.

7. Vortex Mixing Bottle and add 25 µL of the Mixed or Premixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)

8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight at 4°C or 2 hours at room temperature (20- 25°C). *An overnight incubation (16-18 hr) may improve assay sensitivity for some analytes.*

9. Gently remove well contents and wash plate 2 times following instructions listed in the PLATE WASHING section (included in manual).

10. Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)

11. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20- 25°C). DO NOT ASPIRATE AFTER INCUBATION.

12. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.

13. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20- 25°C).

14. Gently remove well contents and wash plate 2 times following instructions listed in the PLATE WASHING section.

15. Add 150 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.

16. Run plate on Luminex 200™, HTS, FLEXMAP 3DTM or MAGPIX® with xPONENT software.

17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor.)

All reagent prep and general instructions can be found in manual.