

Exploring the Immune System in High-risk Pregnant Women

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This thesis is dedicated to my parents

Declaration of Originality

I, Gavin Sooranna declare to the best of my knowledge that the work presented in this thesis is original and entirely my own work. All information derived from published or unpublished works of others has been acknowledged and referenced appropriately.

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Abstract

The immune system during pregnancy undergoes substantial changes to reinforce tolerance of the semi-allogenic foetus until term labour, but disruptions in the immunological balance between innate and adaptive populations can lead to adverse pregnancy outcomes. At present, there are no immunological profiles which can be associated with any aetiology of preterm labour making its prediction difficult. Progesterone is currently being used as a preventative treatment for preterm birth and has been shown to be an effective immune-modulator and suppresses inflammation. In myometrial tissue, aminophylline has been shown to enhance the anti-inflammatory effect of progesterone. This project determined cross-Sectional alterations in systemic and local immune cell phenotypes of women presenting with threatened preterm labour who experienced preterm birth in comparison with term birth pregnancy and secondly, the longitudinal changes in high-risk pregnant women receiving a combination of aminophylline and progesterone intended to prevent preterm labour.

Pregnant participants were recruited at Chelsea and Westminster Hospital, maternal blood and cervical mucus were collected at each visit, and leukocytes were extracted for phenotypic and functional analysis using flow cytometry. Functional assays including: LPS-stimulation and intracellular cytokine staining were performed later on frozen cells.

A cross-Sectional comparison in innate and adaptive cell populations demonstrated that systemic non-classical monocyte and cytotoxic natural killer cell frequencies were lower in women who experienced preterm labour, furthermore, CD4⁺ TERMA cell frequency was also lower implying that a dysfunction in innate immunity during pregnancy may suppress an adequate adaptive immune response to upcoming antigen exposure. Longitudinal comparison showed that lower non-classical monocyte frequency and markers of natural killer cell maturity were observed in women treated with aminophylline and progesterone supplementation. Whereas, HLA-DR activation on non-classical monocytes was decreased in women treated with progesterone only. Also, LPS-induced monocytes showed no decrease in IL-8, TNF- α

and IL-10 secretion in either treatment group. This suggests that aminophylline may have an antagonistic effect to progesterone rather than enhance it, as shown by reduced proportions of non-classical monocytes and decreased cytotoxic potential of an NK cell subset.

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List of Abbreviations and Acronyms

In order of appearance:

CD – Cluster of differentiation	iNKT – Invariant natural killer cells
TCR – T Cell Receptor	PD-1 – Programmed cell protein-1
DP – Double-positive	PD-L1 – Programmed cell death ligand-1
DN – Double-negative	Tim-3 – T cell immunoglobulin and mucin-domain containing-3
ISP – Immature single positive	HIV – Human immunodeficiency virus
APC – Antigen presenting cell	PRR – Pathogen recognition receptors
MHC – Major histocompatibility complex	M1/M2 – Macrophage subset 1/2
TFH – T follicular helper cells	CSF-1 – Colony stimulating factor-1
Treg – T regulatory cells	TEM – Trans-endothelial migration
Cm – Central memory	ILC – Innate lymphoid cells
Em – Effector memory	NCR – Natural cytotoxic receptors
TEMRA – Terminally differentiated T cells	WHO – World Health Organisation
Th2 – Type 2 T helper cells	PTB – Preterm birth
IL – Interleukin	PE – Pre-eclampsia
STAT – Signal transducer and activator transcription	PBMC – Peripheral blood mononuclear cells
INF- γ – Interferon gamma	TLR – Toll-like receptor
GM-CSF – Granulocyte colony stimulating factor	PTL – Preterm labour
TGF- β – Transforming growth factor-beta	LPS – Lipopolysaccharide
ROR γ t – Related orphan receptor gamma	PPROM – Premature rupture of membranes
FoxP3 – Forkhead box P3	sPTB – Spontaneous preterm birth
CTL – Cytotoxic lymphocyte	P4 – Progesterone
Th1 – Type 1 T helper cells	dNK – Decidual natural killer cells
TNF – Tumour necrosis factor	PR – Progesterone receptor
NK – Natural killer cells	pNK – Peripheral natural killer cells
T-bet – T-box transcription factor	AP – Aminophylline
cAMP – Cyclic adenosine triphosphate	CST – Cytometer Setup and Tracking
G – Revolutions per minute	ASF – Area scaling factor
Ca ²⁺ - Calcium 2+ oxidation state	Ig κ – Immunoglobulin-kappa
Mg ²⁺ - Magnesium 2+ oxidation state	V – Volts
PBS – Phosphate buffered saline	MFI – Median fluorescence intensity
PMNC – Polymorphonuclear cells	PMT – Photomultiplier tube
L/D – Live/dead	PROGRAM – Progesterone and aminophylline for the prevention of preterm labour
FACS – Fluorescent-activated cell sorting	FMO – Fluorescence minus one
FCS – Fetal calf serum	TPTL – Threatened preterm labour
sup - Supplementation	PTD – Preterm delivery
CO ₂ – Carbon dioxide	fFN – Fetal fibronectin
DMSO – Dimethyl sulphoxide	

sPTL – Spontaneous preterm labour
 DC – Dendritic cells
 HLA-DR – Human leukocyte antigen
 NKT – Natural killer T cell
 NF κ B – Nuclear factor κ B
 MMP – Metalloproteinase
 VEGF – Vascular endothelial growth factor
 PIBF – Progesterone induced blocking factor
 MAS – Maternal assessment suite
 EDTA - Ethylenediaminetetraacetic acid
 TB – Term birth
 FSC – Forward scatter
 SSC – Side scatter
 mPR – Membrane progesterone receptor
 ZAP70 – Zeta chain associated protein kinase
 70
 GR – Glucocorticoid receptor
 LIF – Leukemia inhibiting factor
 M-CSF – Macrophage colony stimulating
 factor
 MCP-1 – Monocyte chemoattractant protein-
 1
 RDS – Respiratory distress syndrome
 IRD – Idiopathic respiratory distress
 PDE – Phosphodiesterase
 cGMP – Cyclic guanine monophosphate
 COPD – Chronic obstructive pulmonary
 disorder
 HDAC2 - histone deacetylase activation
 PI3K δ - Phosphoinositide-3-kinase- δ

HATs - histone acetyltransferase enzyme
 CREB - cAMP response element binding
 proteins
 PHA – phytohemagglutinin
 Bcl-2 – B-cell lymphoma-2
 AC – Antenatal clinic
 PAMP – Pathogen associated molecular
 pattern
 PRR – Pattern recognition receptor
 MD-2 – Myeloid differentiation-2
 TIR – Toll-like IL-1 receptor
 TNFR – Tumor necrosis factor receptor
 VD – Vaginal delivery
 ESC – Elective caesarean Section
 ELISA – Enzyme linked immunosorbent assay
 mRNA – Messenger ribonucleic acid
 MIP – Macrophage inflammatory protein
 IC₅₀ – Inhibition concentration at 50%
 HP – Healthy pregnant
 NP – Non-pregnant
 hsCRP – High-sensitive C-reactive protein
 NOS2 – Nitric oxide synthase-2
 IL-2R – Interleukin-2 receptor
 Epac1 – Exchange protein directly activated by
 cAMP-1
 PKA – Protein kinase A
 FAP – Fatty acid profile study
 CVF – Cervical vaginal fluid
 HPV – Human papillomavirus

Abstracts Submitted and Presented

1. **Sooranna G, Shah N, Singh N, Johnson M.** 'The Cervical Leukocyte Composition Throughout Pregnancy'. Royal College of Obstetricians and Gynaecologists (RCOG) 2019, London, UK. – Poster
2. **Sooranna G, Shah N, Singh N, Johnson M.** 'The Immune Modulatory Effects of both Progesterone and a Combination of Progesterone and Aminophylline on the Maternal Immune System'. Society for Reproductive Investigation (SRI) 2019, Paris, France. *REPRODUCTIVE SCIENCES*, Vol:26, Page 293A, ISSN: 1933-7191 – Poster
3. **Sooranna G, Shah N, Cocker A, Singh N, Johnson M.** 'Progesterone Suppresses the Inflammatory State of Innate Cells in Peripheral Blood and Cervical Mucus, European Congress for Immunology (ECI) 2018, Amsterdam, Netherlands - Poster

1. Introduction

The maternal immune system during pregnancy is highly complex and still to this day is not fully understood. Not only is it required to allow the acceptance, growth and tolerance of allogenic cells, but must also respond to pathogenic stimuli in order to protect its host from developing severe illnesses [1]. High-risk pregnancy is associated with alterations in the function and phenotype of these cells which cause problems for both the mother and the child. This chapter will introduce cell types from both the innate and acquired immune system and their role during term and preterm pregnancy.

1.1 Components of the Immune System

The mammalian immune system is composed of two subsets: Innate immunity are the first line of defence against foreign bodies, whereas, the acquired immunity develops memory and adaptation responses towards infection allowing a more effective counter attack if reinfection were to occur [2].

1.1.1 Acquired Immune System

1.1.2 T Cell Development

The generation of functional T cells occurs when lymphoid progenitor cells produced in the bone marrow which lack expression of CD4 and CD8 ($CD4^- CD8^-$) migrate to the thymus gland to undergo T cell receptor (TCR) rearrangement into thymocytes with double-positive (DP) $CD4^+$ and $CD8^+$ expressions [3]. The double negative (DN) $CD4^- CD8^-$ thymocytes undergo several developmental stages (Figure 1.1.1.1) with varying expressions of CD44 and CD25 before becoming DP, then the progression from DP into an immature single positive cell (ISP) occurs before subsequently maturing into

either a naive single positive CD4 or CD8 T cell which then migrates into the periphery [4].

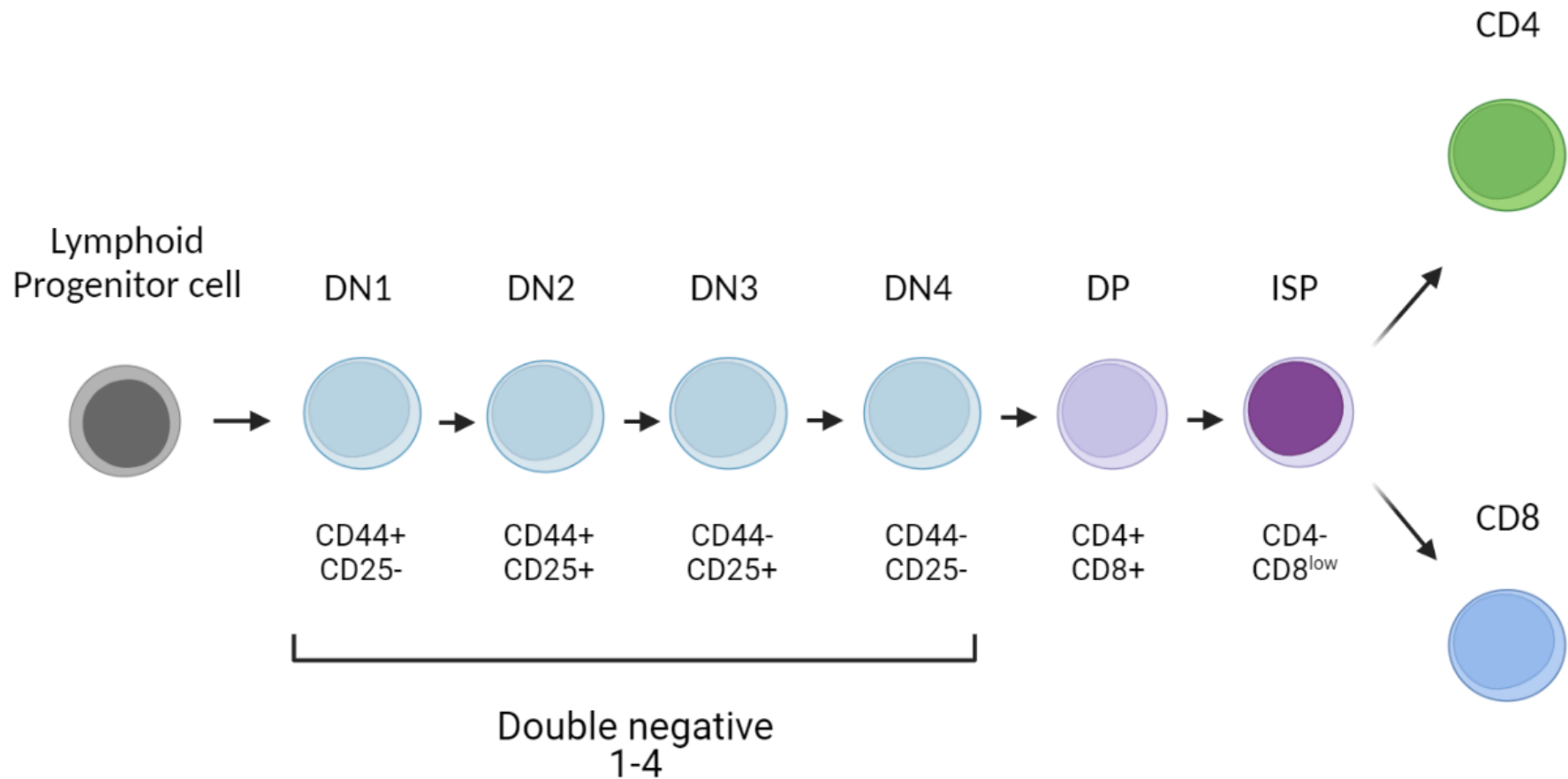


Figure 1.1.1.0 T Cell Development Pathway

The development of CD4 and CD8 T cells from a lymphoid progenitor cell. Each double negative (DN) thymocyte is characterised by the varying surface expression of CD44 and CD25 before differentiating into a double positive (DP) and then an immature single positive thymocyte (ISP). The ISP then matures into a naive single positive CD4 or CD8 T cell [5]. – Adapted from Crompton et al, 2011, Biochemical Pharmacology.

1.1.3 CD4⁺ T Cells

CD4⁺ T cells are key mediators of pathogen recognition and elimination [6]. Their function ranges from recruiting other immune cell types from the innate immune system, suppression of immune responses and B cell and cytotoxic T cell activation [7]. Recognition from pathogen associated molecules presented to them by antigen presenting cells (APC) causes naïve T cells to mature into functional T cells [8]. Activation of naïve CD4⁺ T cells (Figure 1.1.1.1) occurs upon interaction of TCR/CD3 receptor to the major histocompatibility complex (MHC) class II [8]. In addition, co-stimulation between CD28 present on the T cell with its co-responding ligand CD80/CD86 present on the APC surface also occurs and is necessary for T cell activation [8]. T cell receptor CD40L and CD40 on the APC interacts to provide a co-stimulatory signal. Unsuccessful co-stimulation causes impairment of effector cell differentiation [9, 10]. Once activated, the T cell will differentiate into a: Th1, Th2, Th9, Th17, Th22, T follicular helper cells (T_{FH}) or T regulatory (Treg) cell type [11], depending on the influence of specific cytokines and transcription factors [11, 12]. These functional effector cells are categorised based on their cytokine profiles, however, they have plasticity and can convert into other effector subtypes depending on the immune response [13].

1.1.4 Memory T Cells

Antigenic stimulation of naïve T cells from an APC initiates the development of memory T cells. This subset of memory T cells include: central memory (C_m), effector memory (E_m) and terminally differentiated T cells (TEMRA) [8, 14]. The expansion of these memory T cell subsets are based on the surface expression phenotype of CD45RO, CD45RA and CCR7 [15]. These memory cells establish effector responses upon

reinfection with a recall antigen [16] and clonal expansion of CD4⁺ and CD8⁺ effector T cells occurs post antigen presentation as a response to pathogenic stimuli [17, 18]. Additionally, there is literature to suggest that long-lived memory T cells linger in the body after an initial response from antigenic stimulation. These cells remain quiescent but have the capability of self-renewal with the lack of an MHC class II ligand [19].

In addition to the above, CD4⁺ T cells are also described by their expression of transcription factors and cytokine profile into a different effector cell subset called T helper cells: Th1, Th2 Th17 and Treg cells [11]. T helper cells (Th2) function as anti-inflammatory cytokine producers and are mediators of humoral immunity enabling the production of B cell antibodies [20, 21]. They secrete a wide range of cytokines; however, during a Th2 immune response, interleukin-4 (IL-4), IL-5 and IL-13 are the critical proteins responsible for protection against extracellular pathogens [22, 23]. The differentiation pathway of a Th2 cell is dependent upon GATA3 up-regulation which is mediated by IL-4 induced signal transducer and activator of transcription-6 (STAT6) activation [24, 25]. Moreover, IL-2 induced STAT5 activation is also important for cytokine production in Th2 cells [26].

1.1.5 Th17 Cells

Th17 cells have an established role in host defence against extracellular pathogens and are involved in mucosal immunity, especially against fungi [27-32]. IL-17 secretion triggers the production of chemokines and cytokines such as IL-6, CXCL8, CXCL9, CXCL10 and CXCL11 [33]. However, Th17 cells can also produce: IL-21, IL-22, interferon gamma (INF- γ) and granulocyte macrophage colony stimulating factor (GM-CSF) [31, 33]. IL-21 and IL-22 offer mucosal protection by stimulating B cell proliferation [33]. Research into Th17 cells have also demonstrated their plasticity,

which sees them becoming Th1 or IL-10 producing T cells if required [30, 33]. Other research has shown that these cells can lay dormant in tissue mucosa for extensive time periods and that a deficiency of Th17 cells can lead to fungal autoimmune diseases such as *muco cutaneous candidiasis* [32]. The signalling cascade of IL-6 in combination with transforming growth factor- β (TGF β) act via STAT3 to stimulate transcription factor related orphan receptor gamma (ROR γ t), which is the main regulator for Th17 differentiation (Figure 1.1.1.1) [27, 28, 34]. However if only the upregulation of TGF- β is promoted without IL-6 then production of Th17 cells is suppressed and Treg production is promoted [22, 35].

1.1.6 T regulatory Cells

Tregs represent approximately 5-10% of human CD4⁺ T cells or 1-2% of the total lymphocyte population [36]. Treg function is to suppress the immune response of other effector T cells, by direct interaction with these cells to inhibit their activation and, therefore, the synthesis and secretion of pro-inflammatory cytokines such as INF- γ and IL-2, and cross-talk with APC to promote immune tolerance [37, 38]. Stimulated Tregs produce: IL-4, IL-10, TGF β and IL-35 which are anti-inflammatory cytokines [37, 39]. Tregs express high levels of CD25, and are characterised by the transcription factor Forkhead Box p3 (FoxP3) (Figure 1.1.1.1). Several studies have discussed the role Tregs play in the induction of tolerance to fetal antigens [40]. Tregs release IL-10 to inactivate or reduce pro-inflammatory responses [41]. Treg functionality is viewed as being able to counter balance inflammatory responses and to prevent excessive immune responses as seen in sepsis where the excessive inflammation causes damage to tissue [42, 43]. Effector cell function is controlled by the immunosuppressive function Tregs [44].

1.1.7 CD8⁺ T Cells

CD8⁺ cytotoxic lymphocytes (CTL; Th1) are key mediators for the elimination of intracellular infections caused by micro-organisms and viruses [45]. Additionally, they develop a cell-mediated response to type IV hypersensitivity and have also been shown to be involved in tissue rejection [22, 23, 46]. They are producers of INF- γ and tumour necrosis factor (TNF) [47] and their function is to enhance pro-inflammatory cell-mediated immunity [20]. After initial stimulation from an APC the differentiation pathway of a naïve T cell into a CTL is under the influence of IL-12 and INF- γ secreted by natural killer (NK) cells [23]. The primary transcription factor required for Th1 differentiation is T-box transcription factor (T-bet) (Figure 1.1.1.1) [48], which enhances Th1 differentiation as well as suppressing the cell's ability to differentiate to other effector cell types.

1.1.8 Invariant NKT Cell

Invariant Natural killer T cells (iNKT) express a unique T cell receptor containing a V α 24 chain and a V β 11 chain in humans [49]. These cells demonstrate both memory and rapid response characteristics and therefore are considered to be a 'bridge' between the innate and adaptive immune system [50]. However, they do not share antigen recognition through MHC like conventional T cells; instead they recognise glycolipid ligands presented by the MHC class I-like molecule CD1d present on APC [51]. Interestingly upon TCR activation, iNKT cells have been shown to activate T cells, B cells and NK cells, with the addition of being able to secrete both IL-4 and INF- γ in significant amounts [52, 53].

1.1.9 Inhibitory Markers

Increased expression in markers of exhaustion and senescence can diminish the function of immune cell types [54]. In fact, malignant tumours have been shown to induce exhaustion and senescence in CD4⁺ and CD8⁺ T cells in cancer patients to disrupt antitumor immunity [55]. Programmed Cell Death Protein-1 (PD-1) has a key function in modulating T cell activity by inducing apoptosis in antigen-specific T cells whilst inhibiting Treg apoptosis [56]. Programmed Cell Death Ligand-1 (PD-L1) when bound to PD-1 decreases cytokine production of pro-inflammatory producing immune cells [56]. Furthermore, the PD-L1/PD-1 pathway is critical for immune homeostasis by promoting Treg development and inhibiting effector T cell responses [57, 58]. T cell immunoglobulin and mucin-domain containing-3 (Tim-3) is a marker of exhaustion in T cells and increased expression of Tim-3 has been shown to suppress the production of TNF- α and INF- γ in these cells [59]. In autoimmune diseases such as human immunodeficiency virus (HIV), Tim-3 and PD-1 expression is increased on CD4⁺ T cells which impairs their function and their ability to proliferate [60, 61].

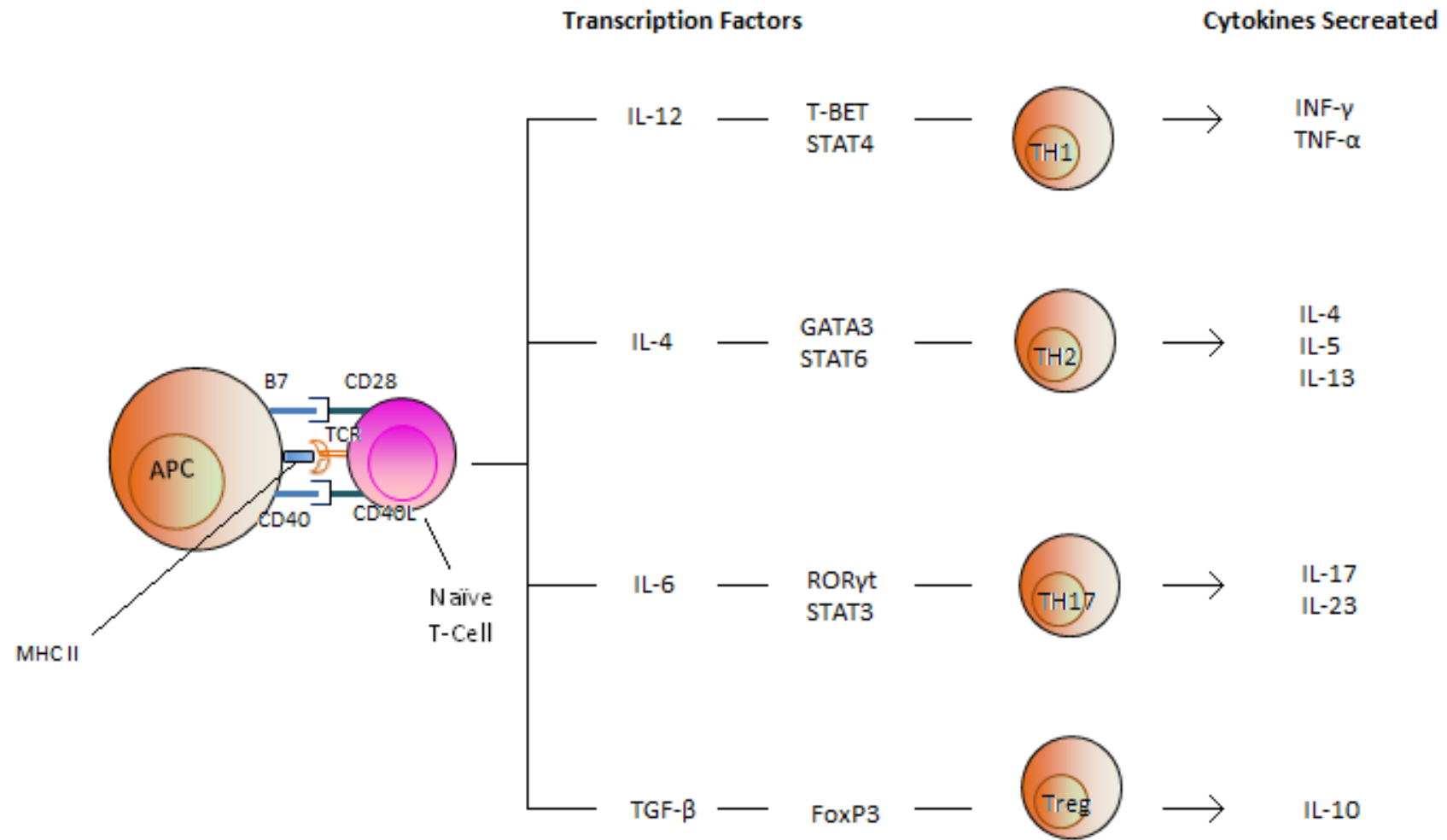


Figure 1.1.1.1: T Cell Differentiation

After activation and co-stimulation by antigens from the APC and naïve T cell, the differentiation of naïve T cell into effector cells is controlled by the influence of cytokines and transcriptions factors [20, 23]. Adapted from Meng et al, 2016, Nature.

1.2.1 Innate Immune System

1.2.2 Monocytes

Monocytes are derived from bone marrow and circulate within the blood equipped with pathogen recognition receptors (PRR), chemokines and inflammatory cytokines to counter against infections [62]. Monocytes are subdivided into: 'Classical', 'non-classical' and 'intermediate' based on their CD14 expression [63]. Classical monocytes are CD14⁺⁺CD16⁻, are phagocytic and produce reactive oxygen species to eliminate microbes [64], furthermore, classical monocytes have been shown to play an integral role in resolving tissue damage by shaping an inflammatory response which initiates the healing process, this includes their ability to migrate into injured tissue and to differentiate into monocyte derived macrophages and dendritic cells (DC) [65]. Whereas, non-classical monocytes are CD14⁺ CD16⁺⁺, have higher MHC II expression and display inflammatory and anti-inflammatory characteristics upon activation. Intermediate monocytes are CD14⁺ CD16⁺ and display inflammatory and phagocytic functions [66, 67]. Monocytes have an established role in tissue repair and defence against pathogens [68] and are the first immune cells to recognise and respond to pathogenic stimuli by triggering an inflammatory response [69]. Monocytes can also migrate into tissue and differentiate into tissue resident macrophages [70].

1.2.3 Macrophages

Macrophages are immune cells which inhabit both lymphoid and non-lymphoid tissues [62]. They differentiate from monocytes into either M1 or M2 macrophages depending on the microenvironment signals [71]. Monocytes stimulated with GM-CSF differentiate into M1 macrophages, these immune cells are inflammatory producing

and defend the host against infections [72-74]. However, the anti-inflammatory M2 macrophages proliferate from monocytes exposed to colony stimulation factor-1 (CSF-1), these cells mediate tissue repair damage by promoting the restoration of blood vessels and the epidermis [72-74].

1.2.4 Neutrophils

Neutrophils are the first immune cells which migrate to sites of acute infection and destroy bacteria, viruses and fungi through phagocytosis [75]. Systemic neutrophils are recruited towards mucosal sites with the greatest production of IL-17 and IL-22 [76]. These cytokines are produced by T cells and act on surrounding epithelial cells to promote the production of neutrophil chemo attractants: IL-8, CXCL1 and CXCL2 [77]. As well as being an abundant cell type in the circulation, neutrophils are also found in tissue [75]; tethering is the first process in which neutrophils attach themselves to the endothelial wall, proteins known as selectins mediate this process and allow neutrophils to roll along the wall of the endothelium detecting any inflammatory signals [78]; chemokines released by the inflamed endothelium induces neutrophil adhesion and activation which is regulated by proteins known as integrins, the signals transferred to effect neutrophil function from these integrins are influenced by the extracellular matrix and control neutrophil respiratory burst activity, adhesion strength, superoxide anion synthesis and even allow neutrophils to cross into the endothelial layer, a process known as trans-endothelial migration (TEM) [79, 80]. In tissue, neutrophils have a key role in regaining homeostasis in infected tissue by promoting a local inflammatory response [80]. However, if neutrophil regulation is not controlled properly by apoptosis, tissue damage can occur from the accumulation of pro-inflammatory cytokine production by these neutrophils [81].

1.2.5 Natural Killer Cells

Human NK cells belong to a group known as innate lymphoid cells (ILC) [82]. They constitute approximately 5-15% of peripheral blood lymphocytes [82]. However, unlike lymphocytes, NK cell subsets are CD3⁻ cells that express differing levels of CD56 [83]. The majority of NK cells comprise of a CD56^{dim}CD16^{bright} subset in the periphery and are more efficient cytokine producers than other members of the NK cell family [84]. NK cells survey the body in search of infected or tumour cells. They have the ability to kill as well as recruit other immune cells in order to protect the host [82, 85]. The ability of NK cells to kill target cells without prior activation is mediated by natural cytotoxicity receptors (NCR); NKp46 (CD335), NKp44 (CD336) and NKp30 (CD337) [86]. NKp46 is the primary receptor isotype involved in killing tumour cells, viruses and bacteria [87]. NK cells have activating and inhibitory receptors located on their surface [88]. Ligands for NK activating receptors will initiate a strong NK response if not restrained by MHC class I co-presentation [88]. Peripheral NK cells are involved in the lysis of apoptotic cells and cells infected with pathogens, releasing cytokines and chemokines [89].

1.3.1 Preterm Birth

The World Health Organisation (WHO) identifies preterm birth (PTB) as birth before 37 weeks (259 days) of gestation and complicates approximately 5-18% of pregnancies worldwide [90]. In 2012, the WHO estimated that around 15 million PTB occur every year and it is the primary cause of neonatal death and the largest cause of mortality amongst children under the age of 5 [90, 91].

1.3.2 The Immune System during Pregnancy and Preterm Birth

Originally, suppression of the maternal immune system was thought to be essential for a successful pregnancy [92]. However, in recent years, the concept that the maternal immune system is not suppressed, rather it is altered to a state of enhanced tolerance towards paternal antigens, has been proposed [93]. Here, the fetal-placental unit may directly modulate the behaviour of the maternal immune system [94, 95]. Another possibility is that the interactions between the innate and acquired subsets are altered in high-risk pregnancy, which leads to adverse pregnancy outcomes. Bounds and colleagues found that preeclampsia (PE; high blood pressure during pregnancy) maybe a consequence of alterations in innate immunity during pregnancy [96]. For example, pre-existing infection, or endothelial dysfunction can lead to an increased expression in innate cell inflammation, furthermore, insufficient trophoblast invasion in the placenta due to excessive inflammation from innate cells can cause inadequate spiral artery remodelling [96]. Another study looked at systemic innate immunity during normal and adverse pregnancy and found that dysregulated cross-talk between the innate and adaptive immune system can lead to an adverse pregnancy outcome, this includes over activation of innate immune responses which can lead to rejection of the foetus, conversely disturbances in innate cell immunity can lead to maternal/fetal susceptibility to infection [97]. These data imply that an exaggerated innate cell phenotype contributes to a severe inflammatory environment and as a consequence the adaptive immune system is unable to respond efficiently enough to promote tolerance during pregnancy.

1.3.3 Acquired Immunity during Pregnancy and Preterm Birth

T cells have been shown to play an important role in pregnancy tolerance between the mother and foetus [98, 99]. Fetal antigens are recognised by maternal T cells through interactions with an APC [100]. Furthermore, antigen-specific T cells derived from the foetus modulates fetal maternal tolerance during gestation [101]. However, poor regulation of effector T cells caused by dysfunctional tregs which mediate the PD-L1/PD-1 apoptotic pathway can contribute to a more pro-inflammatory cytokine environment which may lead to pregnancy complications [98, 102]. Additionally, adverse pregnancy outcomes have been associated with dysregulation of Tim-3 expression [103]. In addition, a significant decrease of Tim-3⁺ CD8⁺ T cells has been demonstrated in miscarriage patients compared to normal pregnant women [104]. Add to this, Meggyes and colleagues found that this pathway may mediate the cytotoxic potential of decidual CD8⁺ T cells to maintain immune tolerance during pregnancy [105]. An imbalance between immune regulators and immune effectors are associated with adverse pregnancy outcomes such as recurrent pregnancy loss and PE [106, 107], it is therefore imperative that effector cell function is controlled properly by the immunosuppressive function of Tregs .

Reportedly, Treg frequencies have been shown to increase as gestation progresses and provide fetal-maternal tolerance due to their suppressive nature [99, 108]. Interestingly, in mouse models and human clinical data, maternal inflammation has been shown to be associated with adverse neonatal outcomes [109, 110]. Experimental work has shown, in both human and mouse pregnancies that a decrease in total Treg populations can be associated with adverse pregnancy outcomes [111]. Another study demonstrated that Treg depleted peripheral blood mononuclear cells

(PBMC) from non-labouring pregnant women had a greater response to both recall antigens and Toll-like receptor (TLR) agonists however, this change was not observed in samples from labouring women, suggesting that spontaneous labour was associated with a loss of Treg functionality [108]. In the same study, Treg phenotype during labour was found to be comprised of a more naïve and less activated subtype [108].

1.3.4 Innate Immunity during Pregnancy and Preterm Birth

The increased expression and activation of monocytes has been associated with preterm labour (PTL) [112]. Intrauterine infections induce PTL from pro-inflammatory cytokine production by the elevated levels of CD14⁺ monocytes interacting with lipopolysaccharide (LPS) induced TLR-2 and TLR-4 [113, 114]. Increased intermediate and non-classical monocytes in the periphery have also been associated with PE [115, 116], likely due to their pro-inflammatory phenotype.

Macrophages have also been shown to influence pregnancy. In normal pregnancy macrophages populate the endometrium and play a key role in placentation and tissue remodelling [117, 118]. However, excessive frequency and activation of these cells are linked with pre-eclampsia [117]. At the maternal-fetal interface, macrophages along with NK cells mediate spiral artery remodelling [119], in fact, in normal pregnancy these macrophages are predominately of the M2 subtype, inducing an suppressive environment to allow the growth of the foetus, on the contrary, an M1 macrophage dominant environment is associated with adverse pregnancy outcomes, suggesting that placental development is hindered [117].

Neutrophils play an important role in the pathology of PTL. In acute chorioamnionitis, neutrophils make up the majority of leukocytes recruited to the fetal membrane;

however the exact pathways that mediate neutrophil migration are still open for investigation [120]. Neutrophils contribute in the release of several pro-inflammatory cytokines during the onset of labour [121, 122]. In the decidua, increased neutrophil frequency and activation is suggested to contribute to premature rupture of membranes (PPROM) [123]. Studies have shown an increased number of neutrophils in decidual tissue in women with chorioamnionitis associated PTL than in women who delivered at term and with spontaneous preterm birth (sPTB) without chorioamnionitis [124]. Neutrophils also play a role in the physiology of pregnancy. Neutrophils exposed to maternal hormones, facilitate the production of fetal antigen specific Tregs. These Tregs produce IL-10 and IL-17 which help to promote angiogenic activities required for placental growth and fetal tolerance [125]. Neutrophil depletion in pregnant mice leads to poor development of the placenta and decreased numbers of healthy foetuses [126-128].

During pregnancy, the increase of progesterone (P4) modifies the uterine environment and alters decidual NK (dNK) cell phenotype despite their lack of a progesterone receptor (PR) [129, 130]. dNK cells differ functionally compared to peripheral NK (pNK) cells [131], they possess a limited cytolytic ability [132], They are by far the most abundant mononuclear cells found in the human decidua [133], and, at the maternal-fetal interface, maintain maternal immune tolerance, demonstrated in a mouse model where their depletion lead to miscarriage [134]. Their function is to promote angiogenesis for placental growth and vascular endothelial growth [135]. NKp46 and NKp30 are expressed on resting dNK cells and are up-regulated on activated ones, while NKp44 is expressed only on activated cells [136]. NKp46 regulates dNK cell function and is able to induce cell cytotoxicity, whereas NKp30 promotes cytokine secretion of dNK cells and NKp44 has an inhibitory function in dNK cells [137, 138].

An increase in maternal peripheral CD56^{bright} NK cells has also been associated with an increased risk of miscarriage [139]. CD69 is a marker for peripheral NK cell activation and it is increased from the 1st to the 3rd trimester of normal, nulliparous singleton pregnancies [139]. As mentioned, there has been an extensive amount of research which has looked into the periphery and its effects on the immune system in pregnancy and PTL. However, the literature surrounding the cervical immune environment has only been briefly touched upon in pregnancy. Hunter *et al.* demonstrated in a cross-sectional study, that PTL had decreased proportions of macrophages in the cervix of women who previously had a sPTB [140]. In this study, the elucidation of the cervical milieu in high-risk pregnancy will be explored longitudinally to help further understand the relationship between the circulatory and local immunity, something which has not yet been reviewed before.

1.4.1 Preventative Strategies to Preterm Birth

1.4.2 Cervical Suture

A cervical suture is a surgical procedure which aims to mechanically support the cervix, the suture or stitch is removed between weeks 36-38 to prevent trauma towards the cervix prior to the onset of labour [141, 142]. A Cochrane database reviewing 15 independent studies involving 3490 pregnant women demonstrated that women who had a cervical suture had a lower chance of delivering their babies preterm and had increased neonatal survival [143]. However, risks associated with this clinical procedure must also be taken into consideration. These risks include: lacerations at the site of surgery, inadvertent membrane rupture and the potential risk of infection from the use of a foreign body [144].

1.4.3 Progesterone

Vaginal P4 is widely used for the management and prevention of PTL [145]. P4 has been shown to be an effective suppresses inflammation whilst maintaining uterine quiescence in both gestational tissue and the immune system [146, 147]. In high-risk groups, although P4 may reduce an individual's risk of spontaneous preterm delivery by 20%, the overall impact on absolute numbers of PTB is much smaller (0.01%). As a result, P4 does not have a significant impact on perinatal mortality, low birthweight, or neonatal death [148]. However, the complex aetiology of PTL, which involves infective and inflammatory processes for some patients, is likely to play a role in the overall neonatal outcome. Alongside P4, there are a number of other preventative strategies which are currently used to decrease the risk of PTL which will be discussed further in Chapters 5 and 6.

1.5.1 Potential Preventative Treatment

1.5.2 Aminophylline

Previous research has shown that in myometrium aminophylline (AP), a drug commonly used for the treatment of asthma, enhances the anti-inflammatory effect of P4 mediated by cyclic adenosine monophosphate (cAMP) following binding to membrane PR [149, 150]. Therefore, in high-risk pregnancy, the longitudinal supplementation of AP alongside P4 might enhance the anti-inflammatory ability of P4 to promote fetal tolerance which may be evident in peripheral and local immune cell changes. The novelty of these findings will be discussed further in Chapters 5 and 6.

2. Research Hypothesis and Aims

2.1 Overarching Hypothesis

High-risk pregnant women will have differences in immune cell phenotype and function which is less suited towards maintaining and supporting pregnancy when compared to healthy pregnant women.

2.1.1 Hypotheses

1. High-risk pregnant women will have an altered phenotype of innate and adaptive immune cells in peripheral blood and cervical mucus when compared to healthy pregnant women.
2. The addition of AP will help to increase the anti-inflammatory effect of P4 *in vivo* by reducing the percentage of pro-inflammatory cell types in peripheral blood and cervical mucus in high-risk pregnant women.
3. AP cultured with LPS-induced peripheral monocytes will modulate a reduction in pro-inflammatory cytokine production *in vitro*.

2.2 Aims

1. To assess the immune profiles in peripheral blood and cervical mucus in high-risk pregnant women and compare the profiles of these who experience PTB versus those who gave birth at term.
2. To assess the longitudinal changes in the immune phenotype in high-risk pregnant women receiving either a combination of P4 and AP or P4 alone to prevent PTB.
3. To identify what effect AP has on LPS-induced monocyte cytokine production.

3. Methodology

3.1 Plasma Separation

Maternal peripheral blood was collected into 10mL Lithium-Heparin coated tubes (BD, Becton Dickinson, UK) and processed within 2 hours of collection. Whole blood was centrifuged at 400g for 10 minutes to separate the plasma. Maternal plasma was aspirated off and stored at -80°C for later use. Equal volume of Dulbecco's phosphate buffered saline (PBS; Sigma-Aldrich) containing Ca^{2+} and Mg^{2+} was used to replace the aspirated plasma and the tubes were inverted.

3.2 Separation of PBMC

Whole blood diluted with PBS, as mentioned above, was layered onto Histopaque (Sigma-Aldrich) in a 1:1 ratio. Density gradient centrifugation at 800g for 20 minutes at room temperature was used to separate whole blood into a buffy coat. The top layer of the buffy coat containing PBMC and polymorphonuclear cells (PMNC) was aspirated off using a sterile Pasteur pipette (Alpha Laboratories, Hampshire, UK) and placed into a new 50mL Corning® Falcon tube (Sigma-Aldrich). The buffy coat was washed using PBS and centrifuged at 400g for 10 minutes at room temperature. 50mL PBS was then added to re-suspend the pellet.

3.3 Viability and Cell Counting

From the 50mL cell suspension, 10µl was aspirated and placed into a 96 well plate (Corning, Flintshire, UK). 10µl of Trypan Blue (Sigma-Aldrich) was then added and mixed via pipetting with the cell suspension. 10µL of this mixture was then placed onto a Kova Glasstic® slide (Hycor Biomedical Incorporated, Edinburgh, UK) and using a

light microscope (Nikon Diaphot, UK) set at x40 magnification, live cells were identified and counted. The average number of cells was counted from 3x9 squares in separate locations found on the Kova slide. Only samples where the viability of cells was greater than 80% were used. The formula (Figure 3.3.1) was used to obtain the total number of cells in the 50mL Falcon tube.

$\text{Total number of cells} = \text{Average number of cells} \times 50 \times 2 \times 10000$

Figure 3.3.1: PBMC count

The calculation to approximate PBMC count whereby 50 represents the volume of the falcon tube containing PBMC, the number multiplied by 2 to factor in the 50:50 distribution of trypan blue to cells and 10000 representing the magnification factor on the light microscope.

3.4 Antibody Staining for Flow Cytometric Analysis

Aliquots of fluorochrome-conjugated antibodies for surface staining of cells were made into a master mix before being pipetted into a 5mL round bottom polystyrene Falcon tubes (Corning, Flintshire, UK). Serial titrations were used to determine required volumes for each antibody. From the original 50mL cell suspension, mentioned above, centrifugation at 400g for 10 minutes at room temperature was performed and the supernatant discarded. The pellet of mononuclear cells was re-suspended with PBS at a concentration of 1×10^7 cells/mL. 200 μ L (2×10^6 cells) and then added to each antibody-containing tube. The cell-antibody mixture was incubated in a dark room covered in aluminium foil for 30 minutes at 4°C and then washed by adding 2mL PBS and centrifuging at 400g for 5 minutes. Once the supernatant had been discarded the remaining pellet was re-suspended in 500 μ L of fluorescent-activated cell sorting (FACS) flow (BD, UK). Acquisition on flow cytometry was performed immediately after and always fresh.

3.5 Intracellular Cytokine Staining

Before starting the assay, LPS was diluted to the following concentration:

Table 3.5.1: Dilution of LPS to the concentration used for stimulation.

Stimuli	Company	Catalogue Code	Stock Concentration	Working Concentration	Cell Type
LPS-O127:B8	Sigma-Aldrich	L4516-1mg	1mg/mL	200ng/ml	Monocytes + Macrophages
1mg of LPS as delivered was diluted into 1mL of RPMI-1640 [1mg/mL]. One of these aliquots was pipetted into 9mL RPMI-1640 sup and divided into 10x1ml aliquots [0.1mg/mL]. This step was repeated with one of the newly made aliquots [0.01mg/mL]. Finally, 49mL RPMI-1640 sup was added to one of these new aliquots giving a stock concentration of [200ng/mL] which was then split into 50x 1mL aliquots and stored at -20°C.					

PBMC separation protocol described in Section 3.2 was followed and cell viability checked. However, Cell pellet was instead resuspended in RPMI supplemented with L-glutamine, 10% FCS and 1% Penicillin and Streptomycin (sup; Sigma-Aldrich) Approximately 1×10^6 cells were aliquoted into a 96-well plate, including one negative control containing no LPS and one positive control containing LPS at 200ng/mL. LPS was titrated and 0.5ng/mL was added to each of the experimental samples. 0.5µL of Brefeldin A (Biolegend, UK) at 1x concentration was added to each well and mixed with a pipette before being placed into a 37°C, 5% CO₂ incubator for 6 hours. Cells were harvested with 200µL of FACS flow and washed with 2mL of FACS flow. Supernatant was discarded and surface staining antibodies shown in Table 3.10.1.6 were added to the cell suspension each at a 1/1000 concentration. Cells were covered in foil and incubated for 30 minutes at 4°C. 2mL of FACS flow was used to wash the cells before being centrifuged at 400g for 5 minutes at room temperature.

Permeabilisation step: The supernatant was discarded and 1mL of Cyto/Fix permeabilisation buffer (BD, UK) was added at 1x concentration to all tubes. Cells were covered in foil and incubated for 40 minutes at room temperature before being washed with 2mL of diluted permeabilisation/wash buffer (BD, UK) and centrifuged at

400g for 5 minutes at room temperature. This wash step was repeated, and supernatant was discarded. An intracellular antibody cocktail was then added at titrated volumes into each tube, covered with foil and left to incubate for 40 minutes at room temperature. A final wash step using 2mL of permeabilisation/wash buffer was performed twice before suspending the cells in 500µL of FACS flow and acquiring on the flow cytometer immediately.

3.6 Cryopreservation of Immune Cells

Freezing media containing 90% heat inactivated fetal calf serum (FCS; Sigma-Aldrich) and 10% sterile dimethyl sulfoxide (DMSO; Sigma-Aldrich) stored at -20°C were thawed prior to use. The freezing medium was added dropwise using a Pasteur pipette to the cell suspension whilst the falcon tube was slowly swirling. The final concentration used was approximately 5×10^6 cells/mL. 1mL aliquots of the cell suspension were dispensed into 1.8mL cryotubes. These were then placed into a cryopreservation box containing 250mL of isopropyl alcohol pre-cooled to 4°C. The box was then transferred to a -80°C freezer where the cells were cooled at -1°C/minute to reduce crystal formation.

3.7.1 Thawing Cryopreserved Cells – Revival Step

Cells were thawed out as quickly as possible in a 37°C water bath until only a small crystal of ice remains. The cells were then transferred using a Pasteur pipette to a 15ml Corning® Falcon tube, 3mL of RPMI-1640 sup was added dropwise to the cell suspension whilst the tube was constantly being stirred to allow the DMSO to be diluted. 3µL of DNase I (Sigma-Aldrich) at a concentration of 10µg/mL was added to the suspension and left in culture for 45 minutes. The cells were then washed with

2mL RPMI-1640 and then centrifuged at 400g for 10 minutes. The cells were handled gently to minimise rupturing/injuring cells.

3.7.2 Frozen Cell Viability

A revival step was implemented to increase frozen cell viability. Conventional thawing using cold PBS (containing Ca^{2+} and Mg^{2+}) demonstrated a poorer yield of live cells (69.2%) when compared with cells that had undergone the revival step (76.3%) shown in Figure 3.7.2.1.

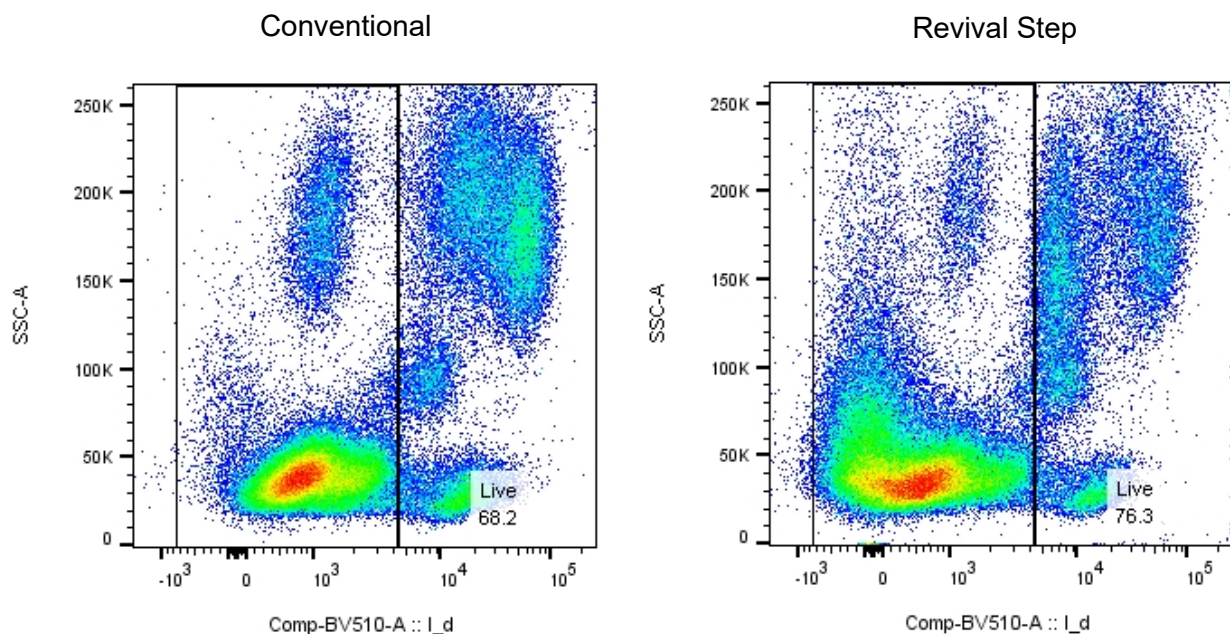


Figure 3.7.2.1: Frozen cell viability

Live dead (L/D) viability stain illustrates a poorer yield of live cells when using the conventional thawing (left) method for PBMC (68.2%) when compared to cells implementing the revival step (right) (76.3%).

3.8 Cervical Vaginal Fluid Collection

Consented women participating in both studies had a high vaginal swab sample taken. A plastic speculum prepared with lubricating gel was used to take the swab. The cervical swab (VWR, UK) was then placed into a sample tube containing 5ml of cold PBS with protease inhibitor and transported to the lab. The cervical vaginal fluid was aliquoted into 1.5mL eppendorfs (FisherScientific, UK) and stored at -80°C. Samples processing and storage occurred within 2 hours of collection.

3.9 Isolation of Cervical Immune Cells

Immediately after the high vaginal swab was taken, a cervical cytobrush (Rovers Medical Services, Netherlands) was placed at the ectocervix and rotated 360° gently five times to ensure no blood contamination to the sample was obtained. The brush was placed into a sterile tube containing 5mL PBS with protease inhibitor. The tube was transported to the lab where it was processed within 2 hours of collection. The tube was vortexed to dislodge aggregated cervical cells and transferred to a new sterile Falcon tube using a 100 micron nylon filter (Corning, Flintshire, UK). 5mL RPMI-1640 medium (Sigma-Aldrich) containing 1% Antibiotic Antimycotic Solution (Sigma-Aldrich) was added to the tube and centrifugation at 400g for 10 minutes at room temperature with brake on was performed. The supernatant was then aliquoted and stored at -80°C. The pellet was re-suspended in 5mL PBS. A 100µL blocking solution was then made which contained 1.8µL of Mouse IgG antibody (Sigma-Aldrich) at a concentration of 1mg/mL and 98.2µL of FACS flow (BD, UK). This solution was then added to the tube containing the pellet and incubated for 10 minutes at 4°C wrapped in aluminium foil. 500µL of FACS flow was added to the tube and the centrifugation step repeated. The supernatant was discarded and the antibody cocktail was added

to the pellet and incubated in a dark room covered in aluminium foil for 30 minutes at 4°C. 500uL of FACS flow was added to the tube and centrifuged at 400g for 10 minutes. The remaining pellet was re-suspended in 500µL of FACS flow and run on the flow cytometer.

3.10.1 Flow Cytometry

Surface and intracellular staining methods mentioned in Sections 3.4 and 3.5 respectively used the following fluorochrome-conjugated antibodies detailed in Tables 3.10.1.1 – 3.10.1.6. The innate cell panel (Table 3.10.1.5) was used to stain peripheral blood and cervical cells whereas; all other panels were used to stain peripheral blood only.

Tables 3.10.1.1 – 3.10.1.6: The following Tables list all antibodies, catalogue numbers, clones and manufacturer used in both projects.

Table 3.10.1.1 - Tregs

Manufacturer	Laser	Antibody	Clone	Category Number
BD	BV421	CD25	M-A251	562442
BD	BV711	CD127	HIL-7R-M21	563165
BD	PE	FOXP3	259D/C7	560082

Table 3.10.1.2 - NK Cells

Manufacturer	Laser	Antibody	Clone	Category Number
BioLegend	FITC	NKp46 (CD335)	9E2	331921
BD	BV421	NKp30 (CD337)	P30-15	563385
Biolegend	BV605	CD16	3G8	302039
BD	BV650	CD56	NCAM16.2	564057
Biolegend	BV711	TCR Vα24-Jα18	6B11	342921
Biolegend	BV785	HLA-DR	L243	307641
Biolegend	PE-Cy5	CD69	FN50	310907

Table 3.10.1.3 - Exhaustion and Senescence

Manufacturer	Laser	Antibody	Clone	Category Number
Biolegend	AF488	CD16	3G8	302022
Biolegend	BV421	CD25	M-A251	356113
Biolegend	BV605	PD-1	EH122H7	329923
BD	BV650	CD56	NCAM16.2	564057
Biolegend	BV711	Tim-3 (CD366)	F38-2E2	345023
Biolegend	BV785	CD127	AO19D5	351329
Biolegend	AF647	CD57	HNK-1	359613
Biolegend	PE	PD-L1 (CD274)	29E.2AE	329705

Table 3.10.1.4 - Effector Cells

Manufacturer	Laser	Antibody	Clone	Category Number
Biolegend	AF488	CD45RO	UCHL1	304212
BD	PerCP	CD8	SK1	565310
Biolegend	BV421	CD28	CD28.2	302929
Biolegend	AF647	CD27	M-T271	356433
BD	AF700	CD3	UCHT1	557943
BD	APC-Cy-7	CD4	RPA-T4	560251
Biolegend	PE	CD45RA	HI100	304107
BD	PECy-7	CCR7	3D12	557648

Table 3.10.1.5 - Innate Cells

Manufacturer	Laser	Antibody	Clone	Category Number
Biolegend	AF488	CD15	H198	301910
Biolegend	BV421	CD45	2D1	368521
Biolegend	BV711	HLA-DR	L243	307643
Biolegend	AF647	CD11b	CBRM1/5	301413
Biolegend	APC-Cy7	CD16	3G8	302017
Biolegend	PE	CD66b	G10F5	305105
Biolegend	PE-Cy7	CD14	HCD14	325617

Table 3.10.1.6 – Intracellular Cytokine Staining Panel

Manufacturer	Laser	Antibody	Clone	Category Number
Biolegend	FITC	TNF-alpha	MP6-XT22	506303
Biolegend	BV421	IL-10	JES3-9D7	501421
Biolegend	APC-Cy7	CD16	3G8	302017
BD	PE	IL-8	G265-8	554720
Biolegend	PE-Cy7	CD14	HCD14	325617

3.10.2 Baseline CST – (Cytometer Setup and Tracking)

A baseline quality check was run either: once every 6 months, a new configuration had been set up on the cytometer, a new lot # of CST beads (BD, UK) or adjustments made by an engineer. The baseline was run in 500µL of FACS flow with one drop of CST beads and was used to set up: optimal voltage, optimal area scaling factor (ASF), optimal target values, tracking of performance and checks linearity of plots.

3.10.3 Daily Performance CST

A daily performance check was run before use every day using the same lot # of beads as the baseline run. The performance check was run in 350µL of FACS flow with one drop of CST beads.

3.10.4 Single Colour Controls

Compensation was performed before every new run using flouochrome-conjugated antibodies attached to mouse immunoglobulin-kappa (Igk) compensation beads (BD, UK). 50µL of FACS flow was added to each antibody compensation tube to negate sticking of compensation beads to the FACS tubes. 1µL of antibody along with one drop each of positive and negative compensation beads were added to each relevant tube. The tubes were covered in foil and incubated for 30 minutes at room temperature. 200µL of FACS flow were added to the tubes, vortexed and run on flow cytometry immediately.

3.10.5 Perfetto Method - Setup

This quality assurance protocol is generally used to ensure a flow cytometer has been fully optimised, calibrated and monitored to deliver the most accurate and sensitivity of fluorescence measurements [151]. This method ensures that longitudinally, any changes in the: optical path, lasers, laser alignment, voltages or detectors which are not picked up by CST will become noticeable. The first Section of the Perfetto method was performed by the engineer upon installation of the cytometer.

3.10.6 Signal Synchronisation/ Area Scaling Factor

Voltages were adjusted on all detectors to 500 volts (V). 1 drop of 1x Rainbow beads (BD, UK) was placed into a round bottom Falcon tube (Corning, Flintshire, UK) containing 350 μ L of FACS flow (BD, UK). Singlets and the bead population were gated. The median fluorescence intensity (MFI) was then measured for both height and area on each detector. The ASF was adjusted until the MFI for height and signal on each detector was equal on a logarithmic scale.

3.10.7 Testing the Laser Delay

The window extension was decreased to zero and the voltages and ASF were adjusted to give an MFI of roughly 100,000 for each detector shown in Figure 3.10.7.1. The laser delay for each detector (from violet to red to yellow/green) was adjusted in 1 μ sec increments until a stable MFI value for each detector was observed. The window extension was then reset to factory settings (10.00).

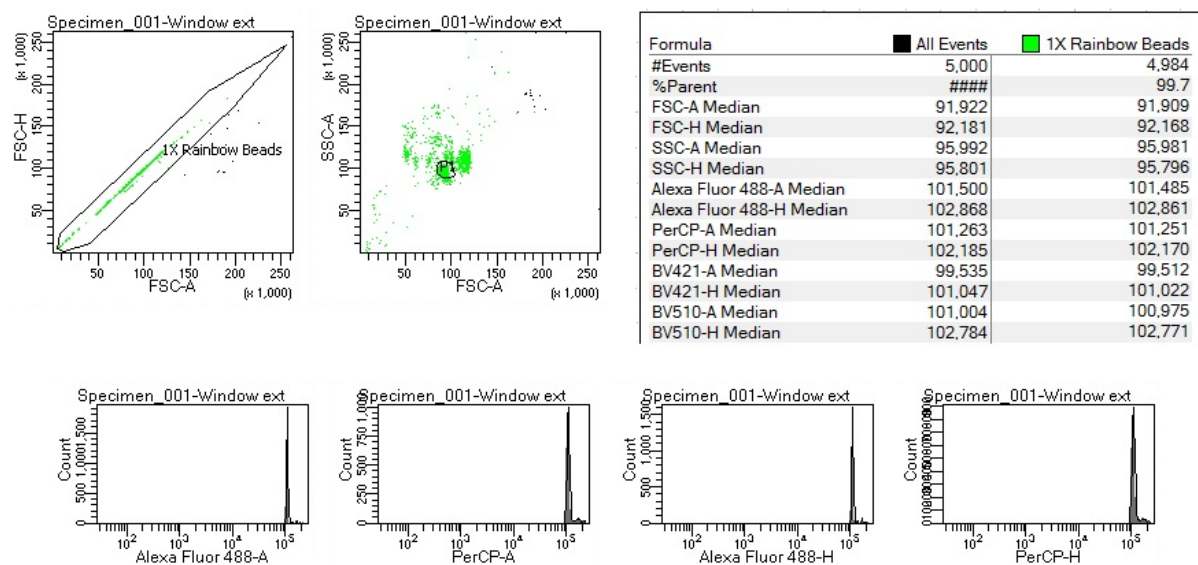


Figure 3.10.7.1: Laser Delay

Singlets were gated on 1x Rainbow peak beads and 5000 events recorded after adjusting the voltages and ASF for each detector until an MFI of roughly 100000 was achieved.

3.10.8 Calibration

To determine the photomultiplier tube (PMT), voltage range, linearity and background all detectors were set to 350V. 1 drop of 8x Rainbow beads (BD, UK) was placed into a round bottom Falcon tube containing 350µl of FACS flow. 5000 events were recorded. Two adjacent peaks were selected and the MFI and %CV (coefficient of variation) were recorded as shown in Figure 3.10.8.1. This step was repeated from 350V up to 750V in 50V increments. The PMT linearity of each channel was calculated by the following equation: $(M2-M1)/M1 = \text{PMT}$.

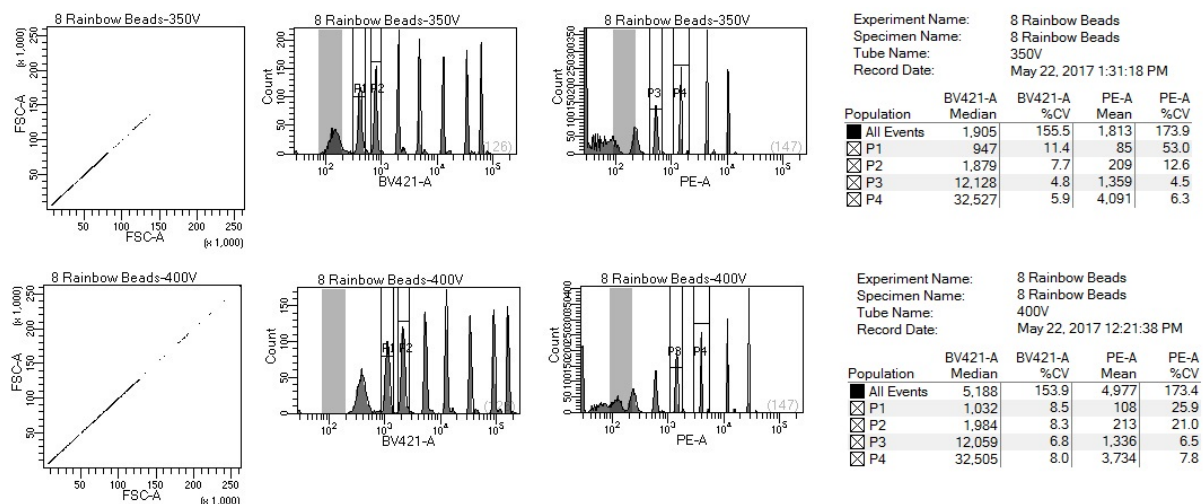


Figure 3.10.8.1: Perfetto Calibration (i)

8x peak Rainbow beads at 350V (top) and 400V (bottom). The mean and %CV (only for M1) were recorded. M1 gated on the left and M2 gated on the right were gated from 350V to 750V to measure PMT voltage. As an example, at 350V for BV421 the %CV = 11.4, M1 = 947 and M2 = 1879. The linearity was calculated to be 0.98 (1879-947/947).

The procedure was repeated using negative CompBeads (BD, UK) however, the MFI was only measured and recorded as shown in Figure 3.10.8.2 The calculation used to plot the signal to background ratio was: (M1/MFI of background) = Ratio of signal to background.

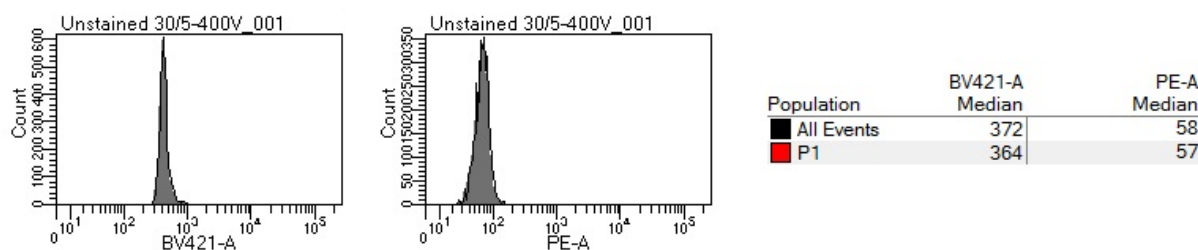


Figure 3.10.8.2: Perfetto Calibration (ii)

The mean of the P1 gated unstained beads were recorded. The signal to background ratio was calculated and plotted against %CV and PMT voltage. As an example, at 350V for BV421

the %CV = 11.4, M1 = 947 and MFI for the background = 364. The ratio was calculated to be 2.60 (947/364).

The PMT linearity, signal to background ratio and the %CV were then plotted for each detector on an Excel spreadsheet. This graph was used to determine the optimal range of voltages for each detector shown in Table 3.10.8.1.

Table 3.10.8.1: The optimal voltage ranges as shown above are obtained by taking the largest signal to background ratio with the lowest CV at the lowest PMT voltage range.

Laser	Channels	Optimal Voltage range (V)
Blue	AF488	450
	PerCP	500-550
Violet	BV421	400
	BV510	400-500
	BV605	450-500
	BV650	450
	BV711	450
	BV786	450-550
Red	AF647	450-500
	AF700	500-550
	APC-Cy7	450
Yellow/Green	PE	450-600
	PE Cy-5	600-650
	PE Cy-7	500-650

3.10.9 Validation of PMT Voltages

Each detector was set to their optimal voltage acquired in the last Section (the lowest voltage was selected in detectors which have a range). Aliquots of fluorochrome-conjugates for each channel were pipetted into a 5mL round bottom Falcon tube. 1 drop each of positive and negative Compbeads were added to the tubes. The bead-antibody mixture was left in a dark room for 20 minutes at 4°C. When each tube was run, the voltages were adjusted so that the MFI of each channel corresponding to their respective fluorochrome was at least double any other MFI from any other channel. This procedure was performed multiple times for each tube as adjusting the voltages

constantly changed the MFIs for each channel detector. Once all the detectors had an MFI of at least double that of any other, 5000 events were recorded for each tube. The files were exported onto FlowJo10.7.0 to the 'Compensation Matrix' shown in Figure 3.10.10.1 where each channel was assessed to make sure no compensation spill over exceeded 40%. A final run of 1x Rainbow beads in 250µL of FACS flow was performed, 5000 events recorded and the MFI obtained for each detector was known as the 'target value'. $\pm 10\%$ of these MFI was recorded as the target ranges (Table 3.10.9.1). To account for any day to day variation subjected to the cytometer, this final step of the Perfetto method was repeated n=5 consecutive days and the average target value over that time period was recorded.

Table 3.10.9.1: The target MFI value and ranges for each channel set up using the Perfetto method on day 0 as shown.

Laser	Channels	Target Values (V)	Target Value MFI	%10 MFI Range
Blue	AF488	450	28671	25804 – 31538
	PerCP	518	3417	3075 – 3759
Violet	BV421	400	32867	29580 – 36154
	BV510	412	20513	18462 – 22564
	BV605	478	3130	2817 - 3443
	BV650	458	1056	950 - 1162
	BV711	445	453	408 – 498
	BV786	450	118	106 - 130
Red	AF647	515	5703	5133 - 6273
	AF700	519	4032	3629 - 4435
	APC-Cy7	535	1696	1526 - 1866
Yellow/ Green	PE	498	8956	8060 - 9852
	PE Cy-5	483	3664	3298 - 4030
	PE Cy-7	440	428	385 - 471

3.10.10 Compensation Matrix at Day 0

The percentage spill over obtained from FloJo10.7.0 for each channel used in the Perfetto set up is shown in Figure 3.10.10.1 No spillage into any channel exceeded 40% except for PerCP into PE Cy-5; however, no panels used would have both these channels open at the same time negating the problem of excessive spill over of these two channels.

	APC-Cy7-A	Alexa Fluor 48...	Alexa Fluor 64...	Alexa Fluor 70...	BV421-A	BV605-A	BV650-A	BV711-A	BV786-A	PE-A	PE-Cy5-A	PE-Cy7-A	PerCP-A
☒ APC-Cy7-A	100	0.1096	2.2409	7.6849	0.1878	0	0.3823	0.2184	11.1576	0.512	1.3108	27.7375	0.1638
☒ Alexa Fluor 488-A	0	100	0	0	0.0168	0.0146	0	0	0	0.0051	0	0	0.156
☒ Alexa Fluor 647-A	15.9694	0.0293	100	25.9555	0.0167	0	0.468	0.2193	0.0731	0.0456	14.5519	1.1565	0.8924
☒ Alexa Fluor 700-A	21.9917	0.5967	4.0671	100	0.5686	0	0.3965	7.8445	2.7759	1.0327	0.6938	4.0654	5.6541
☒ BV421-A	0	0.0246	0	0.0024	100	0.2412	0.0441	0.0196	0.0073	0	0.0098	0	0.0098
☒ BV605-A	0	0	0.027	0.0067	6.1535	100	38.7086	11.47	2.3933	17.0007	9.584	0.7982	1.7428
☒ BV650-A	0.999	0	5.7009	1.8973	8.1243	17.6163	100	30.1371	5.3789	0.1497	6.8895	0.5118	0.9899
☒ BV711-A	11.0498	0.0255	2.507	15.7728	15.8521	0.1144	7.969	100	32.3615	0.0793	0.2668	0.9412	21.4603
☒ BV786-A	8.9205	0.0651	0.0865	0.4541	20.6118	0.303	0.4975	2.715	100	0.045	0.0432	1.1035	0.346
☒ PE-A	0.0025	2.466	0.0049	0.0025	0	2.6863	0.4642	0.1796	0.0295	100	5.8184	0.3132	4.0255
☒ PE-Cy5-A	0.4843	0.1073	6.2436	1.6576	0	0.0198	2.0824	0.8096	0.1634	0.7227	100	2.2605	34.4169
☒ PE-Cy7-A	9.6655	1.2789	0.031	0.331	0	0.1035	0.0207	0.1448	7.9535	3.5251	0.4242	100	4.1006
☒ PerCP-A	3.1944	0	6.0993	4.5012	0	0	18.0378	19.0576	4.931	0.2134	43.28	3.278	100

Figure 3.10.10.1: Compensation matrix

The compensation matrix shown from FlowJo10.7.0 acquired with the target values shown in Table 3.10.9.1 illustrating the percentage overlap for each channel. Channels which had <40% spill over were not both open in the same panel to minimise compensation overlap.

3.10.11 Perfetto Tracking

After each CST run and before any sample acquisition, MFI and voltage was checked for each fluorescent channel using the reference MFI and voltages listed in Table 3.10.9.1 which were acquired on day 0. To allow for any machine fluctuation, the MFI thresholds were set to $\pm 10\%$ from the original MFI targets and voltages were set to $\pm 5\%$ from the original voltages set on day 0. Figure 3.10.11.1 shows the MFI fluctuations from 206 days of study from initial target MFI. Figure 3.10.11.2 shows the consistency in voltages acquired each time before sample acquisition from 206 days of study.

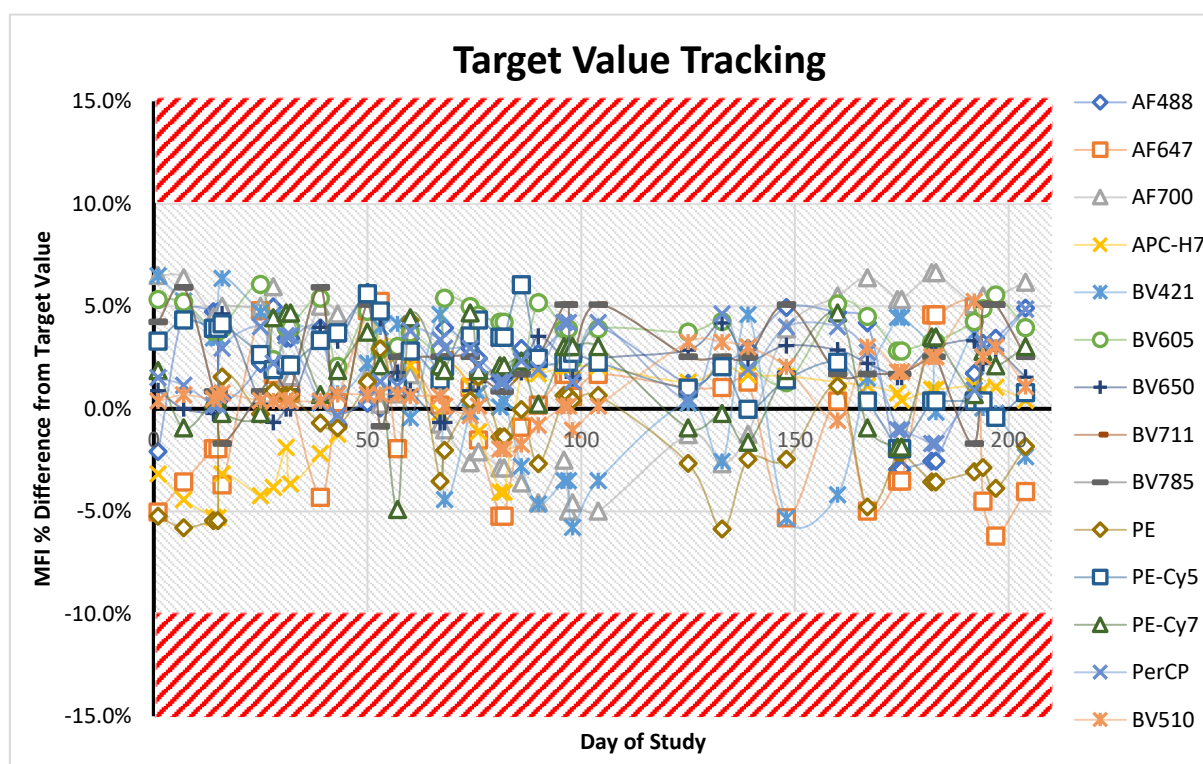


Figure 3.10.11.1: Target value tracking

MFI fluctuations illustrated up to 206 days of study as a percentage change from the original target MFI set on day 0. All recorded data points for each fluorescent channel shows no percentage change greater than $\pm 10\%$ from target MFI.

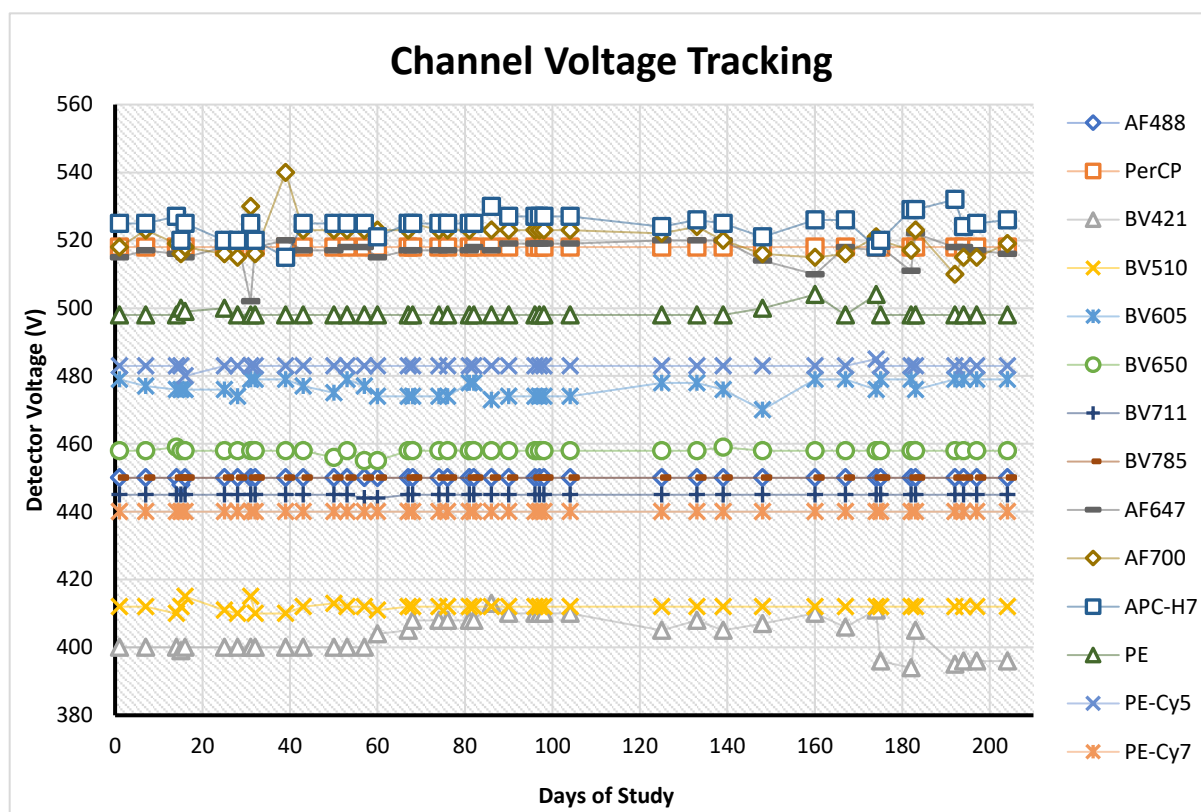


Figure 3.10.11.2: Channel voltage tracking

Voltages recorded before sample acquisition up to 206 days of study. All recorded data points for each fluorescent channel shows no percentage change in voltage greater than $\pm 5\%$ from the original values set at day 0.

3.10.12 Perfetto Validation

Every MFI recorded from 206 days of study for each fluorescent channel was compiled into one graph shown in Figure 3.10.11.3. The graph illustrates that at no point did MFI fluctuate past the $\pm 10\%$ threshold change.

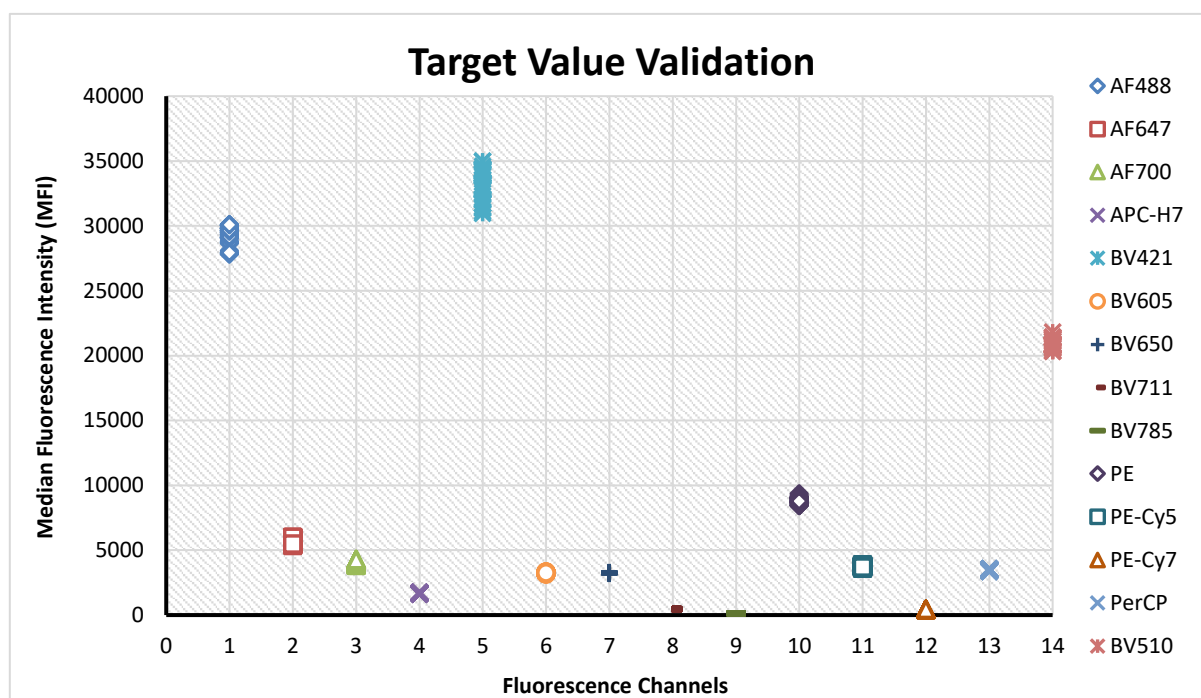


Figure 3.10.11.3: Target value validation

Validation of target values shows no MFI fluctuation over the +/- 10% threshold set on day 0 from 206 days of study.

3.10.13 Intra Nuclear FoxP3 Staining

After PBMC isolation, 2×10^6 cells were transferred into a 5mL FACS tube. LIVE/DEAD fixable violet dead cell stain (Thermofisher, UK) was added to the cell suspension in 1 in 1000 concentration and vortexed. The cells were wrapped in foil and incubated for 15 minutes at 4°C. The cells were then washed using 2mL of FACS Flow (BD, UK) and spun at 500g for 5 minutes at 4°C. The supernatant was discarded and the surface staining antibodies were added at the adjusted concentrations using titrated volumes giving a final volume of ~100µL. The suspension was mixed, placed in foil and incubated for 15 minutes at 4°C. The previous wash step was repeated. The cells were resuspended in 1mL of fixation buffer (BD, UK) at 1x concentration and vortexed. The cells were wrapped in foil and were incubated for 10 minutes at room temperature.

The wash stage was again repeated. The cells were then resuspended in 500µL of permeabilisation buffer (BD, UK) and vortexed. The cells were then covered in foil and incubated for 30 minutes at room temperature before being washed again. Once the supernatant had been discarded the intracellular FoxP3 was added and mixed. The cells were covered in foil and incubated for 30 minutes at room temperature before being washed twice. After the final wash step the cells were resuspended in 500µL of FACS flow and acquired on the flow cytometer immediately.

3.10.14 FoxP3 Optimisation

The FoxP3 staining protocol was developed late during the project after an error was made during cytometric analysis. Unfortunately, this meant all FoxP3 data excluding 2 patients in the PROGRAM cohort had to be removed. Treg data has been phenotyped using CD25 and CD127 instead. The difference was the initial protocol used an intra nuclear FoxP3 fix/perm buffer kit from Biolegend, UK, compared with the new protocol which used a human FoxP3 buffer set from BD Biosciences. Incubation times and concentrations of buffer were identical; therefore, it is unknown as to why the initial buffer did not produce any FoxP3 staining. Figure 3.10.14.1 shows the differences between FoxP3 staining. The new protocol produces a DP cluster of CD25^{high} FoxP3⁺ cells verified by the adjacent FoxP3 histogram, whereas data from the old protocol lacks this population.

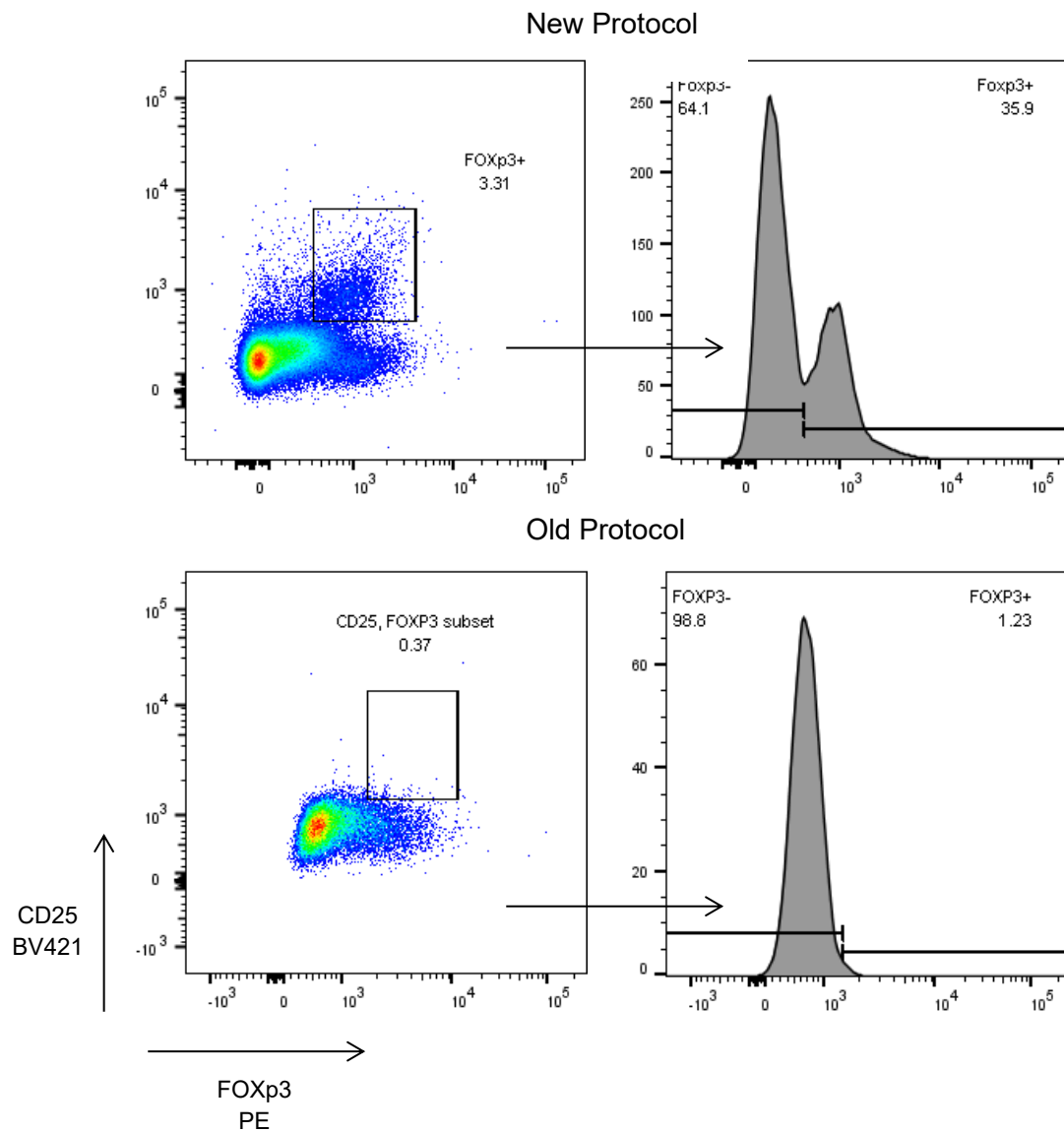


Figure 3.10.14.1: FoxP3 staining

The updated FoxP3 staining protocol (top) showing a cluster of CD25^{high}, FoxP3⁺ cells confirmed by the adjacent histogram showing two distinct populations, whereas the old protocol (bottom) taken from a patient sample shows no DP cells and only one peak on the adjacent histogram showing no staining.

3.10.15 Fluorescence Minus One Controls

Background noise was measured using fluorescence minus one (FMO) controls on bright lasers, such as PE shown in Figure 3.10.15.1 containing cytokine antibodies. For the intracellular cytokine staining each FMO control required one antibody assigned to a specific laser to be left out of the master mix.

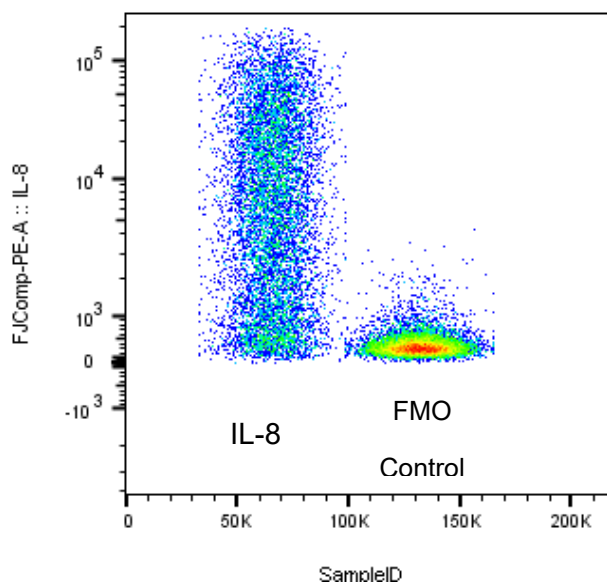


Figure 3.10.15.1: FMO control

FMO control (right) showing no IL-8 staining compared with the staining from the experimental sample (left). Both were stimulated with 50ng/mL of LPS.

3.10.16 Antibody Titrations

Antibodies were titrated based on the manufacturer's recommended volumes in order to find optimal staining with the least background interference. PBMC were separated using the methodology outlined in Section 3.2 and cells were obtained from non-pregnant female volunteers. 10×10^6 cells/mL was divided into 5x200 μ L aliquots for the titrations. A serial dilution of 10 μ L, 5 μ L, 2.5 μ L, 1.25 μ L and an unstained control was used to calculate the stain index shown in Figure 3.10.16.1 The volume which yielded the greatest stain index was used for experiments.

$$\text{Stain Index} = \frac{[\text{MFI1} - \text{MFI2}]}{2 \times \text{SD}}$$

Figure 3.10.16.1: Stain index

Stain index was calculated for each dilution factor and plotted against each other. The highest value obtained was recorded as the optimal volume and was used in experiments.

3.10.17 LPS Titration

Due to the potency of LPS as a TLR-4 agonist, LPS was titrated to minimise a cytokine storm. Dilutions of up to 1000ng/mL down to as low as 0.1ng/mL were tested out and staining was compared between fresh and frozen PBMC. Optimal LPS concentration was determined to be 0.5ng/mL to be used on frozen cells. The stain index shown in Figure 3.10.17.1 was used to verify IL-8 production from CD14⁺ Monocytes. Peripheral blood was taken from the same donor.

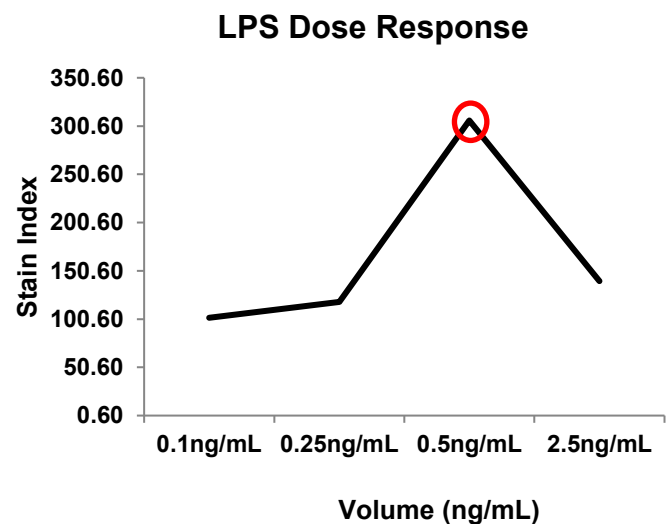
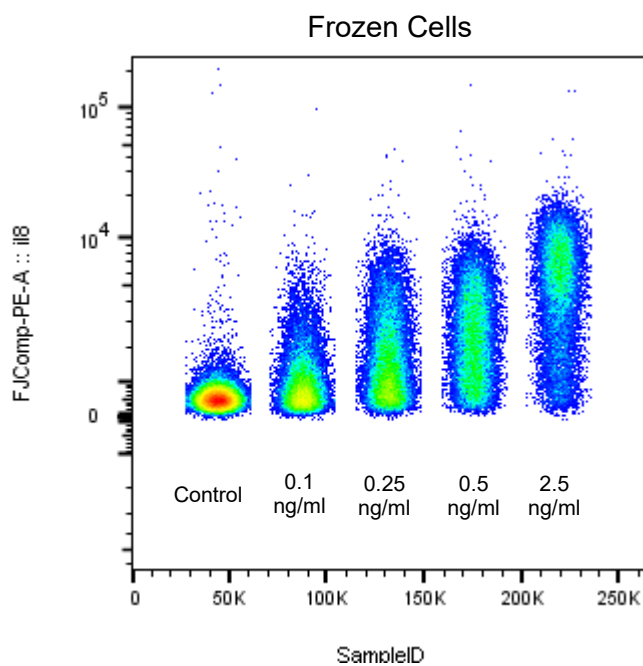
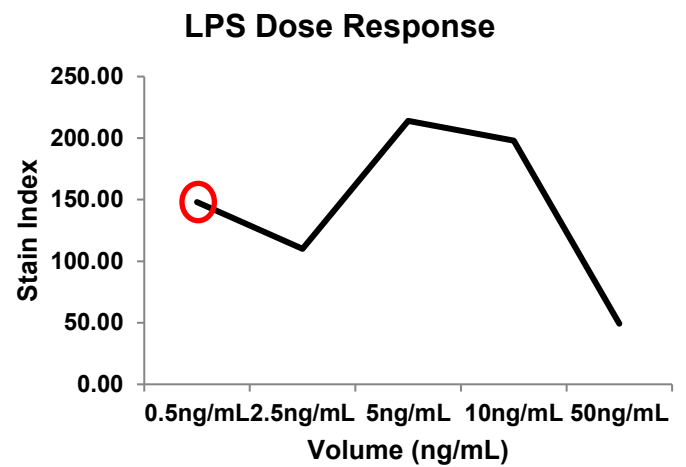
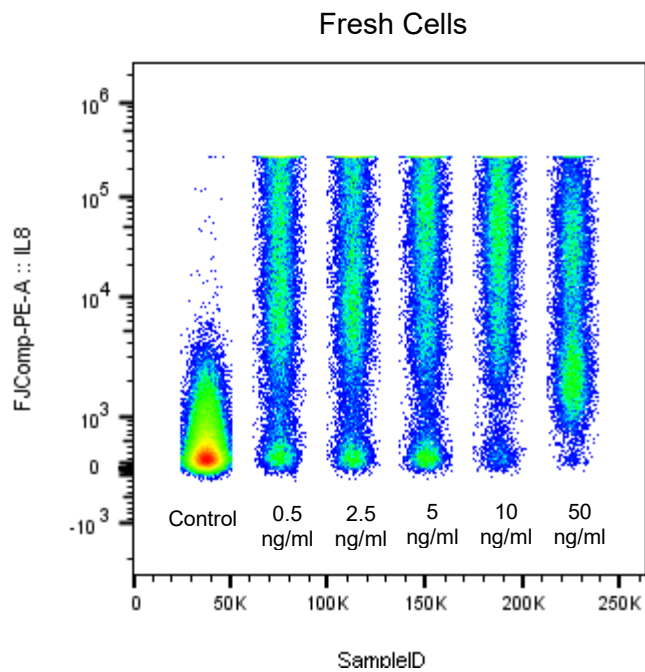


Figure 3.10.17.1: LPS titration and Dose Response

Fresh cells (top) showed the highest stain index at 5ng/mL, however, stain index decreased at 2.5ng/mL then started to increase at 0.5ng/mL. When compared with frozen cells (bottom) stain index was highest at 0.5ng/mL therefore, this was the concentration of LPS chosen for assessing innate cell immune function.

3.10.18 LPS Incubation

Cell viability decreases with cryopreservation for an extended period of time, especially when cells are not stored in liquid nitrogen which was an option not available during this project. In addition, the potency of LPS as a stimulant can cause cell death from prolonged exposure in culture, therefore, a time course was implemented in order to minimise cell death without the loss of sufficient cytokine production. Time trials of 6, 12 and 24 hours were conducted on frozen cells taken from the same donor and incubated with a range of concentrations from 0.5ng/mL to 50ng/mL of LPS. 6 hours was found to yield the highest number of viable cells (97.6%) shown in Figure 3.10.18.1.

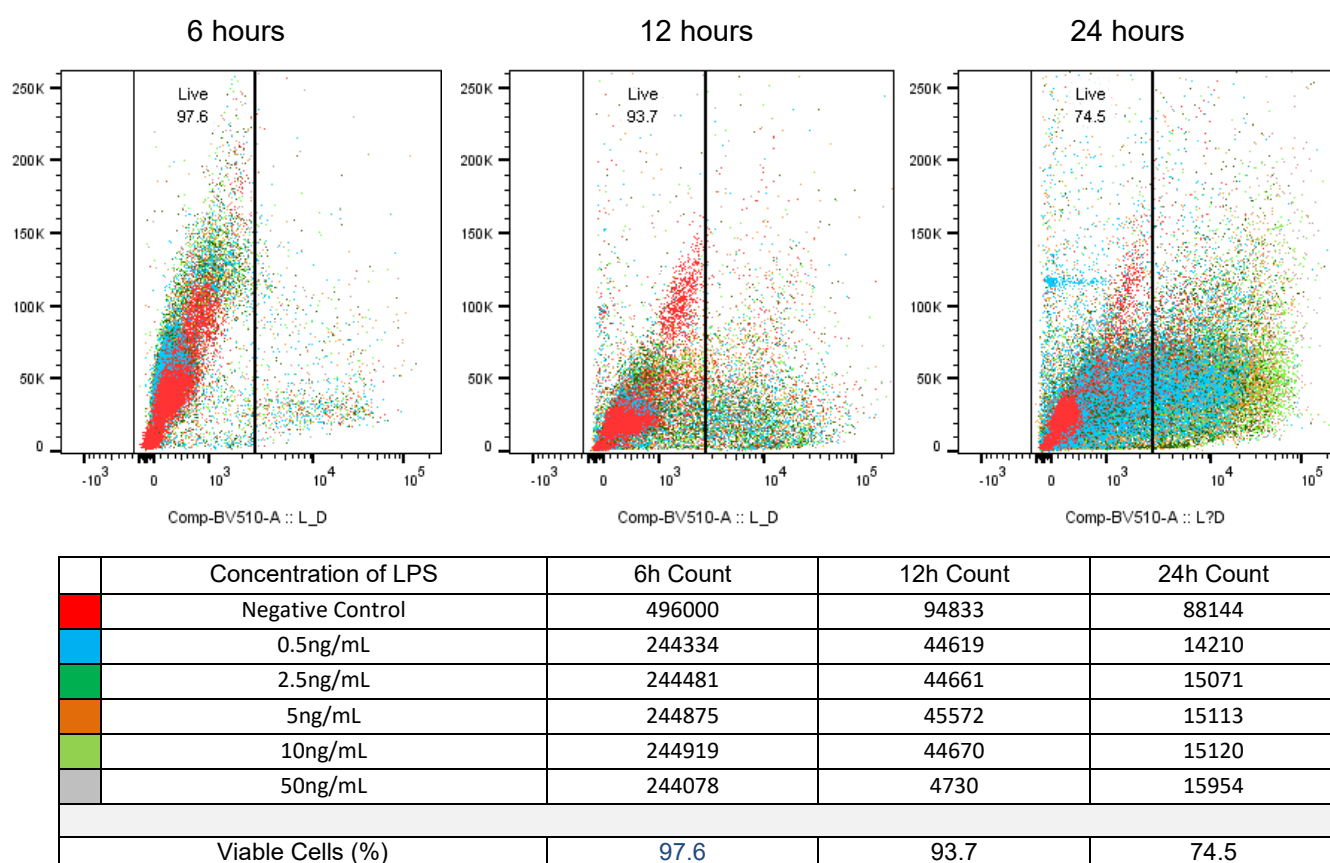


Figure 3.10.18.1: LPS time course

Cell viability 6, 12 and 24 hours (left to right respectively) after LPS stimulation using L/D viability dye was conducted. 6 hours yielded the most viable cells and was therefore used.

3.10.19 Aminophylline Dose Responses

Before starting the assay the following concentrations were made up for theophylline and P4:

Table 3.10.19.1: The dilution of theophylline and P4 to working concentrations of 1000 μ M and 1000nM respectively.

Reagent	Company	Catalogue Code	Stock Concentration	Working Concentration	Relative Molecular Mass
Theophylline	Sigma-Aldrich	A1755	100mM	1000 μ M	210.21 g/mol
Aliquots of 21mg of powdered theophylline was weighed out and placed in an eppendorf and sealed with parafilm as theophylline is very sensitive to moisture in the air. When ready to be diluted to the working concentration of 1000 μ M. 1 mL of deionised water was added to the eppendorf [100mM]. 100 μ L of this solution was diluted in 9.9mL of deionised water [1000 μ M].					
Progesterone	Sigma-Aldrich	P0130	2000 μ M	1000nM	314.46 g/mol
31.4mg of powdered progesterone was dissolved in 50mL of deionised water [2000 μ M]. 25 μ L of the solution was added to 49975 μ L of deionised water giving a working concentration of [1000nM].					

The Am dose response assay was used to determine whether culturing peripheral monocytes with either: P4, Am or a combination of P4/Am at varying concentrations would modulate pro-inflammatory cytokine production *in vivo*. PBMC revival step protocol was followed and cell viability checked. 1×10^6 cells were aliquoted into a 96-well plate including a positive and negative control plus the addition of a combination treatment in which only the supernatant would be collected and stored at -80°C for later analysis. Using the working concentrations for theophylline and P4 shown in Table 3.10.19.1 each experimental well was cultured with the concentrations shown in Figure 3.10.19.1. Reason for chosen concentrations of theophylline and P4 are discussed in Chapter 6 Sections 6.1.7 – 6.1.9 RPMI sup was used to make up all wells to a total volume of 200 μ L. The LPS positive control was made up to a concentration of 50ng/mL. 0.5 μ L of Brefeldin A (Biolegend, UK) was added to each well excluding the supernatant collection well, as this was the condition used to simulate serum

concentrations of P4 and Am *in vivo* from the dosages used during the PROGRAM study. The plate was then placed into a 37°C, 5% CO₂ incubator and left for 6 hours. Cells were harvested with 200µL of FACS flow and washed with 2mL of FACS flow. Supernatant was discarded for all excluding the supernatant collection well which was aliquoted and stored at -80°C. Surface staining antibodies shown in Table 3.10.1.6 were added to the cell suspension each at a 1/1000 concentration. Cells were covered in foil and incubated for 30 minutes at 4°C. 2mL of FACS flow was used to wash the cells before being centrifuged at 400xg for 5 minutes at room temperature. The remaining procedure is identical to the permeabilisation step found in Section 3.5.

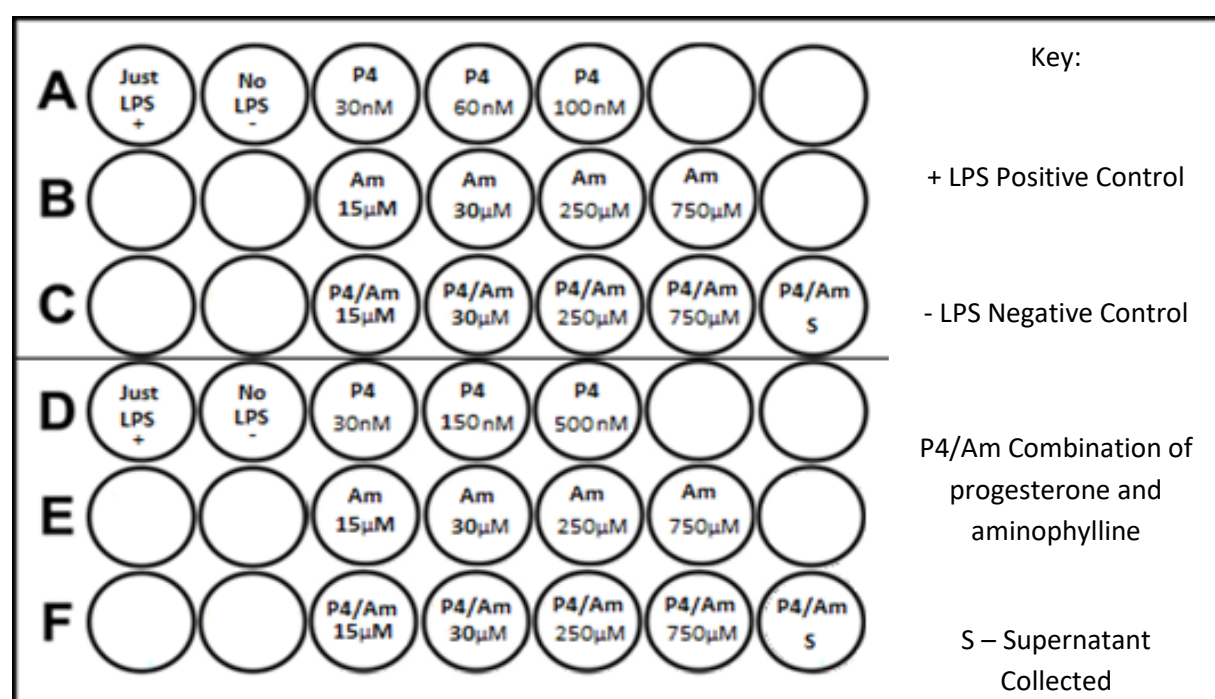


Figure 3.10.19. 1: Aminophylline dose response

96-well plate showing all experimental conditions of the dose response assay. Each of the 'P4'; 'Am' and 'P4/Am' wells contained 1×10^6 leukocytes along with 0.5ng/ml of LPS. The remainder of the 200uL volume/well was made up of RPMI media and the corresponding amount of P4 and Am required to meet the concentrations listed for each of these wells. The LPS + well contained LPS at a concentration of 50ng/mL and the LPS – well contained no LPS. The concentration used for the 'P4/Am S' well was 30nM and 30µM of P4 and Am respectively. Rows A-C were PBMC isolated from healthy pregnant women; Rows D-F isolated from non-pregnant women.

4.0 The Immune System in Women Presenting with Threatened Preterm Labour

4.1 – Introduction

Threatened preterm labour (TPTL) is diagnosed when a woman has painful uterine contractions between 24 and 36⁽⁺⁶⁾ weeks of gestation [152]. Approximately 5% of these women will deliver within 10 days from the onset of the contractions, and the remaining 95% will deliver at term [152]. Tocolytics such as Atosiban, an oxytocin receptor antagonist, or Nifedipine, a calcium channel blocker, are administered to delay the onset of labour by 48 hours to enable the administration of antenatal steroids to promote fetal lung maturation [153]. However, these drugs only have a temporary effect and are ineffective at delaying labour past 7 days [154].

The mechanisms underlying the activation of labour are still to this day not fully understood. Inflammation is responsible for some forms of PTB, with our group showing that the intensity of reproductive tissue inflammation varies between different PTL phenotypes including placental abruption, chorioamnionitis, stretch (multiple pregnancy or polyhydramnios) and idiopathic [155-157]. Babies born preterm are at risk of significant morbidity and mortality [91]. While modern advancements in neonatal medicine have reduced the mortality rates of PTB, these infants are still at risk of developing life-long respiratory and neuro-developmental disability [158-160].

In routine clinical practice, women with history of cervical surgery, previous mid-trimester miscarriage or PTB are monitored in PTB surveillance clinics to reduce their risk of PTL in any subsequent pregnancy. However, 85% of women who give birth preterm do not have any predefined risk factors and so are not identified to be at risk

and their first presentation will be with PTL. For these women presenting in the acute setting with symptoms of PTL, the only clinical tools available to assess their risk of preterm delivery (PTD) are an ultrasound assessment of the cervical length and the fetal fibronectin (fFN) test [161-163]. For these women, the QUIPP app provides a method of triaging women who are at a high-risk of PTL and PTB [164]. This app uses a combination of cervical length and/or quantitative vaginal fFN levels to determine the individual's risk of PTB. Acute management is based on preparing and optimising the health of the neonate by the administration of steroids and magnesium sulphate to the mother in order to reduce the risk of respiratory distress syndrome and cerebral palsy in the neonate [165-167]. It is therefore important to elucidate a way of identifying which women with signs and symptoms of PTL will go onto deliver preterm and whose babies are at risk of the complications of prematurity. It is important to note that, delaying birth in every circumstance is not always the better option due to the heterogeneity of labour, for example, during infection where the baby is subjected to a hostile environment due to inflammatory producing bacteria, delaying labour will not help promote neonatal health or survival. In terms of a treat all strategy, Watson *et al* compared 188 women presenting with TPTL (< 30 weeks) with 'at risk' thresholds for spontaneous preterm birth (sPTB) of 1%, 5% and 10% within 7 days using the QUIPP app. If an 'at risk' threshold of 5% had been used for women presenting with TPTL between 24 and 29⁺⁶ weeks' gestation, then 89.4% (n = 168) of admissions would have been avoided [168]. That being said, while the fFN test is a good triaging tool for women who have symptoms of abdominal pain, it is less effective in identifying asymptomatic women [169]. Therefore, a way of identifying who is at risk of PTL in advance, based on their immune profiles and treating them accordingly to provide the best chance of survival for the mother and the fetus is something which this study aims to achieve.

4.1.1 The Immune Environment during High-risk Pregnancy

One hypothesis is that spontaneous preterm labour (sPTL) is caused by the early breakdown of maternal-fetal tolerance that results in an increase in maternally driven inflammation in the chorio-decidual interface, precipitating the onset of PTL [170]. The inverse may also be true as there is evidence that there is a loss of fetal tolerance for peripheral maternal antigens, again resulting in inflammation at the maternal-fetal interface, activating pro-labour pathways which drive uterine contractions and the onset of PTL [95]. Frascoli and colleagues compared cytokine production and activation of both T cells and dendritic cells (DC) in cord blood to peripheral blood in preterm versus term neonates. Interestingly, higher activation in DC from preterm infants in cord blood was observed, added to this CM cells in preterm infants had a more pro-inflammatory phenotype when compared to term infants [95]. Furthermore, production of INF- γ and TNF- α released by fetal T cells in the preterm infants stimulated contractility in myometrial cells [95].

To support growth and development of the foetus, the maternal immune system is modulated to an anti-inflammatory posture to create an optimal environment for fetal growth and development, with an increase in immune suppressive cells such as Tregs [171]. The Treg influence is reversed with the onset of labour, with the maternal immune system switching to a pro-inflammatory status, promoting pro-labour gene expression [172], with the infiltration of neutrophils, monocytes and macrophages [124, 173, 174]. A possibility of sPTL is that there is a dysfunctional connection between the innate and adaptive immune system causing this shift from anti to pro-inflammatory to occur earlier in the pregnancy [175].

To date, research into TPTL has been sparsely researched in terms of immune cell phenotyping. In the peripheral circulation, early literature has described an increase in systemic white blood cells with gestation during normal pregnancy [176]. However, only some of these immune cell types have been associated with pregnancy complications and PTB. This study will aim to elucidate certain peripheral and cervical immune cell phenotypes which have not previously been looked at in women presenting with TPTL or compared their immune cell profiles with healthy pregnant women.

4.1.2 Acquired Immune System in Preterm Labour

It is well established that the maternal immune system plays a key role in pregnancy, from embryo implantation, fetal-maternal tolerance, protection of the foetus and parturition [77, 177]. As discussed earlier, maintaining maternal tolerance of fetal antigens and fetal tolerance of maternal antigens is essential for the pregnancy to develop until term and an early collapse of this tolerogenic state may be associated with PTL [123]. This is why the adaptive immune system plays such an important role in pregnancy.

Maternal Tregs are thought to be important mediators of fetal tolerance in pregnancy [178]. In the peripheral circulation, maternal Tregs have been shown to increase as gestation progresses in both humans and mice [179, 180]. Recent findings have also shown that Tregs are involved in the establishment of tolerance towards fetal antigens by suppressing inflammatory maternal effector T cells [111]. With that being said Treg activation and altered function is also an important feature of labour onset in both term and PTL. A study published by Schober *et al* examined the suppressive activity of peripheral Tregs in 65 pregnant women during their third trimester who were at risk of

going into PTL [181]. They showed that women who went on to deliver their babies preterm, had significantly lower HLA-DR⁺ expressing Tregs when compared to those who delivered at term at point of hospital admission [181, 182]. They went on to measure the suppressive activity of these Tregs and found that their function was strongly diminished when compared to the women who delivered spontaneously at term [181]. Other studies have showed decreased Treg numbers in the circulation and decidua in women who had recurrent miscarriage when compared to healthy pregnant controls [183, 184].

As gestation progresses, elevation in Em CD4⁺ and exhaustion markers PD-1 and PD-L1 on T cell lymphocytes are observed systemically in normal pregnancy [179]. However, there are data to suggest that changes in the proportions of Em and Cm CD4⁺ and CD8⁺ T cells are linked to pregnancy complications such as preeclampsia [185], which is a condition involving chronic activation of immune cells leading to increased pro-inflammatory production [186]. Exhausted or senescent T cells, expressing inhibitory and exhaustion markers, prevent the optimal control of infection, are poorly proliferative and display a severe lack of function [54, 187]. This phenotype of T cells may influence maternal immune responses to infection and inflammation that are thought to drive some types of PTB. In addition, pregnancy complications have been associated with dysregulation of Tim-3 expression, which is another inhibitory marker, on peripheral effector T cells [103].

Along with the suppressive effects of the adaptive immune system, the cytotoxic responses in the circulation have also been studied in pregnancy. Interestingly, a study found increased peripheral CTL in women who had chronic chorioamnionitis when compared to women who did not [188]. This subset of T cell may have a role in the association of PTB.

Another subset of T cells which may have an interesting role in pregnancy are the natural killer T (NKT) cells. These cells are considered to be a bridge between the innate and adaptive immune system [50]. Specifically, NKT cells expressing the iNKT receptor, V α 24-J α Q, make up a small proportion of peripheral immune cells [138] and stimulation of these cells activates the nuclear factor κ B (NF κ B) signalling pathway leading to cytokine and chemokine production [52]. In the clinical setting, activation of iNKT cells is linked with PE [189]. However, there is conflicting evidence with another study which failed to replicate this finding [49].

4.1.3 Innate Immune System in Preterm Labour

In PTL, there are several underlying causes of pregnancy related complications; however, inflammation and infection represent high-risk factors in the development of PTB [190]. Labour is an inflammatory process [172, 191] which involves an influx of neutrophils, monocytes and macrophages into the reproductive tissues and fetal membranes [124, 173, 174]. Systemically, subsets of peripheral leukocytes become activated [192]. However, early activation of innate immune cells causes them to secrete pro-inflammatory cytokines and chemokines which can compromise the fetal-maternal tolerance and lead to PTL [193]. This phenomenon mainly occurs in the presence of intrauterine infection. In around 40% of PTL cases, intrauterine infection is thought to be the main mediator [194] and in fact, chorioamnionitis occurs following bacterial invasion in gestational tissue [195]. Importantly, neonates born as a result of chorioamnionitis, are at a significantly higher risk of developing cerebral palsy, and the combination of prematurity and infection is an important cause of neonatal mortality [196]. Additionally, these preterm infants possess a reduced pool of innate immune cells in their circulation with impaired function, which restricts T cell activation

increasing their susceptibility to infection [193]. Peripheral neutrophils play an important role in the pathology of sPTL especially in cases of acute chorioamnionitis [197]. This is because migration of neutrophils into the fetal membranes, causing chorioamnionitis, results in an intense inflammatory response, which is associated with the onset of PTL [198, 199].

The function of monocytes in relation to pregnancy and PTB is still being explored. TLR-4 is present on the cell surface of monocytes/ $m\phi$ and its activation initiates the production of pro-inflammatory cytokines via the NF κ B signalling pathway [200]. Two studies have shown that increased activation of TLR-4 receptors on the cell surfaces of CD14⁺ monocytes is associated with PTL [112, 201]. In addition to these findings, elevated circulating non-classical and intermediate monocytes in peripheral blood have been associated with other conditions such as PE in both human and rat models [202]. Therefore, it appears that increased activation of monocytes during pregnancy may cause problems and lead to iatrogenic PTB [113].

Macrophages are tissue resident cells that play a crucial role in late gestation and parturition [191, 203]. In mice models, uterine macrophages increase significantly prior to labour when compared to non-pregnant controls and may have a role in myometrial contractility [204]. Interestingly, depletion of macrophages in mice prevented LPS-induced preterm birth [205]. These macrophages have been shown to be the main source of metalloproteinase-9 (MMP-9) which has a crucial role in remodelling the extracellular matrix around the cervix to prepare for cervical ripening (shortening and opening) that occurs at the onset of labour in both in humans and mice [205, 206]. CCL2 is a chemoattractant which recruits macrophages into sites of inflammation [207] and its elevation in amniotic fluid is associated as an early marker for the onset of labour [208]. A study by Esplin *et al.* demonstrated that CCL2 was higher in the

amniotic fluid of women presenting with TPTL who went on to deliver preterm, when compared to women who delivered at term, both in the presence and absence of infection [209].

Natural killer (NK) cells play a crucial role in pregnancy, they are by far the most abundant cells found in the human decidua [133] and these uNK cells have been well documented in pregnancy. However, pNK cells have a different phenotype and are functionally distinct to uNK cells [131]. The majority of uNK cells resemble cytokine producing CD56^{bright} pNK cells but express surface and transcription markers specific to tissue resident innate lymphoid cells [210]. Normal pregnancy is associated with a decreased percentage frequency in pNK numbers due to a hormone-induced decline in CD16⁺ cells [211, 212]. On the other hand, elevation of CD56^{bright} and CD56^{dim} CD16⁺ NK cells in peripheral blood is associated with recurrent pregnancy loss, despite abnormal levels of P4 [212]. This implies that NK cell numbers are modulated by multiple pregnancy related hormones and not just P4. The ability for NK cells to function without prior activation is mediated by NCR; NKp46, NKp44 and NKp30 [86]. These NCR have been postulated to initiate activation and the production of growth factor vascular endothelial growth factor (VEGF) which aid in the process of angiogenesis in uNK cells [213, 214]. Unlike in uNK cells NKp44 is not expressed in peripheral NK cells [215]. Whilst this is not relevant in the peripheral circulation, in decidua a key function of uNK cells is to support both immune regulation and induce vascular growth of gestational tissue during pregnancy [216]. NKp46, on the other hand, is an important NK cell activating receptor that enables recognition and elimination of virus and cancer cells among others [217]. Recently, excessive NKp46 expression in peripheral NK cells is associated with first trimester losses [218]. In terms of NK cell activation, a study showed that non-pregnant women who had a

history of recurrent pregnancy loss had increased CD69 expression on pNK cells [219]. Another study showed the same increased expression of CD69 in pregnant women with a history of recurrent pregnancy loss when compared to healthy non-pregnant controls [220]. Interestingly, P4 supplementation is not associated with changes in frequency and activation of pNK cells at differing stages of pregnancy, despite findings that P4 up-regulates progesterone-induced blocking factor (PIBF), which is shown to decrease cytotoxicity in CD56^{bright} NK cells [146, 221]. This suggests that P4 supplementation during pregnancy may repress NK cell function.

4.1.4 The Immunological Phenotype of the Cervix

Throughout pregnancy the cervix and its mucus undergoes considerable changes, yet there are very few studies which have looked into how, from a phenotypic point of view, this occurs as gestation progresses. A study conducted in 2016 by Hunter *et al* looked at the cervical leukocyte population in 120 women who previously had a sPTB. The analysis of a single cervical cytobrush sample taken from the cervical canal of these pregnant women between weeks 12-25 of gestation showed that circa 90-100% of the cervical mucus was composed of polymorphonuclear (PMN) cells with the remaining 10% being monocyte/m ϕ [222]. Cervical mucus supernatant was also analysed for cytokine and chemokine concentrations using a multiplex immunoassay. Results of this study showed that lower levels of monocytes and CCL2 were present in the women which gave birth before term. Lower levels of CCL2, a potent monocyte attractant [207], reduce monocyte migration towards sites of inflammation or tissue damage and result in reduced wound healing or infection [207]. In the process of PTL, the frequency and activation of monocytes has been shown to increase in the periphery, in particular, elevated levels of CD14⁺ non-classical monocytes have been

observed along with increased expression of TLR4 [223], a Toll-like receptor responsible for the induction of genes which code for pro-inflammatory responses by monocytes. However, PTL immune cell phenotype locally in the cervix has not been well documented in the literature, in fact phenotypic changes in cervical mucus has mainly been documented with the studies of HIV and other sexually transmitted illnesses where impaired function of these immune cells is associated with an increased risk of transmission and infection [224]. Interestingly, a study conducted by Trifonova *et al* identified the composition of immune cells in the ecto-cervix and the endocervical canal from non-pregnant women. The majority of cells found in the ecto-cervix are macrophages which make up around 37-55% of CD45⁺ cells with approximately 30% of these macrophages being CD14⁺ [225]. A similar composition of macrophages was observed in the endocervical canal. In addition, CD3⁺ T cell lymphocytes make up around 35% of the overall cell composition with a split of 40:60 between CD4⁺ and CD8⁺ cells respectively in the ecto-cervix, whereas, in the endocervical canal around half the numbers of CD3⁺ T cells was observed [225]. Neutrophil composition is only around 10% of CD45⁺ cells [225] with the remaining cells being distributed between NK cells, DC and B-cell lymphocytes in the ecto-cervix [225]. These cell types were similarly abundant in the endocervical canal, with the exception of B cells which were lower [225].

Whilst there are differences in peripheral and mucosal immunity, the observations made in the literature regarding maternal circulation are perhaps indicative of what occurs in the cervix. Consequently, women presenting with TPTL are likely to possess immune profiles which display a more activated immune cell phenotype in cervical mucus, disrupting fetal-maternal tolerance and having a lack of immune cell numbers

required to fight infection. The novelty of this study will help to understand better the cervical milieu during high-risk pregnancy and how it differs from healthy pregnancy.

4.2 – Chapter Hypothesis

- i. High-risk pregnant women presenting with TPTL will have an altered phenotype of innate and adaptive immune cells in peripheral blood and cervical mucus when compared to healthy pregnant women.

4.2.1 – Chapter Aims

The aims of this chapter are as follows:

- i. To study the immune profiles in peripheral blood and cervical mucus in pregnant women who present with TPTL and compare the profiles of these who experience PTB versus those who give birth at term.
- ii. To compare the immune profiles in peripheral and the cervix of women presenting with confirmed TPTL to healthy pregnant controls.

4.3 –Processing and Methodology

4.3.1 Recruitment

Women with confirmed TPTL were recruited at the Maternal Assessment Suite (MAS) of Chelsea and Westminster Hospital. Healthy pregnant controls were recruited among women attending the outpatient antenatal clinic. Informed consent was taken from all participants. Ethics approval was obtained from the Brompton and Harefield research ethics committee (10/H0801/45). A high vaginal swab was taken for the fFN test and any excess fluid was collected into a separate sample tube. A cervical cytobrush (Rovers Medical Services, Netherlands) was used to collect immune cells located around the endocervical OS. 24mL of maternal peripheral blood was collected into 2x10mL Lithium-Heparin tubes along with a 1x4mL EDTA tube. The TPTL group was identified by either a raised fFN of over 50ng/ml and/or a cervical length that gave a QUIPP score of >5% risk of preterm birth within the subsequent week, or premature ruptured membranes. For woman presenting with premature rupture of membranes only peripheral blood was collected. Inclusion and exclusion criteria are listed in Table 4.3.1.1 Term birth (TB) and sPTB were retrospectively categorised from the women presenting with TPTL once they had given birth and data were then analysed. Gestation matched controls with a low risk of PTB were recruited from the antenatal clinic and those who gave birth at term were included in the analysis.

Table 4.3.1.1 - The inclusion and exclusion criterion which were used to identify both women presenting with TPTL and healthy pregnant women who were suitable to recruit for the study.

	Inclusion and Exclusion Criteria
Inclusion Criteria (TPTL)	Recruitment from Maternal Assessment Suite (MAS): 1) Age 18-40 at time of consent 2) Singleton pregnancy 3) Painful uterine contractions between 24-36 ⁺⁶ weeks of gestation 4) +fFn result (>50ng/ml) 5) Women presenting with PPROM or SROM [~]
Inclusion Criteria (Healthy)	Recruitment from Antenatal Clinical: 1) Age 18-40 at time of consent 2) Singleton pregnancy 3) No history of PTB 4) No previous mid-term loss 5) -fFn result (<50ng/ml) 6) No ruptured membranes
Exclusion Criteria	Patients not suitable for the study were determined by: 1) Vaginal bleeding – Risk contaminating the cervical vaginal fluid and cervical immune cells 2) Sexual Intercourse in the last 24 hours - Risk contamination from paternal derived antigens 3) PPROM/SROM [~] - fFN is not taken 4) Cervix is dilated to 3 cms - Women is in active labour already 5) Cervical cerclage – Can interfere with the fFN test causing false positive results. 6) Placenta praevia – Can risk rupturing the placenta with the brush potentially causing haemorrhage.

[~] Peripheral blood still taken but no swabs or cytobrush taken.

4.3.2 Maternal Blood Sample Processing

Maternal peripheral blood immune cells were collected into lithium-heparin tubes and isolated using methods described in chapter 3: Section 3.2 and subjected to flow cytometric analysis using the panels outlined in chapter 3: Tables 3.10.1.1 – 3.10.1.5. All cytometric analysis was run on fresh blood. Maternal plasma was aspirated and stored at -80°C for later analysis and 1×10^7 PBMC were cryopreserved using the methods described in Chapter 3 Section 3.6.

4.3.3 Maternal Vaginal Swab and Cytobrush Processing

Maternal immune cells collected with the cervical cytobrush and isolated using the methods described in chapter 3 Section 3.9 for flow cytometric analysis using the panel outlined in chapter 3: Table 3.10.1.5. Excess mucosa collected from the brush was diluted in RPMI-1640 sup and stored at -80°C. Excess fluid taken from the fFN test was also transferred to an eppendorf and stored at -80°C.

4.3.4 Demographic Data of Women Presenting with TPTL

Table 4.3.4.1 displays the maternal demographic, obstetric and pregnancy outcomes data of all cohorts: TPTL who delivered preterm (sPTB; N=9), TPTL who delivered at term (TB; N=5) and control subjects (Healthy controls; N=4). Due to the pilot nature of this study and the rarity of patients being recruited who met the criteria outlined in Table 4.3.1.1 for this study, it is important to note that these N numbers are too low to draw any meaningful statistical findings from.

Data on immune cell counts and markers of activation were compared between women presenting with TPTL who delivered preterm (sPTB) against women who delivered at term and healthy women. With respect to birth outcomes, the median gestational delivery of the TB women revealed that parturition occurred 17 days on average before the healthy control group, suggesting that the immune response of these women might show both term and preterm characteristics. Furthermore, onset of labour was unknown for a total of 4 women across all 3 groups. This was due to the fact that these women initially presented to MAS at Chelsea and Westminster hospital, however, subsequently delivered at a different hospital.

Table 4.3.4.1 – Maternal demographic, obstetric and pregnancy outcomes data for recruited women presenting with TPTL and healthy pregnant controls.

Demographic and obstetric data	(TPTL) sPTB (n=9)	(TPTL) Term Birth (TB) (n=5)	Healthy pregnant controls (n=4)
Median maternal age	34 (29-40)	31 (26-36)	32 (22-39)
Ethnicity, n (%)			
Other	5 (55.5)	2 (40.0)	2 (50.0)
White	4 (44.4)	3 (60.0)	2 (50.0)
Median gestational age at recruitment, (weeks)	31 ⁺⁴ (24 ⁺³ -35)	32 ⁺¹ (27-35 ⁺¹)	28 ⁺¹ (24-35 ⁺³)
Median fetal fibronectin level (ng/ml)	261 (137-500)	440 (320-500)	6 (4-8)
PROM, (%)	4 (44.4)	1 (20.0)	0 (0.0)
Median BMI at recruitment (kg/m ²)	22 (19-34)	22 (18-24)	24 (20-31)
Parity, n (%)			
0	2 (22.2)	2 (40.0)	3 (75.0)
≥1	7 (77.7)	3 (60.0)	1 (25.0)
Previous PTB, n (%)			
0	5 (55.5)	5 (100.0)	4 (100.0)
≥1	4 (44.4)	0 (0.0)	0 (0.0)
Pre-eclampsia, n (%)			
Yes	1 (11.1)	0 (0.0)	0 (0.0)
No	8 (88.8)	5 (100.0)	4 (100.0)
Gestational diabetes, n (%)			
Yes	1 (11.1)	1 (20.0)	0 (0.0)
No	8 (88.8)	4 (80.0)	4 (100.0)
Pregnancy Outcomes			
Gender of Neonate, n (%)			
Male	3 (33.3)	2 (40.0)	2 (50.0)
Female	4 (44.4)	2 (40.0)	1 (25.0)
Unknown	2 (22.2)	1 (20.0)	1 (25.0)
Onset of Labour, n (%)			
Spontaneous	5 (55.5)	4 (80.0)	2 (50.0)
Induced	0 (0.0)	0 (0.0)	1 (25.0)
No labour	2 (22.2)	0 (0.0)	0 (0.0)
Unknown	2 (22.2)	1 (20.0)	1 (25.0)
Aetiology of Labour, n (%)			
Idiopathic	7 (77.7)	4 (80.0)	2 (50.0)
Infection	0 (0.0)	0 (0.0)	0 (0.0)
Polyhydramnios	0 (0.0)	0 (0.0)	0 (0.0)
Abruptum	0 (0.0)	0 (0.0)	0 (0.0)
Unknown	2 (22.2)	1 (20.0)	2 (50.0)
Median gestational age at delivery, (weeks)	32 ⁺⁶ (27 ⁺² -36 ⁺⁴)	37 ⁺⁶ (37-38 ⁺⁴)	40 ⁺² (39 ⁺³ -41)

Medians (m) followed by their corresponding (min to max) range, whereas, numbers (n) followed by (percentages).

4.3.5 Statistical Analysis

GraphPad Prism version 8.4.3 (San Diego, USA) was used to calculate all statistical analyses. In the TPTL study, the Shapiro-Wilk's test was firstly used to define data distribution. If the distribution of data was found to be parametric then a 1-way ANOVA and a Tukey's multiple comparison test was applied. However, if the distribution of data was non-parametric, then a Kruskal-Wallis 1-way ANOVA and a Dunn's multiple comparison test was applied to measure the variability between true PTL and the controls. All peripheral blood and cervical cell flow cytometry data was collected on BD FACS Diva version 8.0 (San Jose, California, USA) and analysed performed using FlowJo version 10.7.0 (Oregon, USA) expressed as a percentage of positive cells taken from leukocyte populations. Normally distributed data was displayed as mean and standard deviation, whereas non-normal data was displayed as median with a percentile range between the highest and lowest values. All statistical tests were two-tailed, and significance was defined as $p < 0.05$.

A paper by Alvarez and colleagues, demonstrated how flow cytometry data can be published. Flow cytometry data can either be expressed as MFI or by percentage of positive cells [226]. In this study, all flow cytometry data was expressed as a percentage of positive cells. MFI was not used to display flow cytometry data due to the high susceptible to experimental conditions, for example laser fluctuation over time [226]. Despite using the Perfetto method to minimise laser fluctuations, it is much easier to interpret flow cytometry data as a percentage rather than MFI for the studies represented in this thesis.

4.4 – Results

4.4.1 Treg Analysis

The peripheral Treg population was phenotyped as lymphocyte sized using forward scatter (FSC) and side scatter (SSC) parameters and were CD3⁺ CD4⁺ CD25^{high} and CD127^{low}. HLA-DR expression was used to measure activation (Figure 4.4.1.1).

No significant change in Treg composition was observed in the sPTB group (Figure 4.4.1.1.2). Additionally, HLA-DR activation was also shown to not change between groups.

4.4.1.1 Treg Gating

Channel	PerCP	BV421	BV510*	BV711	BV786	AF700	APH-H7	PE~
Antibody	CD8	CD25	Live/Dead	CD127	HLA-DR	CD3	CD4	FOXP3
Concentration	1/40	1/80	1/200	1/40	1/80	1/80	1/80	1/40

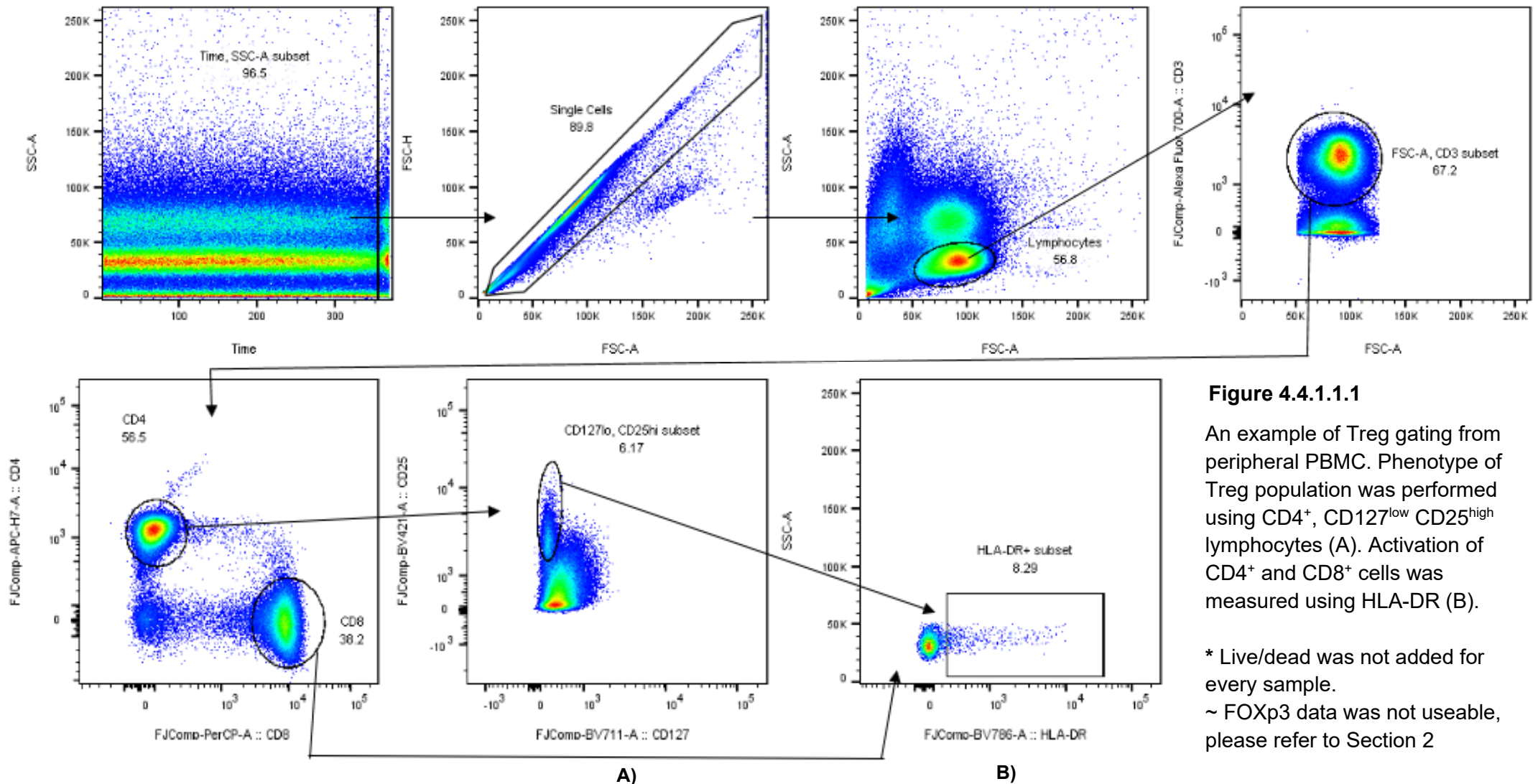


Figure 4.4.1.1.1

An example of Treg gating from peripheral PBMC. Phenotype of Treg population was performed using CD4⁺, CD127^{low} CD25^{high} lymphocytes (A). Activation of CD4⁺ and CD8⁺ cells was measured using HLA-DR (B).

* Live/dead was not added for every sample.
~ FOXP3 data was not useable, please refer to Section 2

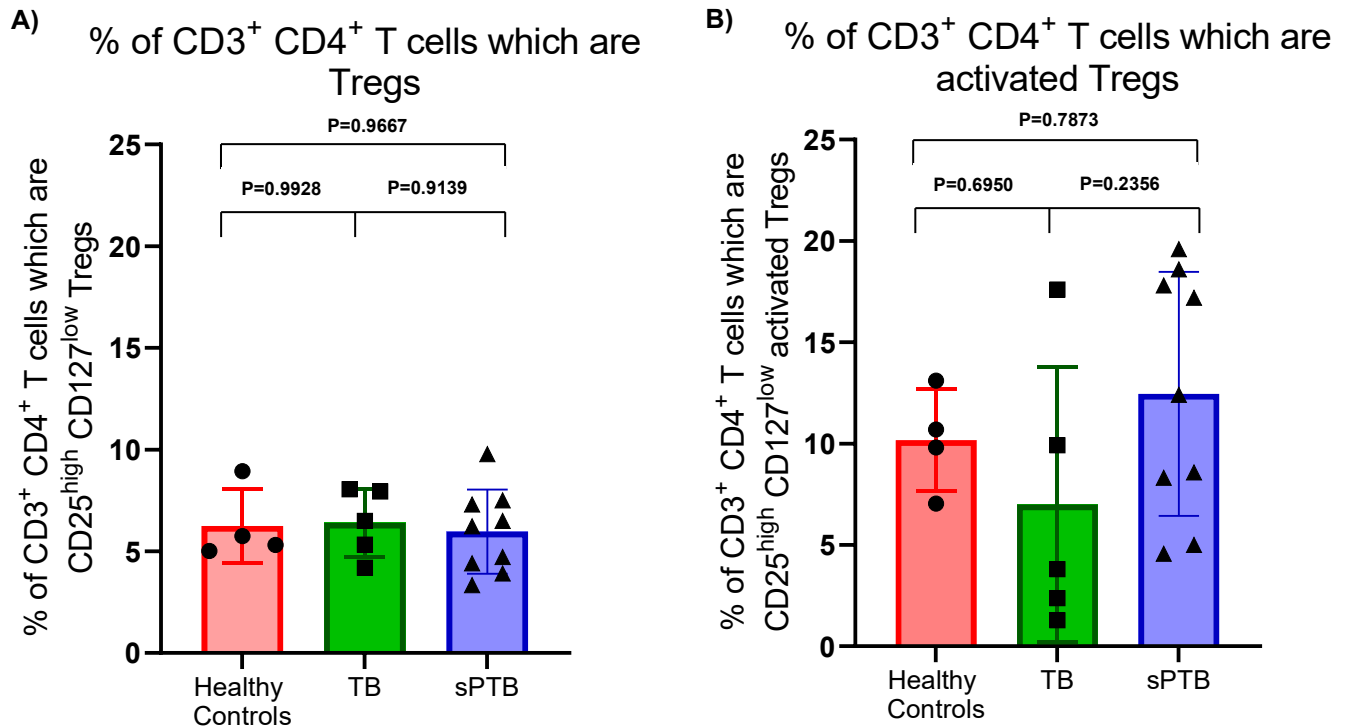


Figure 4.4.1.1.2: Percentage frequencies of CD3⁺ CD4⁺ T cells which are CD25^{high} CD127^{low} Tregs and HLA-DR activated Tregs.

These graphs compare CD3⁺ CD4⁺ lymphocytes which are CD25^{high} CD127^{low} Tregs (A) and activated Tregs (B) between sPTB patients (N=9), TB (N=5) and healthy controls (N=4) at point of hospital admission. Statistical analysis was undertaken using a 1-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P < 0.05.

4.4.2 Effector Cell Analysis

Effector T cells were defined as lymphocyte sized using FSC and side scatter SSC parameters and were CD3⁺. T-helper cells and CTL were differentiated by CD4⁺ and CD8⁺ respectively. Memory subsets were identified as: memory (CD45RO⁺ CD45RA⁻), transitioning memory (CD45RO⁺ CD45RA⁺), naïve (CD45RO⁻ CD45RA⁺), central memory (CM; CD45RA⁻ CCR7⁺), effector memory (Em; CD45RA⁻ CCR7⁻) and terminally differentiated CD45RA (TEMRA; CD45RA⁺ CCR7⁻) populations. For CTL alone the Em population was additionally divided into the following subsets using

CD27 and CD28 surface expression: Em1 (CD27⁺ CD28⁺), Em2 (CD27⁺ CD28⁻), Em3 (CD27⁻ CD28⁻) and Em4 (CD27⁻ CD28⁺) (Figure 4.4.2.1).

The percentage of CD4⁺ T cells was similar across all three groups and no significant differences were observed in naïve, transitioning or CD45RO⁺ memory cells from the CD4⁺ subsets. However, Cm CD4⁺ cells were significantly higher in the sPTB cohort when compared to the TB ($p=0.0104$) but no change in frequency was observed in the healthy controls ($p=0.8336$). In addition, TEMRA CD4⁺ cells were significantly lower in the sPTB cohort compared to the TB group ($p=0.0389$) (Figure 4.4.2.2.2). No difference was observed in Em CD4⁺ cells across all groups.

CTL data showed similar results to the CD4⁺ cells with no alterations in frequencies across the 3 groups. No significant differences were observed in naïve, transitioning or CD45RO⁺ memory subsets. However, central memory cells, like the CD4⁺ subset were significantly higher in the sPTB cohort when compared to the TB group ($p=0.0439$) (Figure 4.4.2.2.3). Again, CM frequency between the sPTB and healthy controls was unchanged ($p=0.9932$). CTL cells had no differences in EM 1-4 cell expression between groups and indifferent in TEMRA cells (Figure 4.4.2.2.4).

4.4.2.1 Effector Cells

Channel	AF488	PerCP	BV421	BV510*	AF647	AF700	APC-H7	PE	PE-Cy7
Antibody	CD45RO	CD8	CD28	Live/Dead	CD27	CD3	CD4	CD45RA	CCR7
Concentration	1/40	1/40	1/40	1/200	1/40	1/80	1/80	1/40	1/40

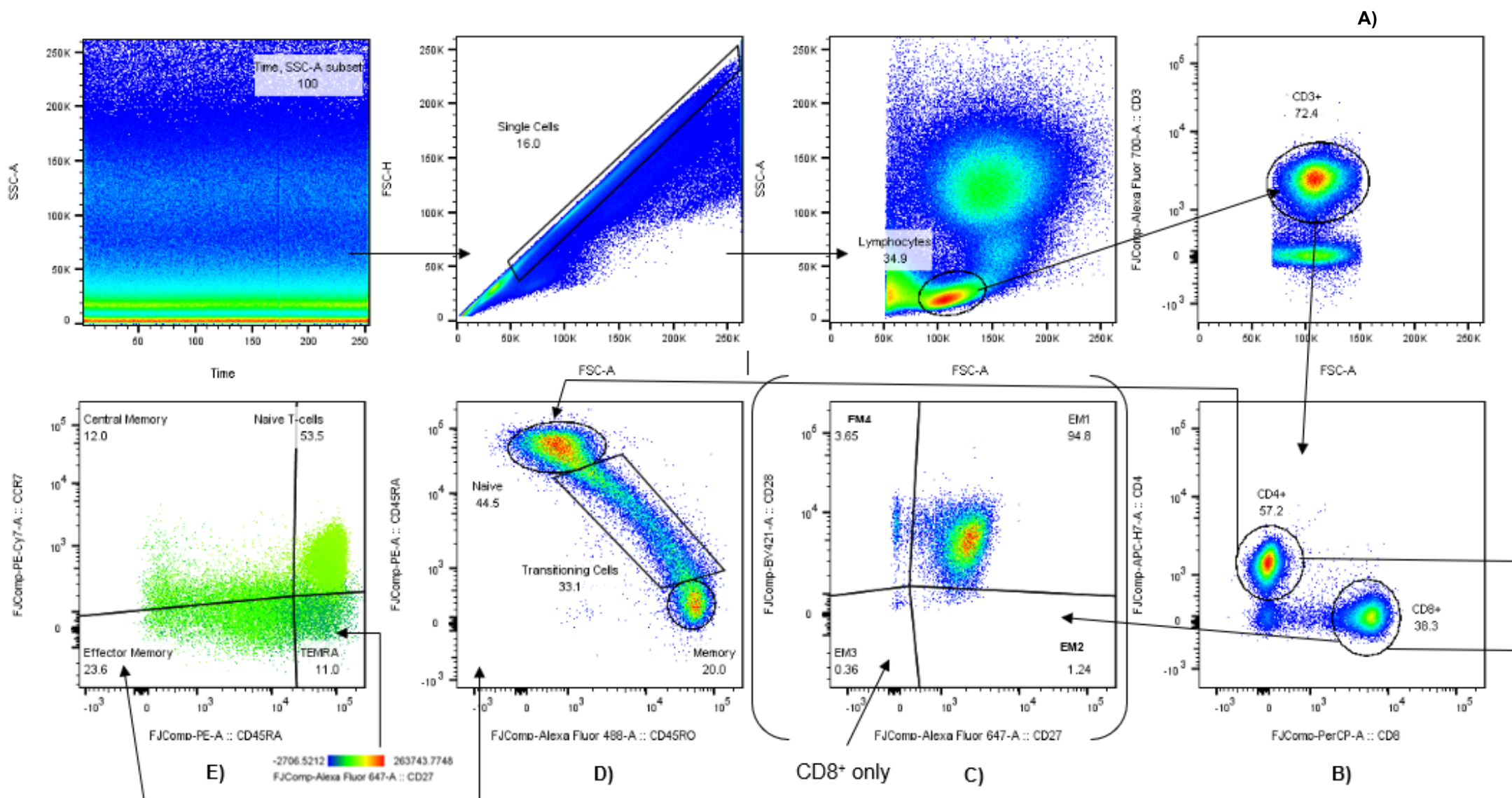


Figure 4.4.2.2.1: An example of effector cell gating. CD3 was used to separate T cells from NK cells, B cells and innate lymphoid cells (A). T-helper cells and cytotoxic T cells were differentiated using CD4 and CD8 (B). Effector memory cells 1-4 expression on CD8⁺ T cells using CD27 and CD28 was used (C). CD45RA and CD45RO were used to distinguish naive and memory T cells (D). Central memory, effector cells, TEMRA and naive cells were phenotyped using CCR7 and CD45RA expression. A colour axis was used to identify TEMRA cells based on their lack of CD27 expression in both CD4⁺ and CD8⁺ cell (E). Dark green areas show no expression of CD27 and light green shows areas of higher CD27 expression.

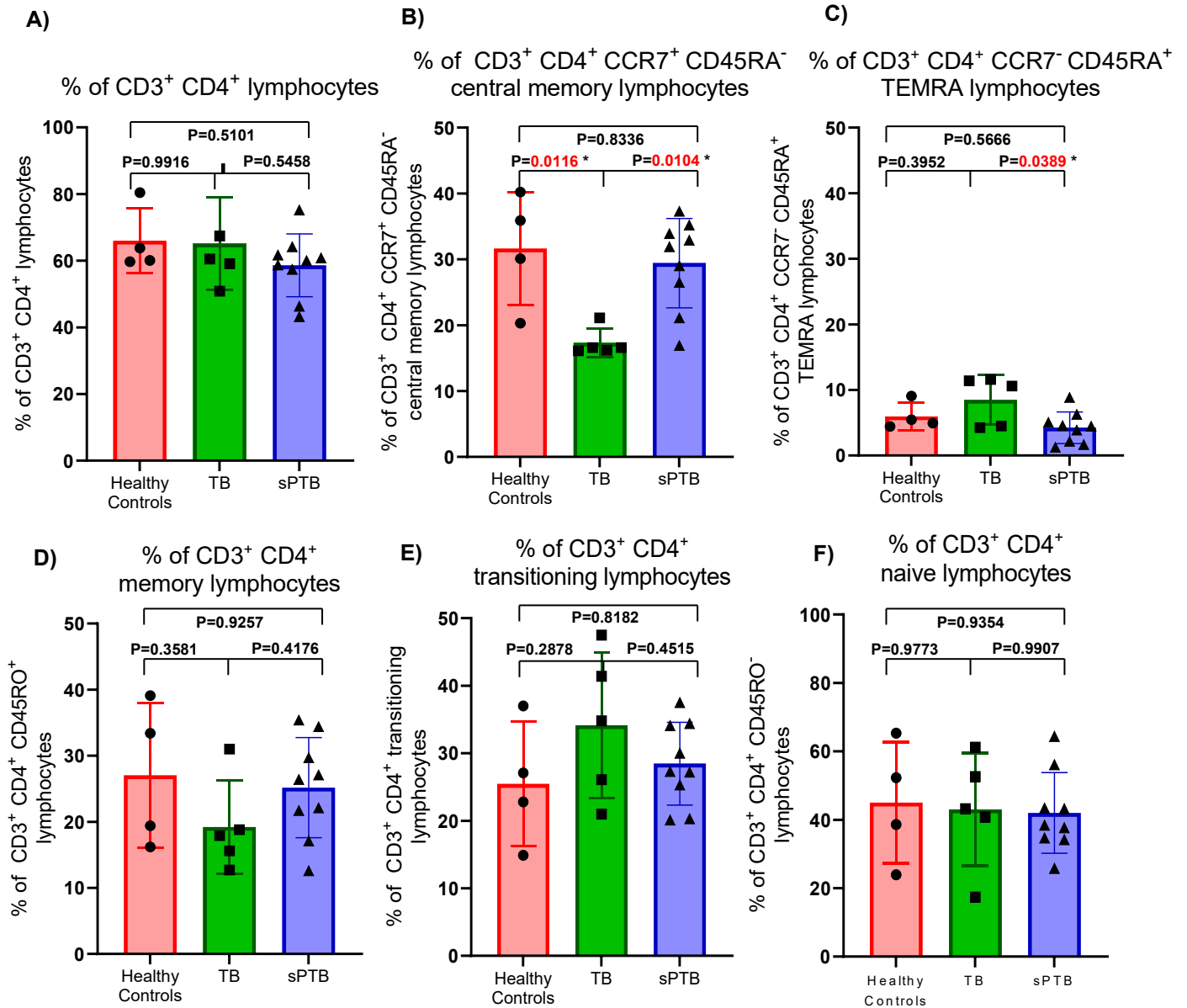


Figure 4.4.2.2.2: Memory subsets of CD3⁺ CD4⁺ T cell lymphocytes.

These graphs show frequencies of CD3⁺ CD4⁺ lymphocytes in peripheral blood (A), Cm (B), TEMRA (C), CD45RO⁺ memory (D), transitioning (E) and naïve (F) T cells between sPTB patients (N=9), TB (N=5) and healthy controls (N=4) at point of hospital admission. Statistical analysis was undertaken using a 1-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P < 0.05 (*).

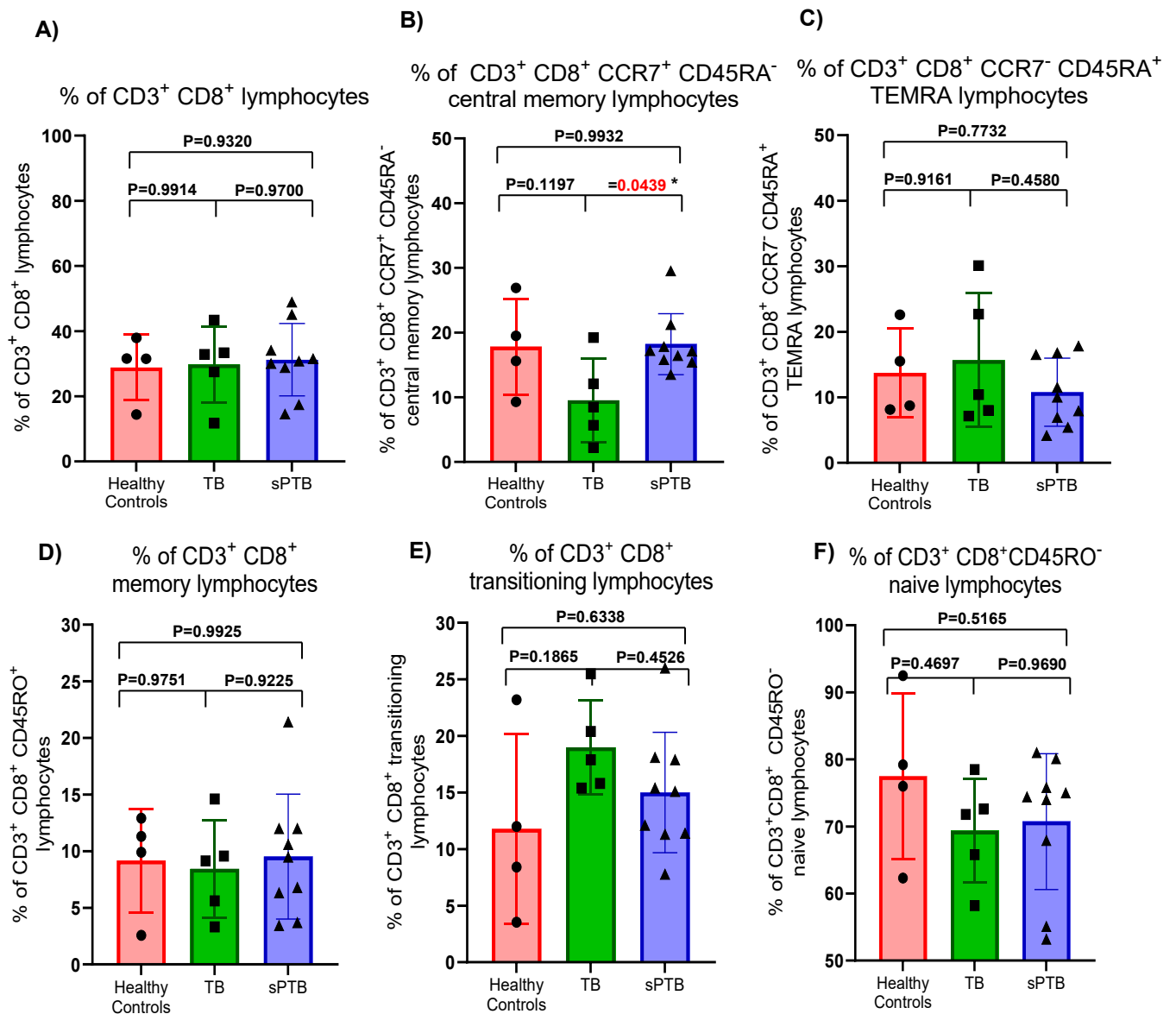


Figure 4.4.2.2.3: Memory Subsets of the CD3⁺ CD8⁺ T cell lymphocytes.

These graphs show frequencies of CD3⁺ CD8⁺ lymphocytes in peripheral blood (A), Cm (B), TEMRA (C), CD45RO⁺ memory (D), transitioning (E) and naïve (F) T cells between sPTB patients (N=9), TB (N=5) and healthy controls (N=4) at point of hospital admission. Statistical analysis was undertaken using a 1-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P < 0.05 (*).

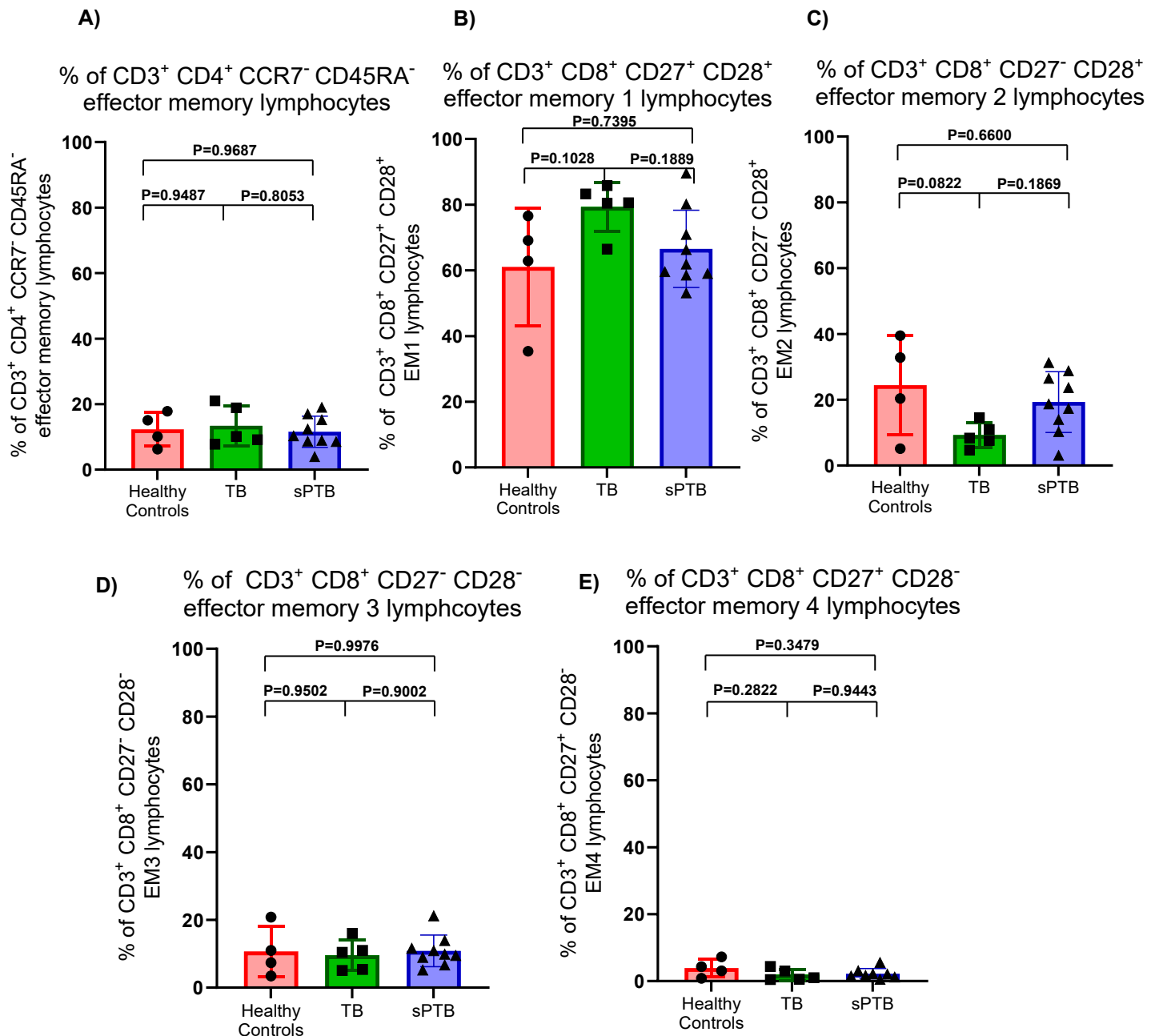


Figure 4.4.2.2.4: Effector memory expressions on CD3⁺ CD4⁺ and CD8⁺ T cell lymphocytes.

These graphs show the EM expression in CD3⁺ CD4⁺ lymphocytes (A), EM 1 (B), EM 2 (C), EM 3 (D) and EM 4 (E) expressions in CD3⁺ CD8⁺ T cell lymphocytes between sPTB patients (N=9), TB (N=5) and healthy controls (N=4) at point of hospital admission. Statistical analysis was undertaken using a 1-way ANOVA and a Tukey's multiple comparison test was applied, Significance was defined as P < 0.05 and all p-values are shown.

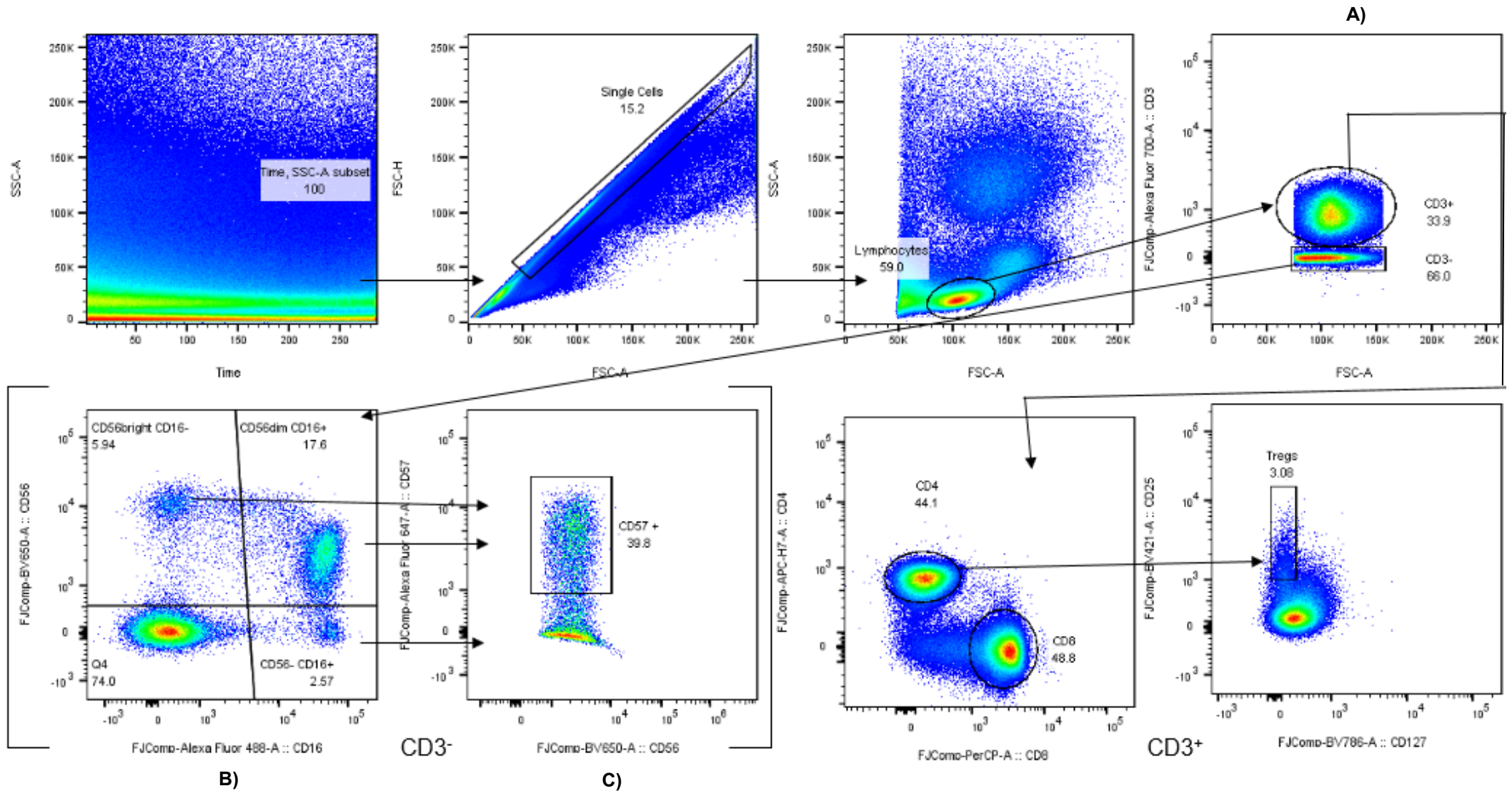
4.4.3 Exhaustion and Senescence Analysis

NK cells and T cells were gated as lymphocyte sized based on FSC and SSC expression and were differentiated using CD3^{-/+} respectively. Expression of CD57, PD-1 and Tim-3 on peripheral CD4⁺ and CD8⁺ T cells was used to measure the frequency of senescence and exhaustion respectively in these populations. CD57 expression on CD56⁺ NK cells was also used to measure NK cell maturity (Figure 4.4.3.1).

Assessment in differences between exhaustion and senescent markers on T cells and NK cells are shown in Figure 4.4.3.1.2 between groups. No significant difference was observed in the number of senescent T-helper and CTL between sPTB and controls. PD-1 expression did not change in the sPTB group when compared to the TB group in both T-helper and CTL cells. However, healthy controls had significantly lower levels of PD-1 expression on both T-helper and CTL when compared to the TB group ($p=0.0498$ and 0.0079 respectively). No change was observed in Tim-3 expression in either T-helper or CTL cells from the sPTB when compared to the TB and healthy controls. CD57⁺ expression on both subsets of CD56⁺ NK cells showed no change across all groups.

4.4.3.1 Exhaustion and Senescent Cells

Channel	AF488	PerCP	BV421	BV510*	BV605	BV650	BV711	BV786	AF647	AF700	APC-H7
Antibody	CD16	CD8	CD25	Live/Dead	PD-1	CD56	Tim-3	CD127	CD57	CD3	CD4
Concentration	1/80	1/40	1/80	1/200	1/40	1/80	1/40	1/40	1/40	1/80	1/80



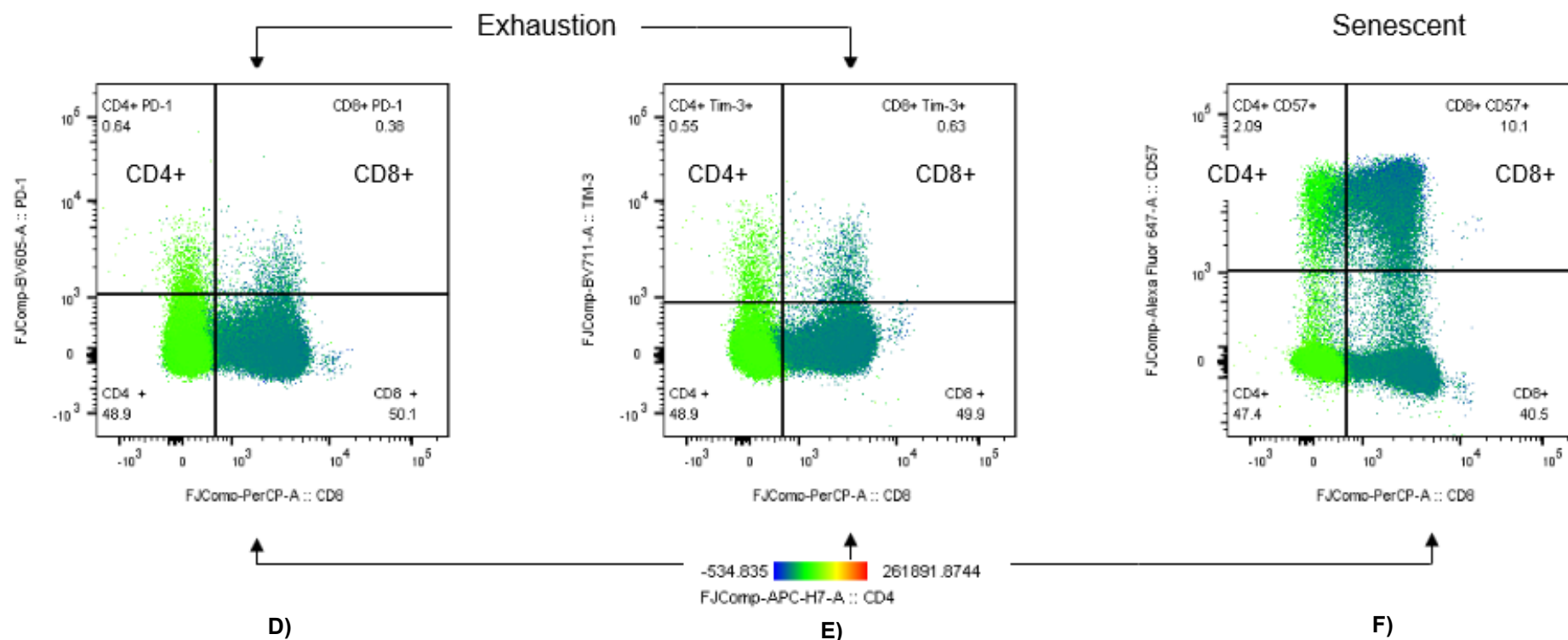


Figure 4.4.3.1.1

An example of peripheral blood NK cell and T cell gating showing exhausted and senescent cell populations. NK cells and T cells were differentiated using CD3 (A). CD57 was used as a marker for senescence on the largest NK cell population ($CD56^{\text{dim}}$ $CD16^{\text{bright}}$) found in peripheral blood (B). Exhaustion was measured based on PD-1 and Tim-3 expressions in both $CD4^+$ and $CD8^+$ T cells (C and D respectively). CD57 was also used to measure senescence in NK cells and T cells (C and F respectively). $CD3^+$ T cells were differentiated using CD8 and a colour axis indicating CD4 expression on each $CD3^+$ cell. Dark green shows no expression of CD4 and light green shows areas of high CD4 expression.

* Live/dead was not added for every sample.

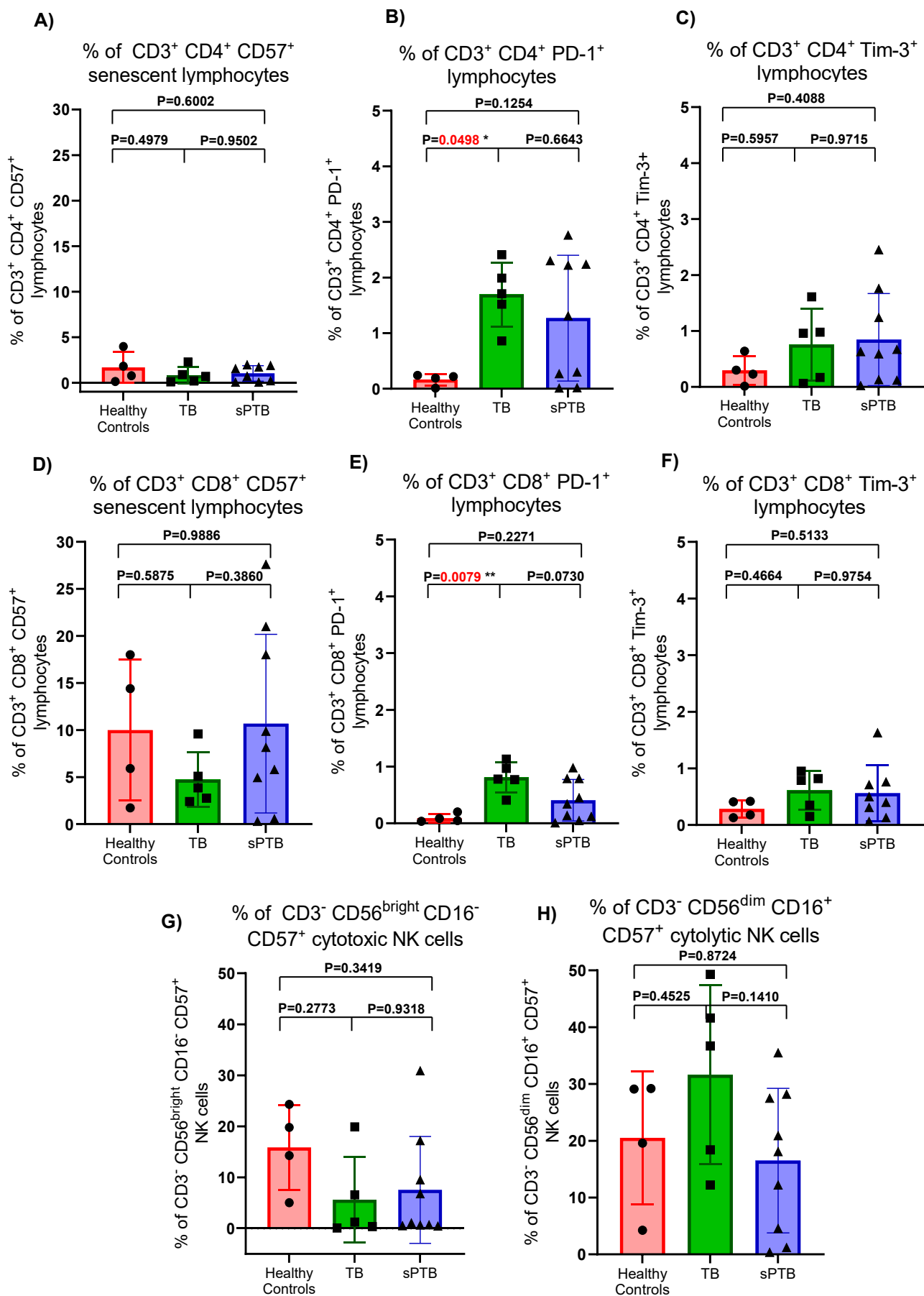


Figure 4.4.3.1.2: CD3⁺ CD4⁺ and CD8⁺ T cell and CD3⁻ NK cell exhaustion, senescent and maturity associated marker expressions.

These graphs show the expression of CD57, PD-1 and Tim-3 in CD3⁺ CD4⁺ T cell lymphocytes (A, B and C respectively) and CD3⁺ CD8⁺ T cell lymphocytes (D, E and F respectively). CD57 expression was measured in CD3⁻ CD56^{bright} CD16⁻ NK cells (G) and CD3⁻ CD56^{dim} CD16⁺ NK cells (H). These markers of exhaustion, senescence and maturity were compared between sPTB patients (N=9), TB (N=5) and healthy controls (N=4) at point of hospital admission. Statistical analysis was undertaken using a 1-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P <0.05 (*), P <0.01 (**).

4.4.4 Peripheral Monocyte Analysis

Peripheral monocytes were identified as monocyte sized using FSC and SSC and CD45⁺. Classical monocyte (CD14⁺⁺CD16⁻), intermediate monocyte (CD14⁺CD16⁺) and non-monocyte (CD14⁺CD16⁺⁺) frequencies were identified. HLA-DR was used to measure activation (Figure 4.4.4.1).

The sPTB cohort showed a tendency to have a higher composition of classical monocytes when compared to the healthy controls but no different from the TB group (p=0.0596 and p=0.4407 respectively). The sPTB group had significantly lower activated classical monocytes when compared to the TB cohort (p=<0.0001). Composition of intermediate monocytes did not change between all groups, though, the sPTB group had significantly lower activated intermediate monocytes when compared to the TB cohort (p=0.0005). Frequency of non-classical monocytes was significantly lower in the sPTB cohort when compared to the healthy controls (p=0.0300) and these were significantly less activated when compared to the TB group (p=0.0003) (Figure 4.4.4.1.2).

4.4.4.1 Monocyte Gating – Peripheral Blood

Channel	AF488	BV421	BV510*	BV711	APC-Cy7	PE	PE-Cy7
Antibody	CD15	CD45	Live/Dead	HLA-DR	CD16	CD66b	CD14
Concentration	1/40	1/40	1/200	1/40	1/40	1/40	1/40

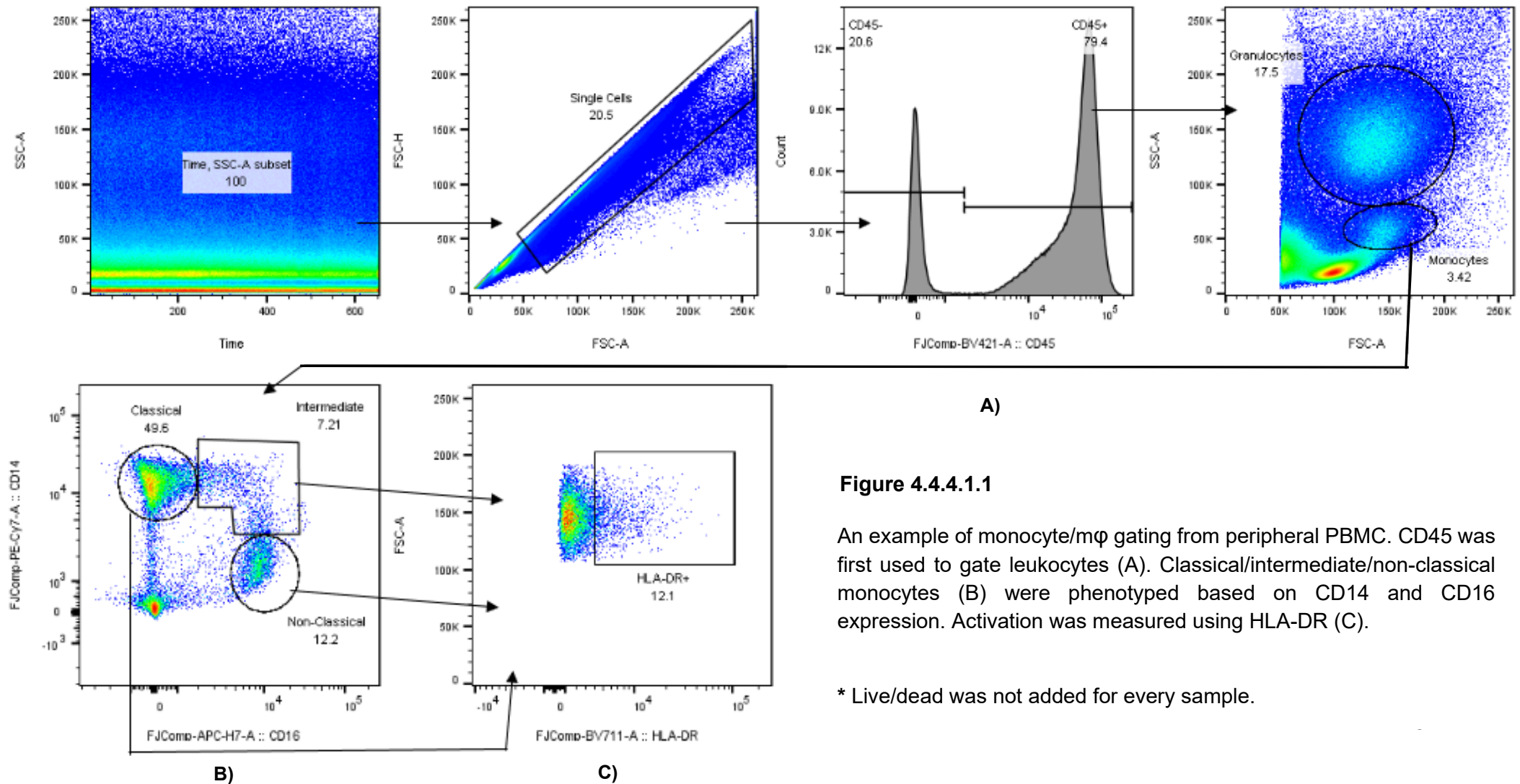


Figure 4.4.4.1.1

An example of monocyte/mφ gating from peripheral PBMC. CD45 was first used to gate leukocytes (A). Classical/intermediate/non-classical monocytes (B) were phenotyped based on CD14 and CD16 expression. Activation was measured using HLA-DR (C).

* Live/dead was not added for every sample.

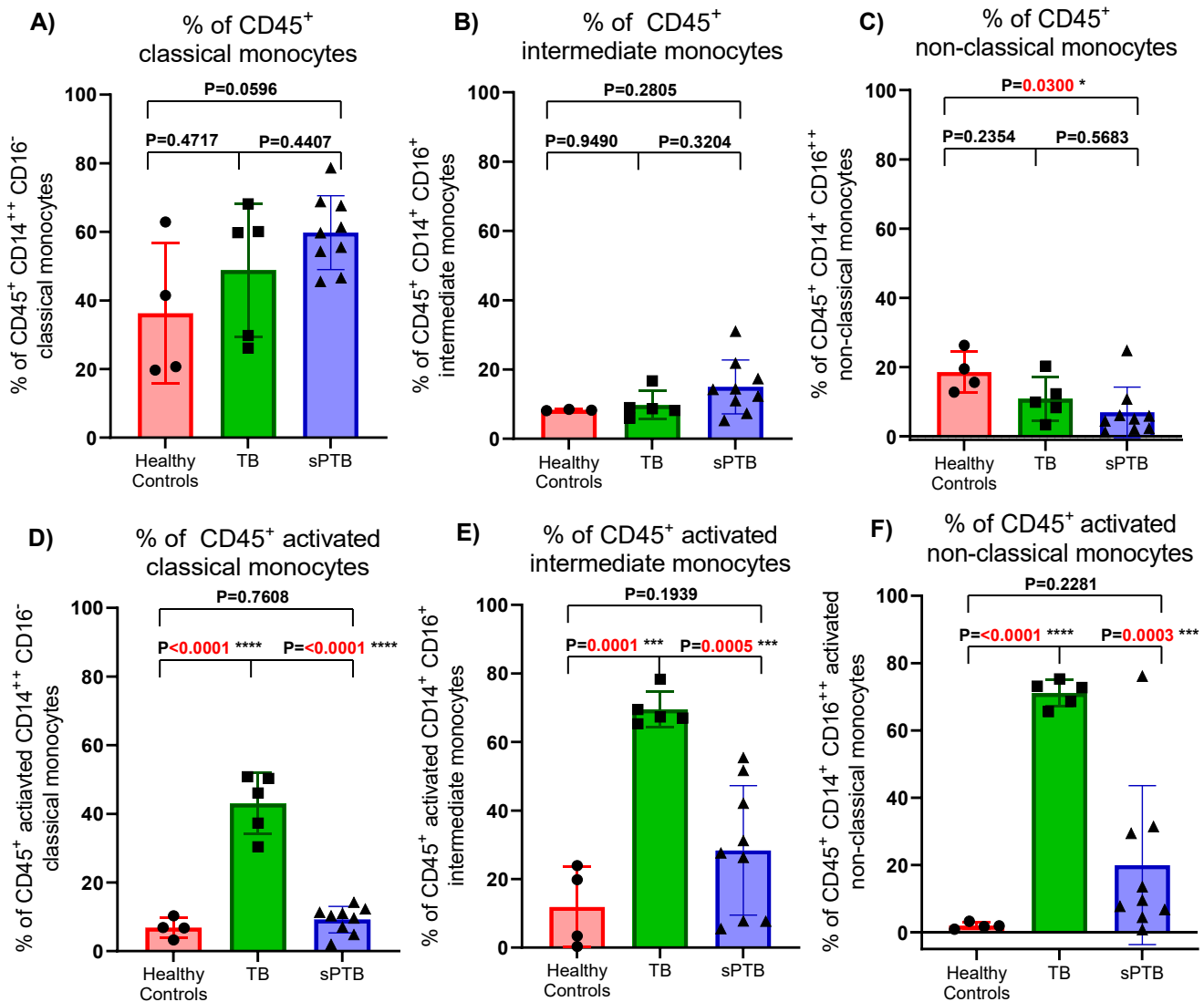


Figure 4.4.4.1. 2: Peripheral CD14 CD16 monocyte subsets and HLA-DR activation

These graphs show levels of CD14⁺⁺ CD16⁻ classical monocytes (A); classical monocyte activation (D), CD14⁺ CD16⁺ intermediate monocytes (B); intermediate monocyte activation (E); CD14⁻ CD16⁺ non-classical monocytes (C) and non-classical monocyte activation (F) between sPTB patients (N=9), TB (N=5) and healthy controls (N=4) at point of hospital admission. Statistical analysis was undertaken using a 1-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) and P < 0.0001 (****).

4.4.5 Peripheral Neutrophil Data Analysis

Neutrophils and eosinophils were identified as granulocyte size using FSC and SSC and CD45⁺ CD15⁺. Neutrophils were then identified as CD16⁺ and eosinophils and CD16⁻. HLA-DR was used to measure activation (Figure 4.4.5.1).

Neutrophil levels did not change between all groups (Figure 4.4.5.1.2), but the proportion of activated neutrophils was observed to be significantly lower in the sPTB cohort when compared to the TB group ($p=0.0138$). Healthy controls also had significantly lower activation of neutrophils when compared to the TB cohort ($p=0.0065$).

4.4.5.1 Granulocyte Gating – Peripheral Blood

Flow cytometry panel and initial gating strategy including time gate, singlet cells and CD45⁺ leukocytes were identical to that of the monocyte gating found in Section 4.4.4.1

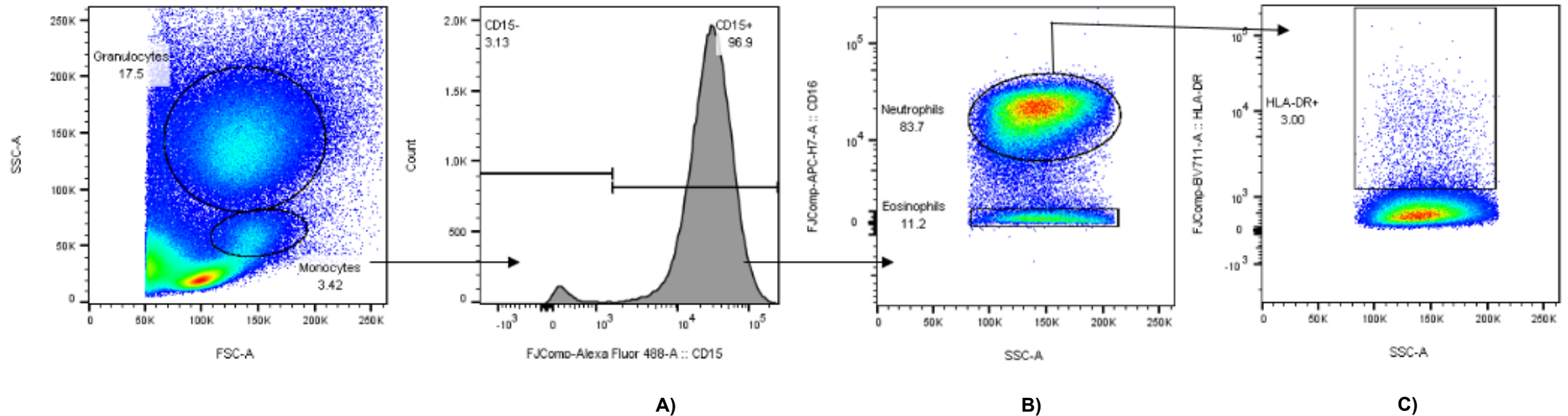


Figure 4.4.5.1.1: An example of neutrophil gating of peripheral PBMC. Basophiles were gated out using CD15 (A); neutrophils were phenotyped as CD15⁺ CD16⁺, whereas eosinophils are CD15⁺ CD16⁻ (B). Activation was measured using HLA-DR (C).

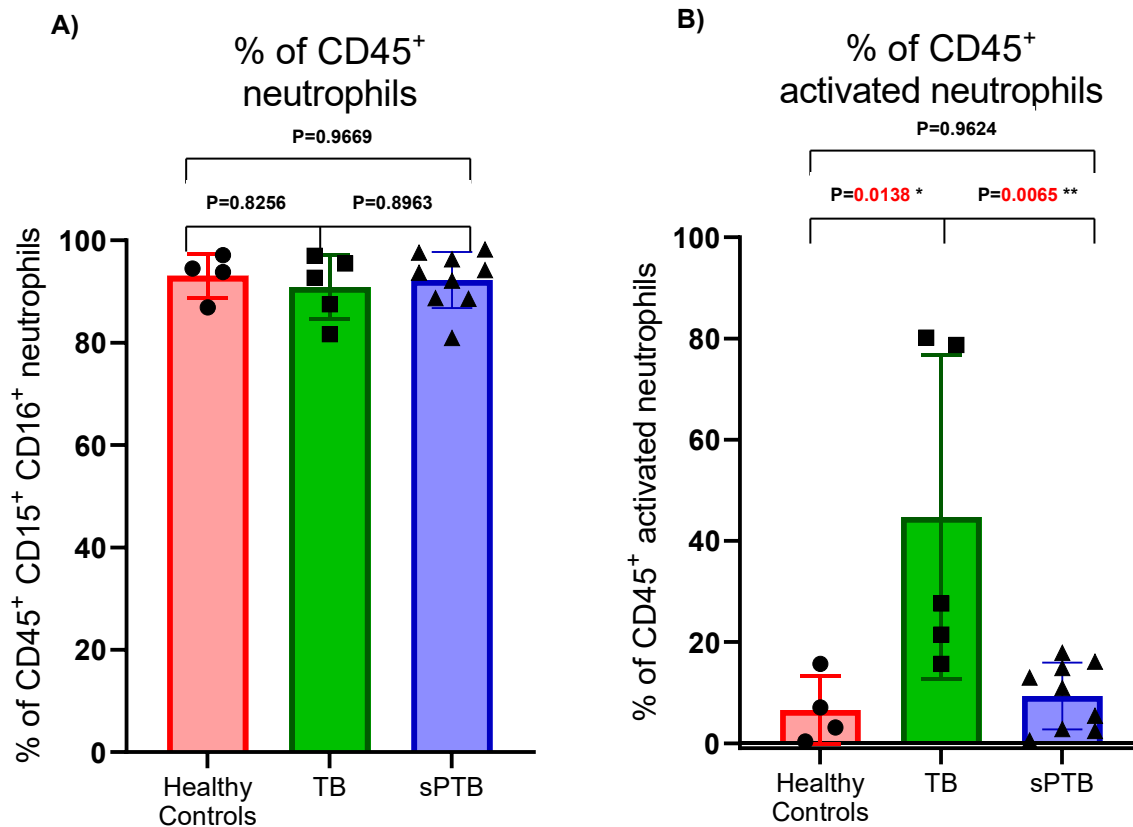


Figure 4.4.5.1.2: Peripheral CD15⁺ CD16⁺ neutrophil frequencies and HLA-DR activation.

These graphs show change of neutrophils (A) and neutrophil activation (B) between sPTB patients (N=9), TB (N=5) and healthy controls (N=4) at point of hospital admission. Statistical analysis was undertaken using a 1-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P values <0.05 (*) and P<0.01 (**).

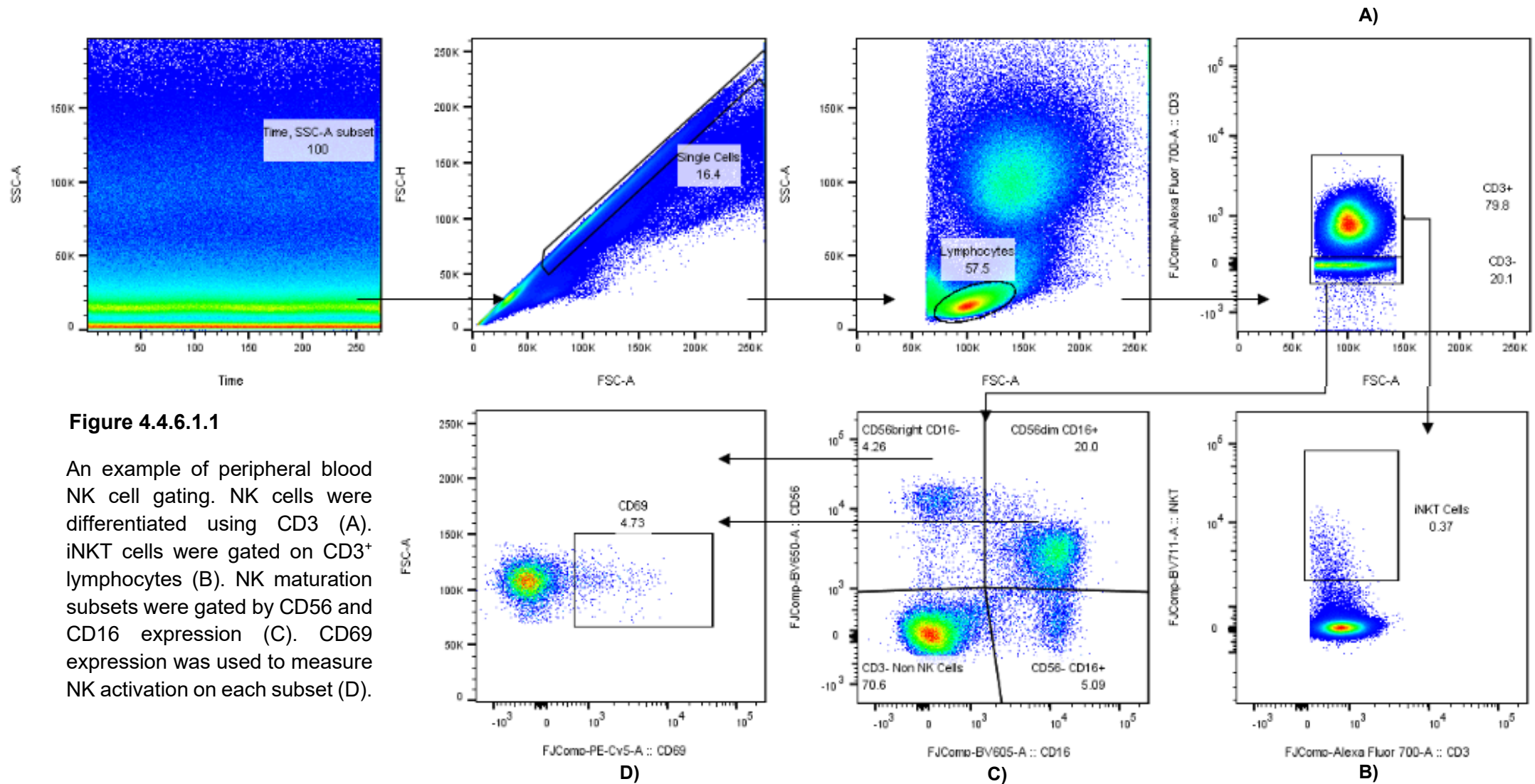
4.4.6 Natural Killer Cells

NK and NKT cells were defined as lymphocyte sized, CD3⁻ and CD3⁺ respectively. The two most abundant NK cell maturation subsets found in peripheral blood were defined as: cytotoxic (CD56^{bright} CD16⁻) and cytolytic (CD56^{dim} CD16⁺). CD69 was used to measure activation (Figure 4.4.6.1.1) and the natural cytotoxic receptors (NCR) NKp30 and NKp46 were used to measure NK cell function. NKp46 was also used to verify CD56⁻ 16⁺ cells were functional NK cells and not monocytes (Figure 4.4.6.1.2).

The sPTB cohort had significantly lower levels of cytotoxic NK cells when compared to the healthy cohort ($p=0.0184$), however, this was not seen when compared to the TB group ($p=0.8861$) at point of hospital admission. Similarly, cytolytic NK cells in the sPTB group had a tendency to be lower and approaching significance when compared to the healthy cohort ($p=0.0622$). However, no change was observed when compared to the TB group ($p=0.3723$). Frequency of CD69⁺ activated cytotoxic and cytolytic cells in the sPTB did not show any significant differences between both the TB and healthy controls (Figure 4.4.6.1.3). No observable difference between all 3 groups was found in the NKp30 receptors between different NK cell subsets; however, NKp46 expression in the sPTB group was lowest and approaching significance in the cytolytic NK cells when compared to the healthy controls ($p=0.0515$), but this difference was not as significant when compared to the TB group (Figure 4.4.6.1.4). Finally, no difference was observed in iNKT cell composition in each group (Figure 4.4.6.1.5).

4.4.6.1. NK Cells

Channel	AF488	BV421	BV510*	BV605	BV650	BV711	AF700	PE-Cy5
Antibody	NKp46	NKp30	Live/Dead	CD16	CD56	TCR (Va24-Ja18)	CD3	CD69
Concentration	1/40	1/40	1/200	1/80	1/80	1/40	1/80	1/40



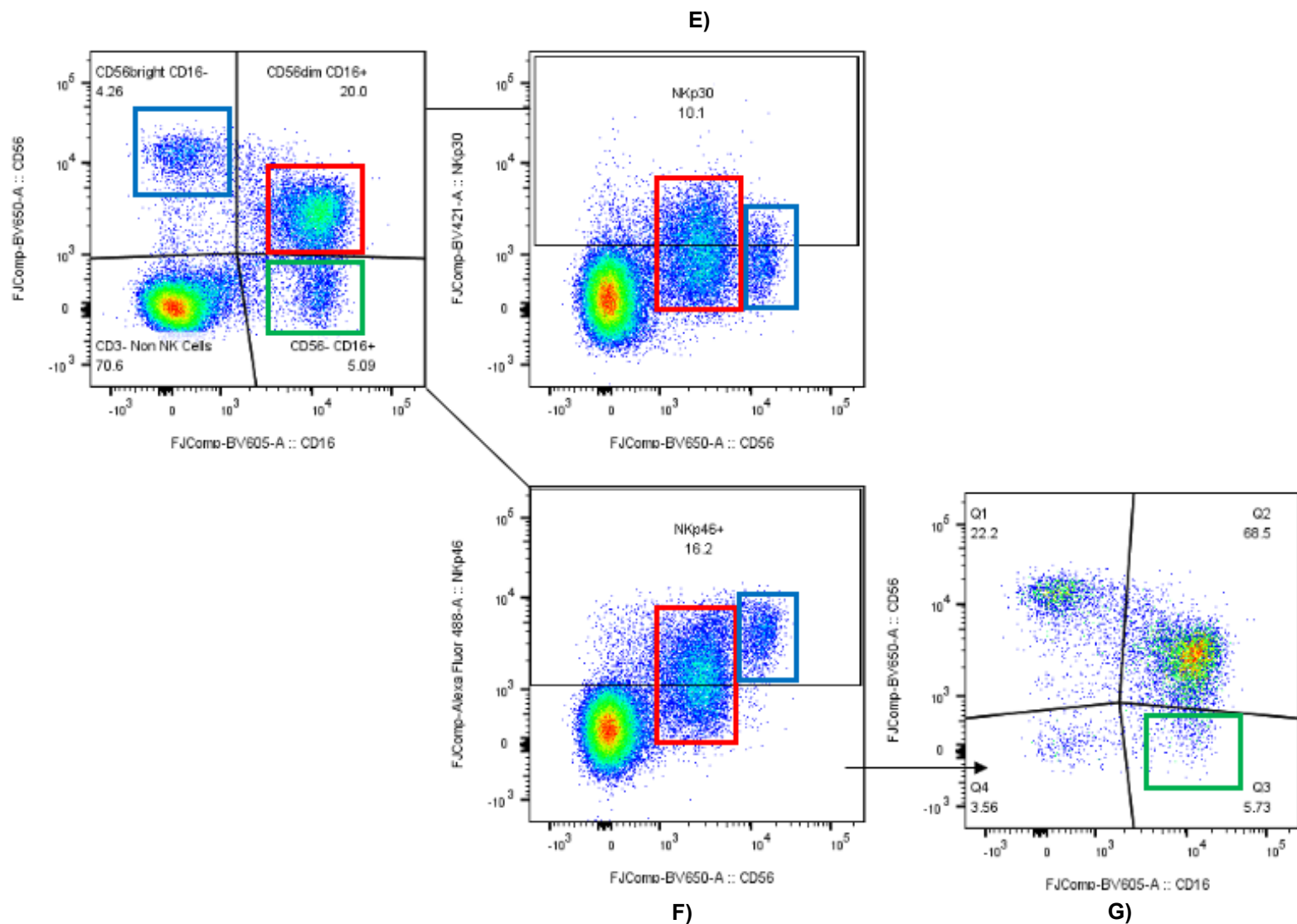


Figure 4.4.6.1.2: NCR expression NKp30 (E) and NKp46 (F) was measured for each NK cell population. CD56⁻ CD16⁺ cells were gated using NKp46 to verify they were NK cells and not monocytes (G).

* Live/dead was not added for every sample.

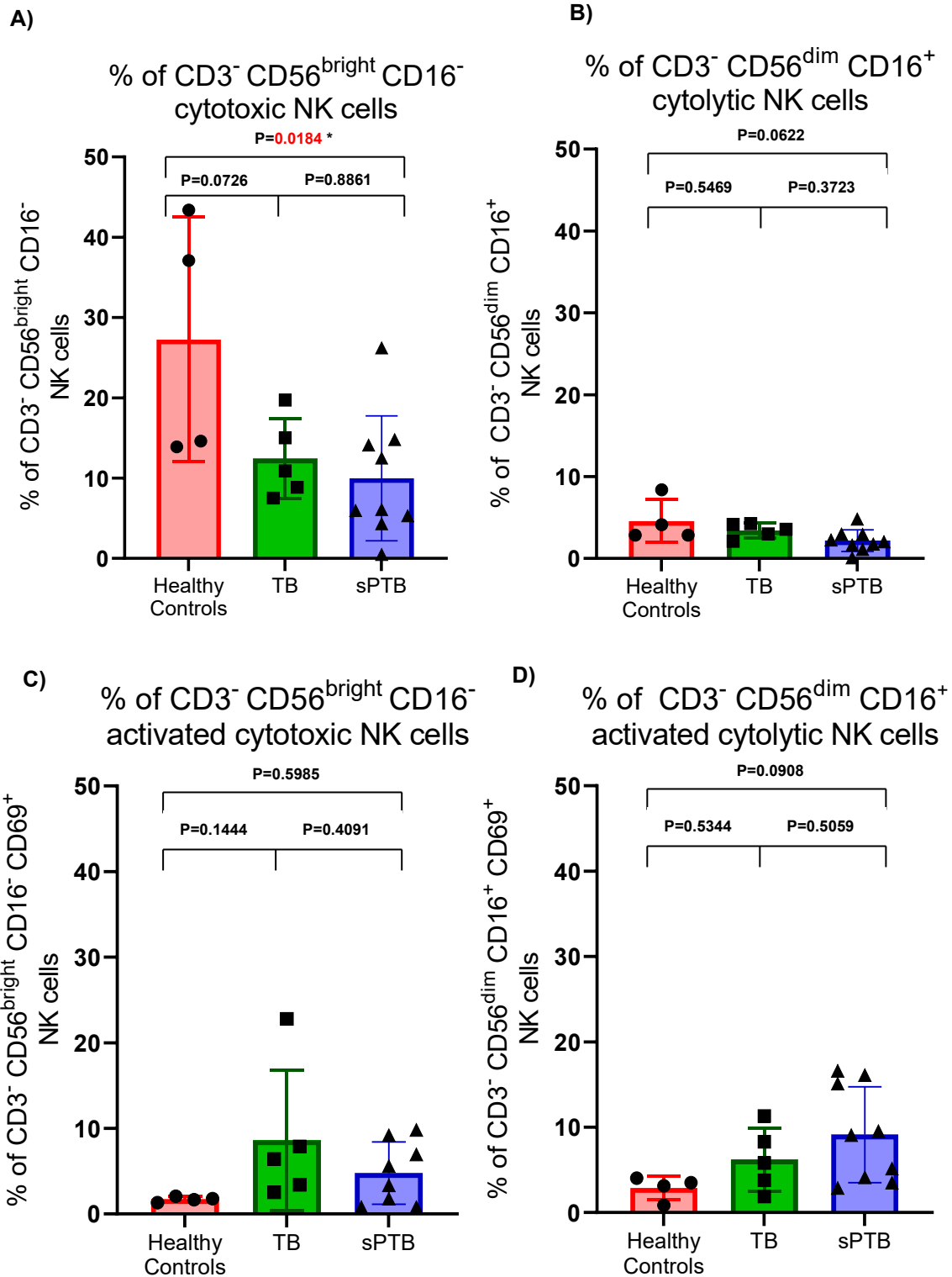


Figure 4.4.6.1.3: CD56 CD16 expression on NK cell frequency and comparison of CD69 in subsets

These graphs show cytotoxic (A), cytolytic (B) and activation of cytotoxic (C) and cytolytic (D) NK cells between sPTB patients (N=9), TB (N=5) and the healthy cohort (N=4) at point of hospital admission. Statistical analysis was undertaken using a 1-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P < 0.05 (*).

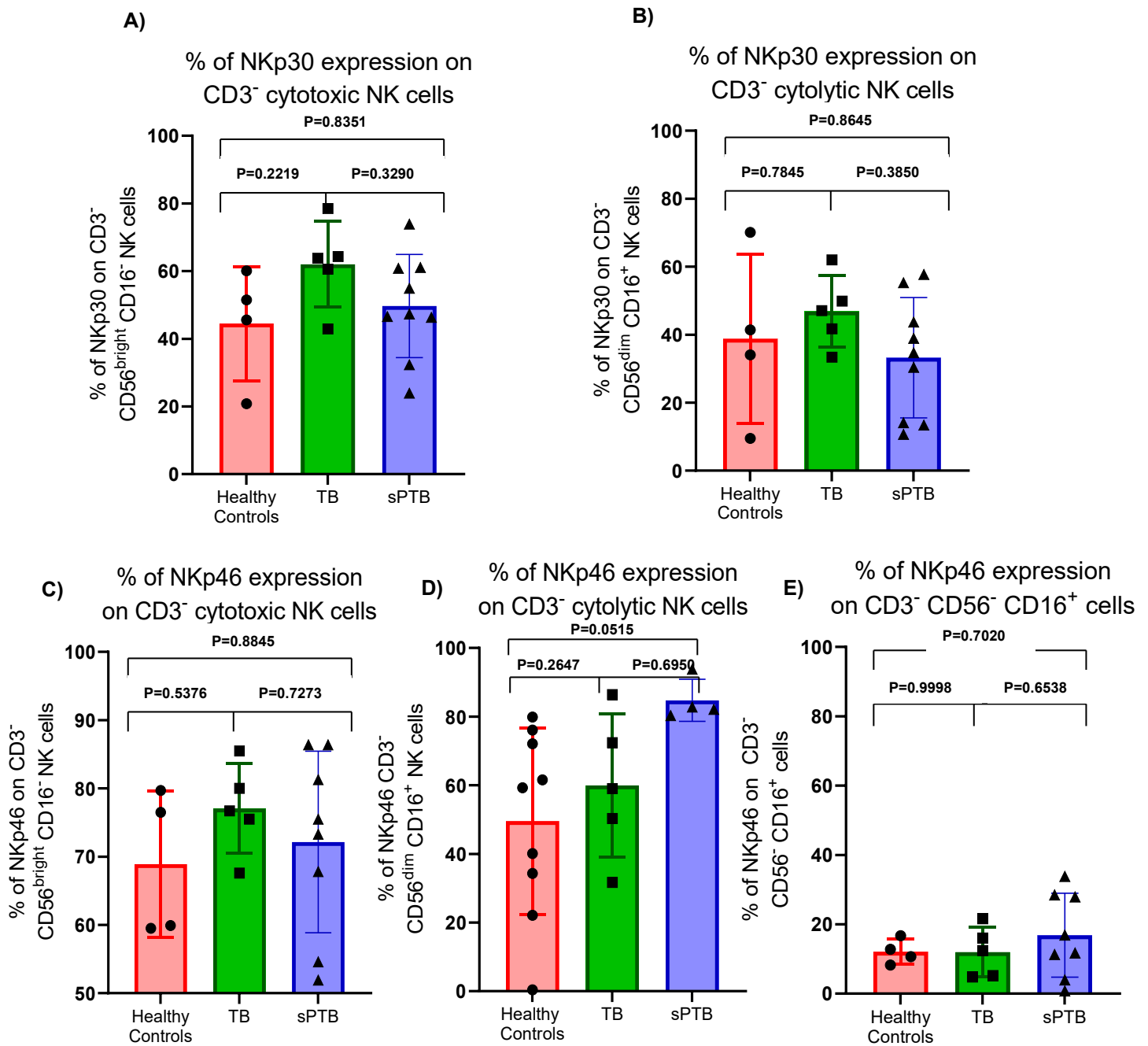


Figure 4.4.6.1.4: NKp30 and NKp46 expression on NK cell maturation subsets

These graphs show the expression of NKp30 on cytotoxic (A) and cytolytic (B) NK cells. NKp46 expression on cytotoxic (C) and cytolytic (D) NK cells and on CD56⁻ CD16⁺ cells (E) between sPTB patients (N=9), TB (N=5) and healthy controls (N=4) at point of hospital admission. Statistical analysis was undertaken using a 1-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P < 0.05.

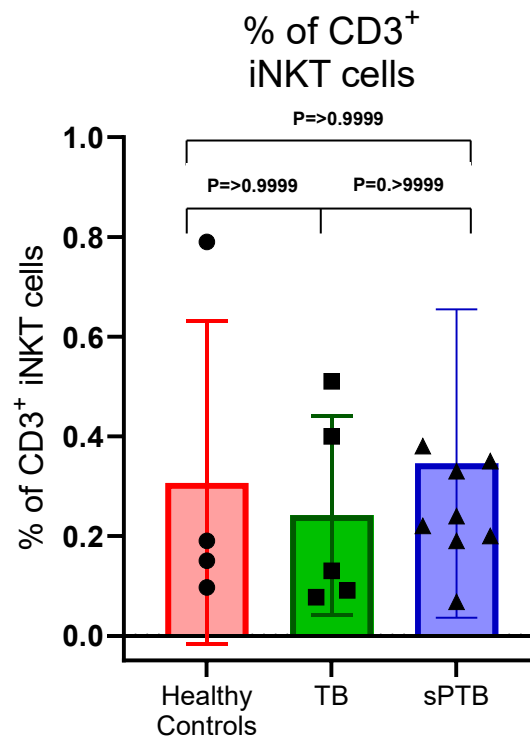


Figure 4.4.6.1.5 CD3⁺ iNKT cell frequency

This graph shows the CD3⁺ iNKT cell population between sPTB patients (N=9), TB (N=5) and healthy controls (N=4) at the point of hospital admission. Statistical analysis was undertaken using a Kruskal-Wallis 1-way ANOVA and a Dunn's multiple comparison test was applied. Significance was defined as $P < 0.05$.

4.4.7 Cervical Swab Cells

Cervical immune cells were gated based on CD45⁺ expression. Granulocytes and monocytes/m ϕ were differentiated using CD15 and CD14. Neutrophils and eosinophils were differentiated using CD66b and CD16. HLA-DR was used to measure activation (Figure 4.4.7.1.1).

Due to the difficulty in isolating cervical immune cells from cytobrush samples, statistical data was not able to be performed due to insufficient N numbers. Useable data for each cohort was as follows: sPTB (N=3), healthy controls (N=1) and TB (N=1). Therefore, one example from each group has been chosen to illustrate the flow cytometry analysis of each immune cell type found in cervical mucus. Cervical cells

like the peripheral blood data was represented as percentage of CD45⁺ cells. as detailed in Section 4.3.5.

The proportion of cervical monocytes was the lowest in the sPTB case (0.79%) intermediate in the TB case (6.02%) and highest in the healthy control (8.58; Figure 4.4.7.1.2). The opposite result was shown in the proportions of cervical granulocytes, where the sPTB group had the highest (95.0%), followed by the TB (91.9%) and lowest in the healthy control group (89.1%). Eosinophil and neutrophil counts were intermediate in the sPTB group (39.0% and 50.1% respectively) compared to the healthy control (3.01% and 95.0% respectively) and TB cases (11.8% and 83.1% respectively). In terms of activation, the highest granulocyte activation was observed in the sPTB case (91.6% and 7.29% respectively), followed by the TB case (0.24% and 0.42% respectively) then lastly lowest activation of granulocytes was in the healthy control (0.029% and 0.039% respectively) (Figure 4.4.7.1.3). However, monocyte activation was the lowest in the sPTB cohort (0.82%), followed by the healthy control (1.80%), the highest activation was found in the TB monocytes (64.0%) (Figure 4.4.7.1.4).

4.4.7.1 Innate Cells – Cervical Cell Gating

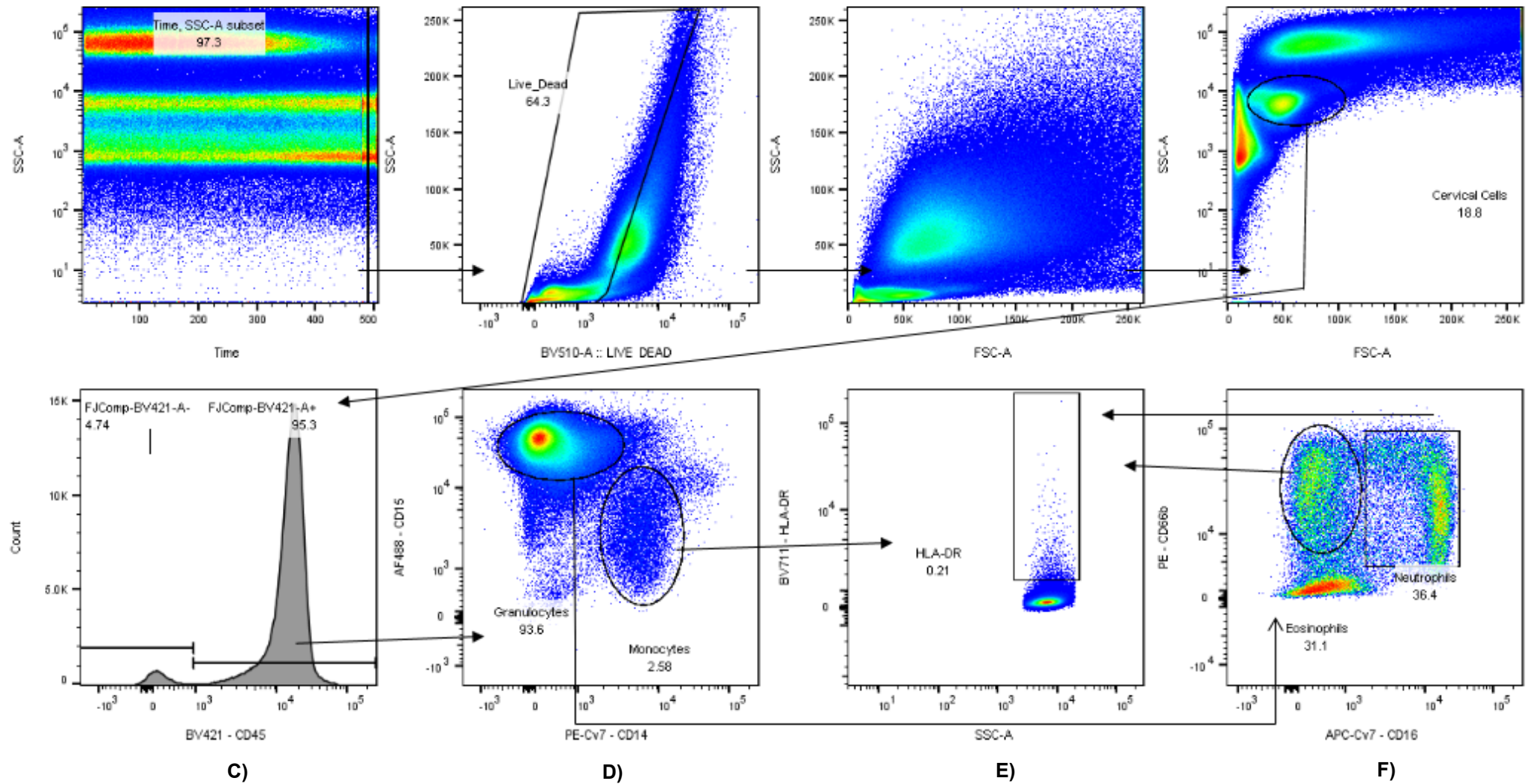
Channel	AF488	BV421	BV510*	BV711	APC-Cy7	PE	PE-Cy7
Antibody	CD15	CD45	Live/Dead	HLA-DR	CD16	CD66b	CD14
Concentration	1/40	1/40	1/200	1/40	1/40	1/40	1/40

The gating strategy for cervical cells was different from peripheral cells. Firstly, the voltage on both FSC and SSC had to be dropped considerably from the PBMC collection. SSC had to be viewed on a logarithmic scale shown in Figure 4.4.7.1.1 due to the vast quantity of squamous epithelial cells collected in every brush sample. The majority of cytobrush samples contained little or no leukocytes because they were obscured by the huge number of epithelial cells. Aggregation of epithelial cells occurs frequently; therefore, no singlet gate was implemented in this gating strategy.

Figure 4.4.7.1.1

A)

B)



An example of cervical cell gating. Live/dead gate (A) was used to exclude as many epithelial cells as possible. Logarithmic scale for SSC (B) differentiated leukocytes from epithelial cells. CD45 used to verify leukocytes (C). CD15 and CD14 (D) were used to distinguish innate cell populations. Activation was measured using HLA-DR (E) and eosinophils were separated from neutrophils by using CD66b and CD16 expression (F).

* Live/dead was not added for every sample.

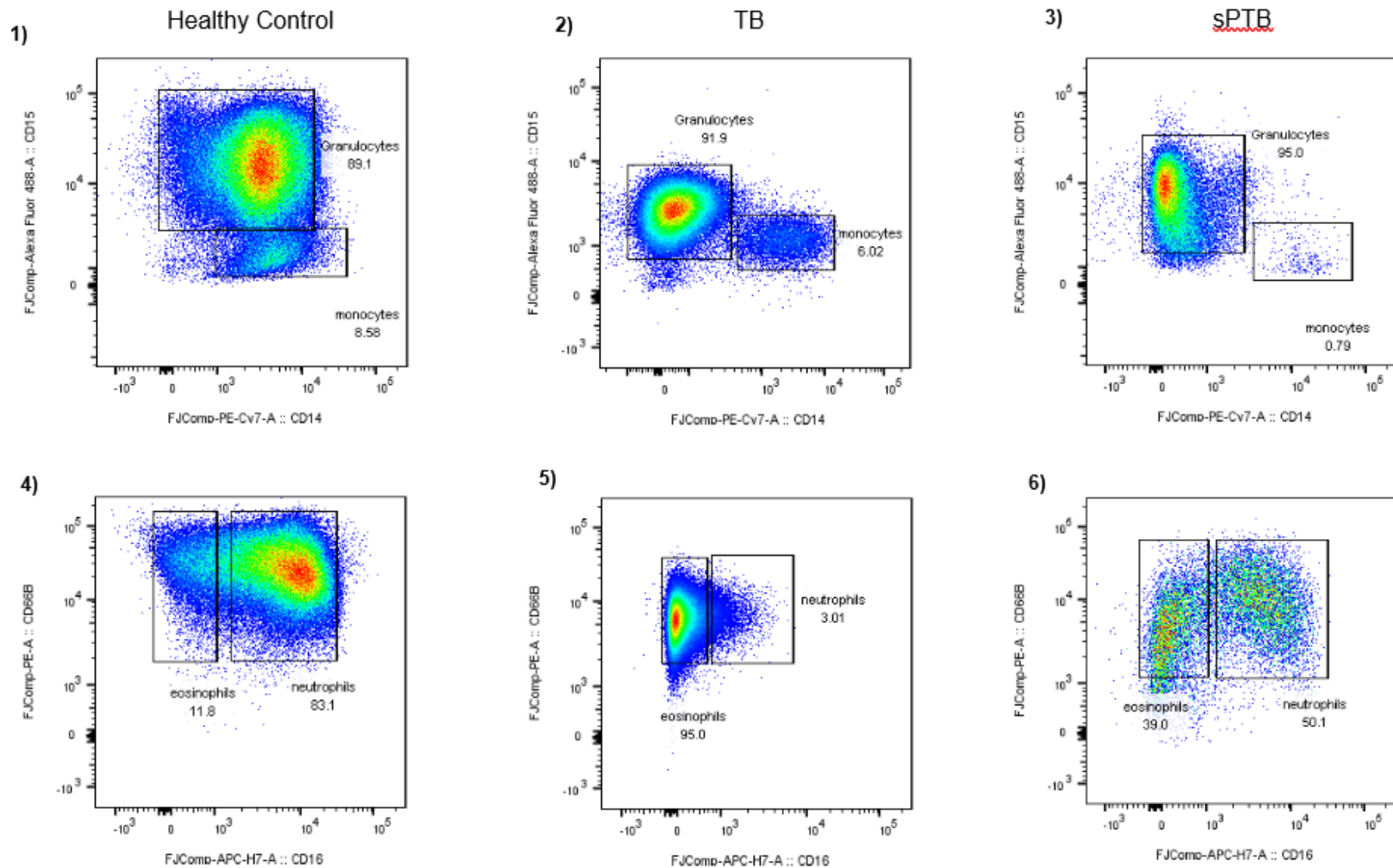


Figure 4.4.7.1.2: Innate cell phenotype was gated from CD45⁺ cells using CD15 and CD14 to differentiate granulocytes and monocyte/mφ (1, 2 and 3). sPTB showed considerably lower cell counts (3; 33,696) compared to healthy control (1; 133,515) and TB (2; 155,476) group. Granulocytes were differentiated using CD66b expression and CD16 (4, 5 and 6). sPTB group (6) showed an intermediate number of eosinophils and neutrophils (39.0% and 50.1% respectively) when compared to the healthy (4; 11.8% and 83.1%) and TB groups (5; 95.0% and 3.01% respectively).

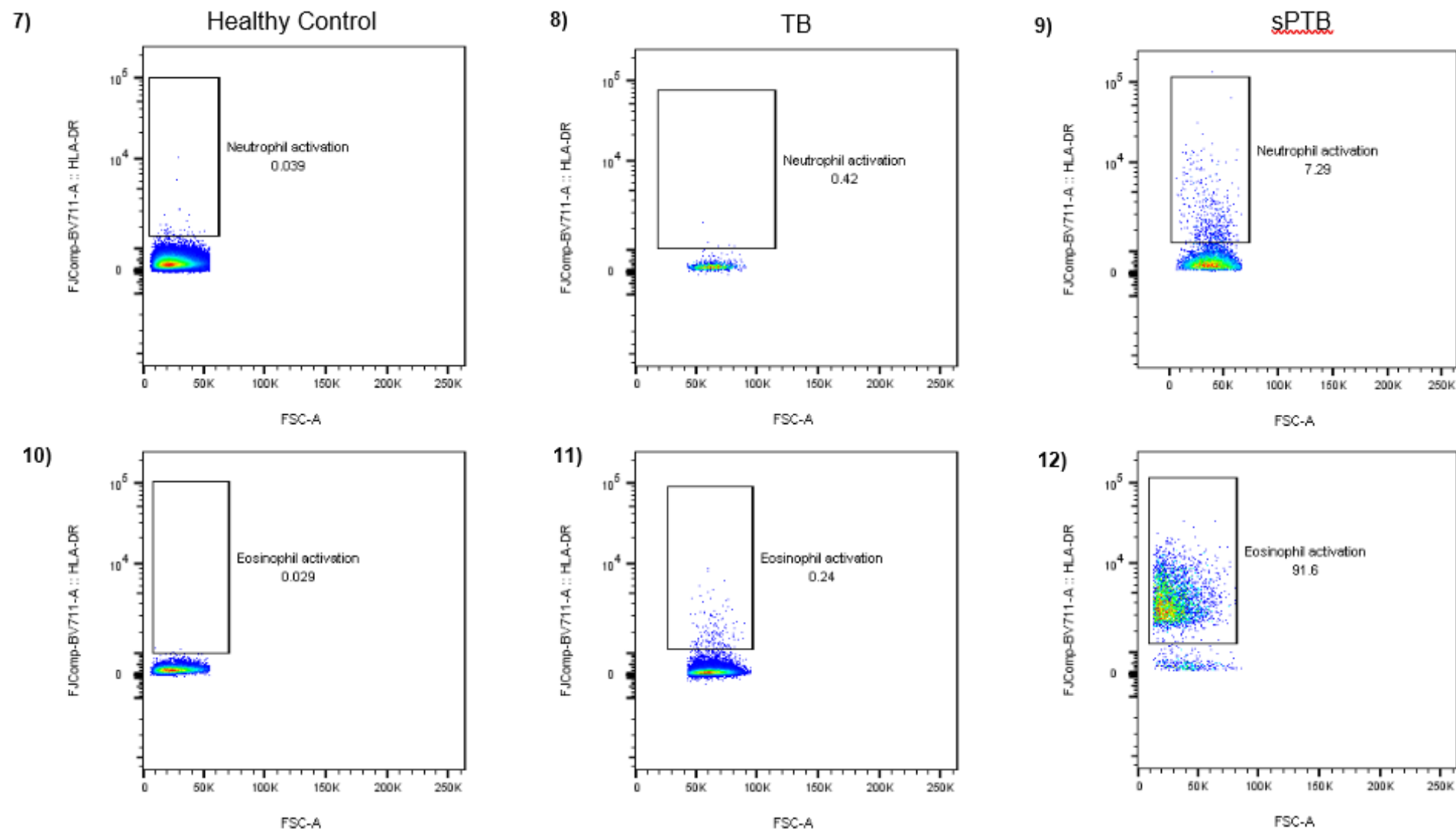


Figure 4.4.7.1.3: Activation of neutrophils was measured using HLA-DR (7,8 and 9). sPTB group had a higher proportion of activated neutrophils (9; 7.29%) and eosinophils (12; 91.6%) when compared to the healthy control (7 and 10; 0.039% and 0.029% respectively) and TB (8 and 11; 0.42% and 0.24% respectively) groups.

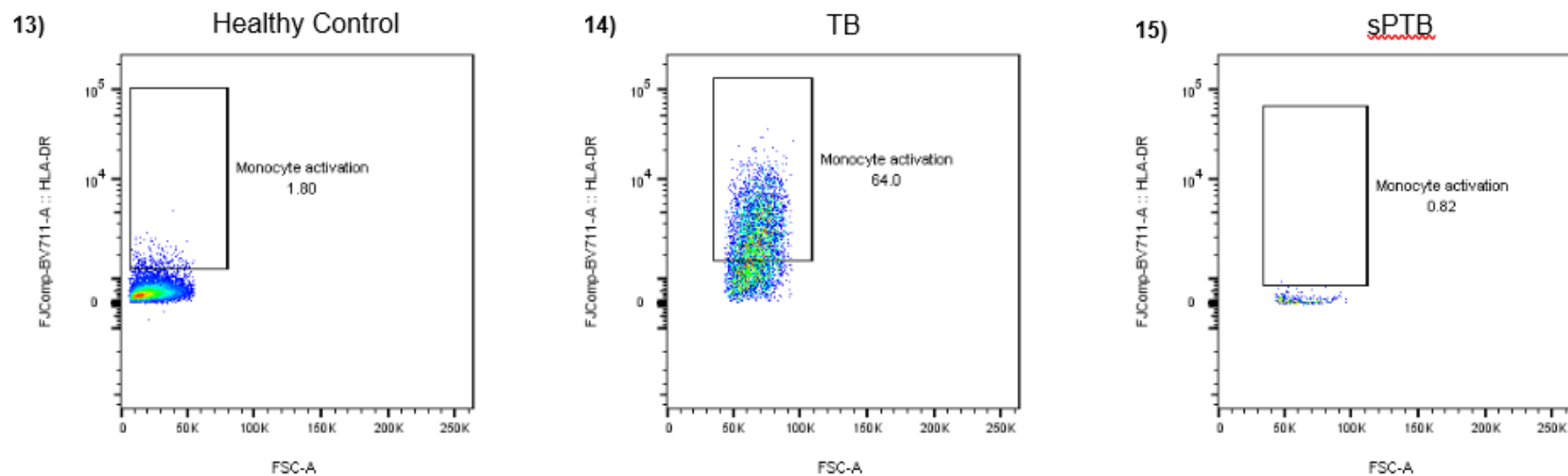


Figure 4.4.7.1.4: Activation of monocytes using HLA-DR (13, 14 and 15). sPTB group had the lowest proportion of activated monocytes (15; 0.82%) when compared to the healthy control (13; 1.80%) and TB (14; 64.0%) groups.

4.5 – Discussion

In this chapter, the phenotypic differences between both cervical and peripheral immune cells between sPTB, TB and healthy pregnant women was studied. As discussed earlier, labour is thought to be mediated by an inflammatory response [172] with the premature activation of the inflammatory pathways leading to preterm birth [123]. This study has looked at women who present with TPTL as well as healthy controls and compared the phenotype of the immune cells between those who gave birth preterm and term.

An initial observation between the sPTB and TB cohorts was that each of these women were classified as ‘high-risk’ based on the inclusion criteria for the study, however the mean interval difference of 9 days was observed in the sPTB cohort from recruitment to delivery, whereas the TB cohort had a mean interval of 40 days between recruitment to delivery (Shown in Table 4.3.4.1). Because of the large gestational time differences between these two groups, it is possible that the stratification of women presenting with TPTL may not be stringent enough which may explain the surprisingly large number of phenotypic differences observed between the sPTB and TB groups. Added to this, only 5% of women presenting with TPTL will go on to deliver within 10 days from the onset of painful uterine contractions [152]. Because of the small percentage of women who go on to deliver preterm, it is possible that many of these TPTL women are misclassified as ‘high-risk’ when they are not.

4.5.1 - Adaptive Cell Phenotypic Changes seen in sPTB Women

Tregs: There is evidence to suggest that in idiopathic PTL functional peripheral Tregs decline at the maternal-fetal interface. However, no change in Treg proportions or

HLA-DR activation was shown in the sPTB cohort when compared to the other groups despite having 7 out of the 9 women experiencing an idiopathic PTB. Surprisingly, the literature regarding peripheral Treg activation has shown that women who delivered at term had higher HLA-DR activation of Tregs whereas; preterm labouring women had decreased HLA-DR expression [181]. These data do not correlate with the data presented in this chapter. The biggest difference between the studies is the stage in labour when recruitment took place. In the study by Schober *et al*, the PTL patients were those where tocolytic treatment failed and they delivered spontaneously [181]. The sPTB patients in this chapter had a mean interval from recruitment to birth of 9 days, and so their peripheral blood was obtained at a much earlier stage of the labour process. Similarly, Schober *et al* measured activation in women who were in labour, whereas women recruited for the TPTL study were not. Also, peripheral Tregs were identified based on their CD45RA expression rather than using both CD45RA and HLA-DR, which is a better method of identifying activated Tregs [181], these dissimilarities may explain why the results are not consistent.

T-Cells: Interestingly, lower CD4⁺CD45RO⁺ memory and higher CD4⁺CD45RA⁺ naïve cells in the peripheral blood have been associated with PTL [227], however, in this study, the frequency of both memory and naïve CD4⁺ T cells found in the sPTB group was analogous to the healthy controls, suggesting that PTL in these women is not driven by a response to recall antigens. Similarly, CD8⁺ memory and naïve populations were comparable between all 3 groups. C_m and E_m of CD4⁺ and CD8⁺ T cells did not differ between the sPTB and healthy women. C_m T cells differentiate into E_m upon chronic antigen exposure from infection, whereas E_m are initiators of cytokine production in the presence of secondary antigen presentation [228, 229]. Intriguingly, sPTB women had lower CD4⁺ TEMRA cells in peripheral blood when compared to the

TB group. This subset has no published data on its function in regards to pregnancy [185]. However, it has been shown that these cells upregulate cytotoxic molecules such as Granzyme B and perforin and provide protection against viruses [14] which suggests that the sPTB group maybe more susceptible to viral infection. On the other hand, no differences were observed between CD4⁺ and CD8⁺ Cm and Em T cells between the 3 groups which imply that the memory responses seen in the T cell populations would respond similarly to systemic infection across all groups.

No change in Tim-3 expression on both CD4⁺ and CD8⁺ T cells was observed in the sPTB cohort when compared to the TB and healthy controls. In normal pregnancy, Tim-3 expression on CD8⁺ T cells has been shown to decrease during the second trimester, but to return to normal during the third trimester whereas, CD4⁺ T cells showed no gestational change [179]. Additionally, no change in PD-1 expression on sPTB CD4 and CD8 T cells when compared to the healthy controls was observed. Tim-3 and PD-1 are indicators of cellular exhaustion and inhibition [230]. Cells with high expression of these markers prevent the optimal control of infection, decrease cellular proliferation and have a severe lack of function [54, 187]. This implies that higher PD-1 and Tim-3 expressions in T cells may cause an anergic response to infection, however, with the findings gathered here, this conclusion cannot be assumed for the sPTB cohort.

4.5.2 - Innate Cell Phenotypic Changes seen in sPTB Women

Monocytes: As pregnancy progresses towards delivery, the expansion of inflammatory cell numbers occurs in the circulation [231, 232]. This is associated with an increase in the proportions of non-classical and intermediate monocytes, with decreasing proportions of classical monocytes with advancing gestation [232, 233]. Interesting, in

the sPTB cohort this was reversed. Classical monocytes derived from bone marrow, mature in the circulation and differentiate into intermediate and non-classical monocytes [232]. Therefore, the higher frequency of classical monocytes found in the sPTB cohort may suggest a failure of differentiation into intermediate and non-classical subsets. Non-classical monocytes can differentiate further into M2 macrophages which invade the decidua and aid in maintaining feto-maternal tolerance [115, 234]. M2 macrophages have also been found to aid in spiral artery remodelling, promote anti-inflammatory responses and are abundant in tissues of healthy pregnant women [235, 236]. The lower frequency of non-classical monocytes in the sPTB cohort could suggest that an insufficient proportion of M2 macrophage differentiation occurs, meaning these women are less likely to have the necessary immune responses to continue pregnancy if an inflammatory event occurs. Furthermore, sPTB is associated with lower frequencies of cervical monocytes/ $m\phi$ which show little to no activation potential. This supports the findings in literature, whereby the failure to detect monocytes in cervical mucus has been shown in women who go on to deliver preterm [140].

Granulocytes: Elevation of granulocytes during pregnancy is normal [237]. Peripheral blood neutrophils levels were similar across all groups. A higher frequency of cervical neutrophils was found in the sPTB patients. Furthermore, these neutrophils displayed higher activation. However, this data cannot be relied upon due to the low sample size analysed. But, with increased activation of neutrophils comes additional production of chemokines and cytokines [238]. Neutrophils can produce several pro and anti-inflammatory cytokines which include: INF- γ , TNF- α , IL-4 and IL-10 respectively, in response to pathogen associated molecular patterns (PAMPs) or host factors [239]. IL-8, a potent chemokine produced predominantly by macrophages, functions to

recruit and activate neutrophils into sites of inflammation [240, 241]. These aforementioned cytokines and chemokines have been reported to be increased in the myometrium and decidua during parturition in both preterm and term labour [242, 243] as they activate MMPs which can degrade the collagenous fibre network in the cervix [244]. This suggests that the cervix could be being primed early for labour before reaching term in the sPTB women. An additional consequence of increased cytokine production from activation is the migration of neutrophils into the matrix of the amnion [197]. This could occur from a chemotactic gradient produced by increased production of IL-8 from these neutrophils. In normal pregnancy, neutrophils are absent in the amnion, [197]. To add to this, it appears that a higher percentage of activated cervical eosinophils was additionally observed in the sPTB patient. Although the cervical data is only based on an N of 1, it cannot be relied upon, however, infiltration of eosinophils into the cervix occurs during cervical ripening [245] and postpartum remodelling [246]. This suggests that these findings may support the idea that the cervical milieu found in-the sPTB women are being primed to be ready for labour.

NK Cells: sPTB women showed significantly lower CD56^{bright} pNK cells. This subset of NK cells have limited cytotoxicity, however produce both inflammatory and anti-inflammatory cytokines upon activation from monocyte derived cytokines (monokines) [247], these NK cells are highly proliferative and have an immunoregulatory role in producing cytokines to defend the host from infection [248, 249]. This suggests that the contribution of this NK cell subset to regulate immune responses may have been compromised in the sPTB cohort. However, in the peripheral circulation, cytokine production by CD56^{bright} NK cells will support other effector mechanisms following target recognition by producing INF- γ and other pro-inflammatory cytokines [250]. Furthermore, CD56^{dim} NK cells had a tendency to be lower in the sPTB when

compared to the healthy controls. These NK cells are more cytolytic than their CD56^{bright} counterpart and aid in killing infectious cells by degranulation [251]. The combination of a declining frequency of cytolytic CD56^{dim} NK cells as well as loss of cytokine producing NK cells in the sPTB women is likely to make them more susceptible to infection. In terms of activation, increased CD69 expression on NK cells induces cytotoxicity and increases cell lysis, which has been shown to interfere with placental development [252]. However, no difference in CD69 expression on sPTB CD56^{bright} or CD56^{dim} NK cells was observed when compared between the TB and healthy controls.

No change in CD57 expression was observed in sPTB women in both CD56^{bright} and CD56^{dim} NK subsets. CD57 is used as a marker to measure NK cell maturity [253]. With increased CD57 expression, more cytotoxic potential of these NK cells is witnessed. With that being said, NK cells with low CD57 expression exposed to an inflammatory response, secrete more INF- γ than those with higher expression [253]. A decreased mature population of NK cells in pregnancy could indicate lower cytotoxic potential of these NK cells, or that an abnormal response to an inflammatory stimulus occurs initiating a cytokine storm that could drive PTL. There is no current data on the expression of CD57 on pNK cells in relation to sPTB, however, Vassiliadou and colleagues found that CD57 expression on dNK cells was higher in women with spontaneous abortion when compared to healthy pregnant controls [254]. This may suggest that the increased cytotoxic phenotype of these dNK was inhibiting trophoblast function.

NKp30 expression on NK cells did not change in the sPTB women. The function of NKp30 is to up-regulate dendritic cell (DC) maturation [255]. Naïve DC do not possess the ability to differentiate naïve T cells into T-helper cells. Furthermore, HLA-G (an

antigen which promotes fetal tolerance) stimulates the production of mature DC-10, the subset of DC that promotes T suppressor 1 (Tr1) cells production of IL-10 that supports pregnancy [256]. Therefore, the lack of mature DC could lead to a deficiency of functional adaptive immune cells, as mature DC have also been established as mediators of fetomaternal tolerance and infection control [257]. Then again, NKp46 expression was somewhat lower on CD56^{dim} NK cells. NKp46 is an activating receptor that regulates interactions with DC and T cells in response to infection [217, 258]. A study has shown raised NKp46 expression on CD56^{dim} NK cells is associated with normal pregnancy [259]. The decreased expression of this NCR on the most abundant NK cell subset in the sPTB cohort suggests that the cross-talk between the innate and adaptive immune systems may be compromised.

4.5.3 - Adaptive Cell Phenotypic Changes seen in TB Women

T Cells: Cm CD4⁺ and CD8⁺ T cells were decreased in the TB cohort when compared to the sPTB and healthy women. However, frequencies of transitioning CD4⁺ and CD8⁺ T cells were no different in comparison, implying that these women had less exposure to secondary antigens.

Like the sPTB cohort, a similar expression frequency of Tim-3 was found in both CD4⁺ and CD8⁺ T cells. However, significantly higher PD-1 was observed in this cohort when compared to the healthy controls. PD-1 is essential to prevent excessive activation of T cells, but over expression causes exhaustion and diminished IL-2, INF- γ and TNF- α cytokine production [260]. At the maternal-fetal interface, the PD-1/PD-L1 pathway is an important regulator of fetal tolerance [261]. Therefore, in pregnancy an exhaustive CD4 and CD8 T cell phenotype may hamper maternal immune responses during infection and inflammation.

4.5.4 – Innate Cell Phenotypic Changes seen in TB Women

Monocytes: With advancing pregnancy, activation of monocytes has been documented to increase in a similar fashion to that observed in sepsis [232, 262]. This degree of excessive activation in non-classical and intermediate monocytes has also been reported to be greater in preeclampsia [115, 232]. The TB cohort had considerably higher activated monocytes than the other two groups, but, also had significantly higher activated classical monocytes as well. With higher activation of CD14⁺ monocytes comes an induced TLR-4 response [263]. This TLR-4 response induces pro-inflammatory cytokine production [263]. Whilst non-classical monocytes are pro-inflammatory by nature [115], classical monocytes display a more suppressive response, with intermediate monocytes exhibiting both attributes [264]. Therefore, the high activation of all 3 subsets could imply that a response to excessive inflammation is being controlled to enable the continuation of pregnancy. Higher activation of monocyte/m ϕ in the TB patient was also found in cervical cells when compared to the HP patient. However, this is based on an N of 1, therefore is not at statistical significance. fFn has been shown to increase MMP-9 in the amniotic fluid [265] and MMP-9 production in murine experiments, but is mainly produced by activated macrophages. Therefore, the increased activation of monocytes in cervical mucous may explain the high concentrations of fFn found in the TB cohort (440ng/ml).

Granulocytes: It is important to mention that cervical leukocyte data for each of the 3 groups was based on an N of 1. Therefore, no statistical relevant conclusions can be drawn from the cervical data. An observation of the data showed a higher percentage of activation in monocytes/m ϕ but decreased granulocyte activation in cervical mucus from the TB patient when compared to the sPTB patient. This implies that fewer pro-

inflammatory cells are present in the cervix in these women when compared to the sPTB patient. However, in the peripheral blood, neutrophil activation was significantly higher when compared to the sPTB and healthy controls. Activated granulocytes have the ability to migrate towards gestational tissue during term and PTL [266]. That being said, there is no evidence to suggest that these activated granulocytes will migrate to the cervix. But, what maybe being demonstrated in the TB group is that the higher systemic activation of neutrophils could be a response to infection, whereas, cervical neutrophil activation in the sPTB patient may mediate cervical ripening. Also, the low number of cervical samples analysed heavily reduces the reliability of the data, more examples of cervical immune profiles would need to be collected to increase the strength of the data.

4.5.5 – Chapter Conclusion

The results highlighted in this chapter show the phenotypic differences in the immune systems between women presenting with TPTL delivering their baby preterm against those who go on to experience labour at term (Figure 4.5.5.1). The sPTB women demonstrated poorer differentiation in systemic monocytes and reduced NK cell subsets capable of responding to infection. These data suggest that this group of patients may have an inherent poor response to infective pathology making them more susceptible to infection driven PTL. This in turn leads to an altered adaptive immune response as seen in memory T cell subsets with increased proportions of Cm T cells but reduced TEMRA. This change in distribution of memory T cells is associated with an improved potential to home to lymphoid tissues but poor effector functions. However, the preliminary nature of the study prevents any firm conclusions being

drawn. A larger sample size of both peripheral and cervical samples from each cohort is needed.

Considering the disparity between immune profiles of the study groups, longitudinal analysis of these high-risk women would be beneficial to provide a clearer picture of how the immune system changes throughout the pregnancy.

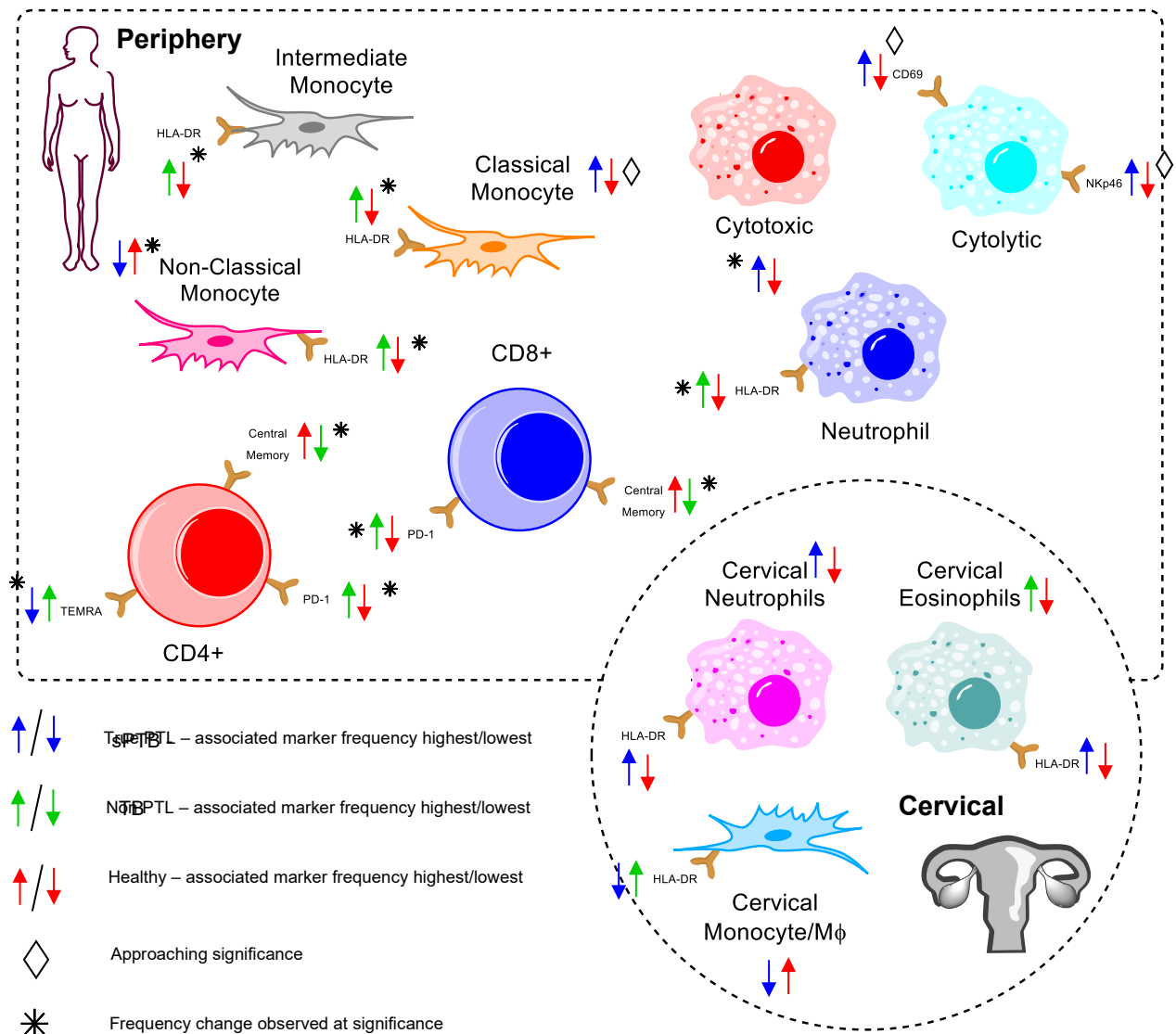


Figure 4.5.5.1 – Summary of the phenotypic changes observed in the immune cells and receptors from each study group.

The Figure condenses all of the phenotypic variances which were observed in percentage of positive cells between each of the study groups which showed a significant difference or were close to significance featured in this chapter. The arrows indicate highest/lowest frequencies observed for each of the groups and the asterisk denoted if the difference was significant.

sPTB (blue), TB (green) and healthy cohort (red).

5. The Immunological Longitudinal Study of a Combination of Progesterone and Aminophylline for the Prevention of Preterm Labour in High-risk Women

5.1 Introduction

In the previous chapter, the understanding of the immunological profiles of high-risk pregnant women who go into PTL was explored in a cross-sectional pilot study. The data suggested that phenotypic adaptations in monocytes, T cells, granulocytes and NK cell populations in the circulation and cervical mucus is different in women who go on to deliver their babies preterm when compared to those who deliver at term. The question of how these immune functions change over the course of high-risk pregnancy and how do current preventive treatments work to maintain the systemic tolerance associated with normal pregnancy will be explored in this chapter.

PTL is managed by treating those at highest risk, with cervical cerclage, pessary, supplementary P4 or offering screening to moderate-risk groups and if indicated, initiating preventative treatment. Women are identified as being at risk of PTL based on the presence of risk factors such as a previous PTB, mid trimester pregnancy loss [267] and previous cervical surgery, such as loop excision or cone biopsy [268]. These patients may be offered a cervical suture, which is history indicated in patients with a previous PTB or mid trimester pregnancy loss, inserted prophylactically at 12 weeks after screening for Down's syndrome, or when the cervical length is seen to shorten before 24 weeks of gestation [269]. Thereafter, they are offered serial transvaginal cervical length assessment often in combination with fFN measurement to determine their risk of PTL [270]. P4 may be offered alone or in combination with a cervical suture to those found to be at increased risk to reduce the risk of PTL [145, 271].

5.1.1 Progesterone

P4 has been shown to be an effective immune-modulator and in reproductive tissues to suppresses inflammation whilst maintaining uterine quiescence [146]. Hassan *et al* showed in a multicentre, randomised double blind study that the administration of vaginal P4 gel to women who were identified in the mid-trimester as having a short cervix reduced the risk of delivering before 33 weeks of gestation by 45% [272]. In addition, the use of P4 may have neonatal benefits, with a study showing that vaginal P4 use in women with a cervical length between 10-20mm is associated with a 61% reduction in the rate of respiratory distress syndrome (RDS) [272, 273].

However, the literature to date is conflicting regarding P4 usage, with some studies demonstrating no difference or only a small reduction in PTB with its use in high-risk singleton pregnancies, and no impact on reducing poor neonatal outcomes [148, 274, 275]. The OPPTIMUM study conducted in 2016 showed that giving a 200mg daily dose of vaginal P4 was not associated with a significant reduction in PTB or adverse neonatal outcomes compared with the placebo group in a sample size of 618 women. Additionally, no long-term harm or benefits were associated with administration of vaginal P4 during pregnancy in infants followed up to the age of 2 [276]. However, in a meta-analysis, including data from OPTTIMUM, Romero *et al* showed that in singleton pregnancies with a short cervix (≤ 25 mm) that administering vaginal P4 reduced the risk of neonatal mortality and morbidity without detrimental effect on neurodevelopment [277]. More recently, the PROGRESS study showed that giving 100mg of vagina P4 did not show any difference in the treatment of respiratory distress syndrome when compared with the placebo group in a sample size of 402 women [148]. Extensive work suggests that P4 has a modest effect on the risk of PTB

suggesting that a more effective therapeutic intervention is required if we are to progress in the prevention of PTL. The addition of a drug administered alongside P4 may help to enhance its capabilities. This part of the thesis will document the longitudinal immunological changes associated with the addition of Aminophylline with P4 in high-risk pregnant women which has not yet previously been documented.

5.1.1.1 Mechanism of Action for Progesterone

The action of P4 is mediated by the PR which targets genes to regulate transcription [278]. P4 is regarded as an essential endocrine support hormone for pregnancy, it can act on a range of different tissues, but the exact site where it acts to maintain pregnancy is not known. In reproductive tissue, two isoforms of P4 receptor known as PRA and PRB have been demonstrated to facilitate its genomic actions [279]. Specifically, in myometrial tissue these isoforms have been shown to be active in response to P4 [280]. In myometrial cells however, *in vitro* up-regulation of PRA expression induces P4-induced pro-inflammatory gene expression. The opposite effect was observed with PRB upregulation [280, 281]. In terms of the interaction between P4 and immune modulation, the membrane progestin receptor (mPR) is the most likely mechanism of action, interestingly; CD4⁺ and CD8⁺ T cells isolated from pregnant women have been shown to contain mPR [282]. Furthermore, these mPR affect T cell function and T cell mediated immune responses by raising Ca²⁺ intracellularly whilst decreasing zeta chain associated protein kinase 70 (ZAP70) [146, 283].

5.1.1.2 The Effect of Progesterone on Acquired Immune Cells

P4 has important anti-inflammatory immune effects which aid in the maintenance of tolerance during pregnancy [284] but, it may also dampen pro-inflammatory innate responses both systemically and locally [147]. However, P4 can also bind to the glucocorticoid receptor (GR) and PR [285], once bound it influences the NFκB signalling pathway to reduce a pro-inflammatory response [147, 286]. Interestingly, in murine models P4 up regulates the production of peripheral and local Tregs to aid fetomaternal tolerance [287]. In non-pregnant human derived PBMC, a study demonstrated that culturing these immune cells with P4 *in vitro*, induced a reduction of FoxP3⁺ cells [288]. In contrast to this finding, there is evidence to suggest that P4 increases Treg production in humans as well. Lee *et al.* demonstrated in human cord blood that P4 mediates Treg proliferation by suppressing the production of Th17 cells via down-regulation of the transcription factor STAT3 [289]. Furthermore, the suppressive effect of P4 induced Tregs is significantly increased [290]. These data suggests that, P4 maybe a regulator of FoxP3⁺ expression [291].

The addition of P4 to human T cells *in vitro* has been shown to increase naïve T cell differentiation into Th2 type cells which produce more anti-inflammatory cytokines such as IL-4 and IL-10 [147, 292]. Additionally, P4 inhibits the proliferation of T cells mediated by DC and promotes suppressive immature DC proliferation [279]. P4 is also shown to up-regulate T cells which produce leukaemia inhibitory factor (LIF) and macrophage colony-stimulating factor (M-CSF) [293], on the other hand, a study has shown that decidual CD4⁺ T cells which lack the ability to produce these proteins have been shown in women with recurrent pregnancy loss [294]. Tim-3 has been shown to downregulate pro-inflammatory responses from Th1 T cells supporting peripheral

tolerance [295]. In mice, it has been shown that mifepristone (a PR antagonist) inhibits T cell proliferation in a similar manner to Tim-3 [296]. Arenas-Hernandez *et al.* showed in mice models that sPTB is associated with increased numbers of effector memory activated T cells expressing Granzyme B and perforin in the decidua, however, treatment with P4 decreased the number of adverse pregnancy outcomes by reducing the pathological inflammation induced by these T cells [297]. There is also evidence to suggest that P4 acts to reduce the sensitivity of memory T cell antigens to stimulation [298].

5.1.1.3 The Effect of Progesterone on Innate Immune Cells

As mentioned in the previous chapter, NK cells play a crucial role in pregnancy and are the most abundant immune cell found in the decidua and are involved with the development of successful placentation [299, 300]. However, their function has been shown to be modulated by P4. For example, a study has shown that P4 stimulates stromal cells in the endometrium causing uNK cell proliferation [301]. Additionally, P4 upregulates angiogenic factors in the endometrium, promoting blood flow and NK ingress into gestational tissues [302]. In mice models, Kuanget *al* demonstrated that the absence of P4 in early gestation is associated with a lack of uNK cells [303]. In the circulation, NK cells during pregnancy have been shown to express a less cytolytic phenotype which is believed to be associated with pregnancy related hormones such as P4 and oestrogen [211]. Elevated levels of P4 during pregnancy are shown to reprogram chemokine receptors in peripheral blood NK cells when they are recruited into the uterine environment [304]. Another study has shown that P4 can stimulate HLA-G expression on 1st trimester trophoblasts *in vitro*, these trophoblasts mediate NK cell inhibition to avoid rejection of the foetus via HLA-G [279, 305, 306]. On the

other hand, P4 does not affect uNK cell activity directly due to their lack of P4 surface or nuclear receptors [307]. It is therefore postulated that P4 acts indirectly through GR to influence NK cell function [308, 309]. In terms of NK cell activation, CD69 expression and INF- γ production by uNK cells are both down-regulated by P4 [310].

P4 has also been shown to affect monocyte function. For example, monocyte chemoattractant protein-1 (MCP-1) which is a potent monocyte chemoattractant [207], is decreased in the cervicovaginal fluid of non-pregnant women taking P4 containing contraception [311]. Migrating monocytes to tissue sites will differentiate into tissue resident macrophages or dendritic cells. In turn, these tissue resident macrophages will differentiate into either an M1 or M2 phenotype depending on the response to tissue injury or wound healing respectively [312]. P4 has been shown to influence the differentiation of these macrophages into phenotypes which promote tolerance by up-regulating immunosuppressive cytokines such as IL-4 and IL-10 in the uterus, this change in phenotype may promote trophoblast invasion [313, 314]. Furthermore, Menzies *et al.* demonstrated that P4 suppresses LPS-induced macrophage activity however, this was shown in mice [315].

In terms of the ability of P4 to mediate neutrophil activity, the data are inconsistent with an *in vitro* study showing that incubation of systemic neutrophils with estradiol and P4 inhibited their degranulation [316] and the administration of vaginal P4 decreased levels of cervical neutrophil numbers [317]. On the other hand, Molloy and colleagues demonstrated that P4 supplementation reduced neutrophil apoptosis in both men and women, whilst also increasing the oxidative burst activity of neutrophils [318].

5.1.2 Aminophylline

Theophylline has been used as a bronchodilator since the 1930s [319]. AP, (a theophylline derivative) has been used for decades as a safe therapeutic agent for the treatment of acute asthma in both pregnant and non-pregnant women [320]. The action of AP, enhances the anti-inflammatory effect of P4 mediated by cAMP following the binding to membrane P4 receptors [320]. As well as reducing uterine contractility [321], AP has been found to improve neonatal outcomes by reducing respiratory distress syndrome (RDS) in preterm infants [321]. A later study by Armanian *et al* demonstrated that AP administered to extremely preterm neonates showed decreased incidences of bradycardia and apnea [322]. Add to this, AP has been reported to decrease uterine contractility *in vitro* and *ex vivo* and to increase the concentration of phosphatidylcholine, a pulmonary surfactant phospholipid in fetal lungs which reduced the risk of RDS [323]. A study where the combined intravenous, maternal administration of AP with magnesium sulphate was associated with a reduced rate of intraventricular haemorrhage in neonates born before 30 weeks of gestation [324]. A further study investigated the use of antepartum AP for the treatment of idiopathic respiratory distress (IRD). This study concluded that perinatal death was significantly lower and in addition, the frequency of IRD was three times less in the AP group compared to the control group [325]. *In vitro* studies have shown reduced contractility of human myometrial strips from the administration of AP [323]. Furthermore, caffeine and AP have a similar mode of action and caffeine is used to reduce the risk of bronchopulmonary dysplasia in extremely preterm infants [326]. It is important to note that these publications are old and should be critically appraised of the validity of the results; however, there have been very few recent findings to elucidate the function of AP.

More recent work from our group has shown that AP can potentiate P4 action to lessen inflammation induced PTB in murine models and also in cultured primary myometrial cells [149]. However, there are no current human *in vivo* studies which investigate the systemic efficacy of combining AP and P4 to assess tolerability in preventing PTL.

5.1.2.1 Mechanism of Action for Aminophylline

The mechanism of action of AP on human smooth muscle is still not fully understood. What is known is that AP binds as a non-selective inhibitor to phosphodiesterases (PDE) 3, 4 and 5 [327]. PDE can be clustered into 11 separate gene families categorised by their ability to hydrolyse cAMP and cGMP [328], two important second messengers that regulate a multitude of intracellular processes [329]. Table 5.1.2.1.1 shows each of the PDE which AP specifically inhibits, and the changes associated when inhibited.

Table 5.1.2.1.1: Illustrates functional changes in PDE inhibition from human-based studies, in particular, PDE3, 4 and 5 which are targeted by AP.

Phosphodiesterase Family	Substrate	Functional Changes of PDE Inhibition
PDE3	cAMP + cGMP	• Vasodilation from increased intracellular cAMP for treatment of heart failure [330]. • Relaxation of the bronchus and of the pulmonary arteries [331]. • Enhanced left ventricular relaxation whilst increasing the uptake of Ca^{2+} and activity of Ca^{2+}ATPase in cardiac sarcoplasmic reticulum [332].
PDE4	cAMP	• The most common PDE present in epithelial cells, smooth muscle, cardiovascular tissue, brain and immune cells (including monocytes, neutrophils, dendritic cells, lymphocytes and macrophages) [333, 334]. • Reduction of airway induced inflammation in Chronic obstructive pulmonary disease (COPD) [335]. • Reduction in TNF-α and increased production of IL-10 in LPS-induced amniochorionic explants. PDE4 inhibition also decreased matrix metalloproteinase-9 (MMP-9) expression and activity a protein implicated in the rupture of membranes [336].
PDE5	cGMP	• Used to treat patients with pulmonary arterial hypertension [337]. • Successful and tolerable treatments used for erectile dysfunction [338].

Theophylline and AP have the same mechanisms of action. As shown in Figure

5.1.2.1.1, theophylline inhibits PDE 3, 4 and 5 and the inhibitions of these PDE slow down the hydrolysis of cAMP and cGMP into AMP and GMP respectively [328].

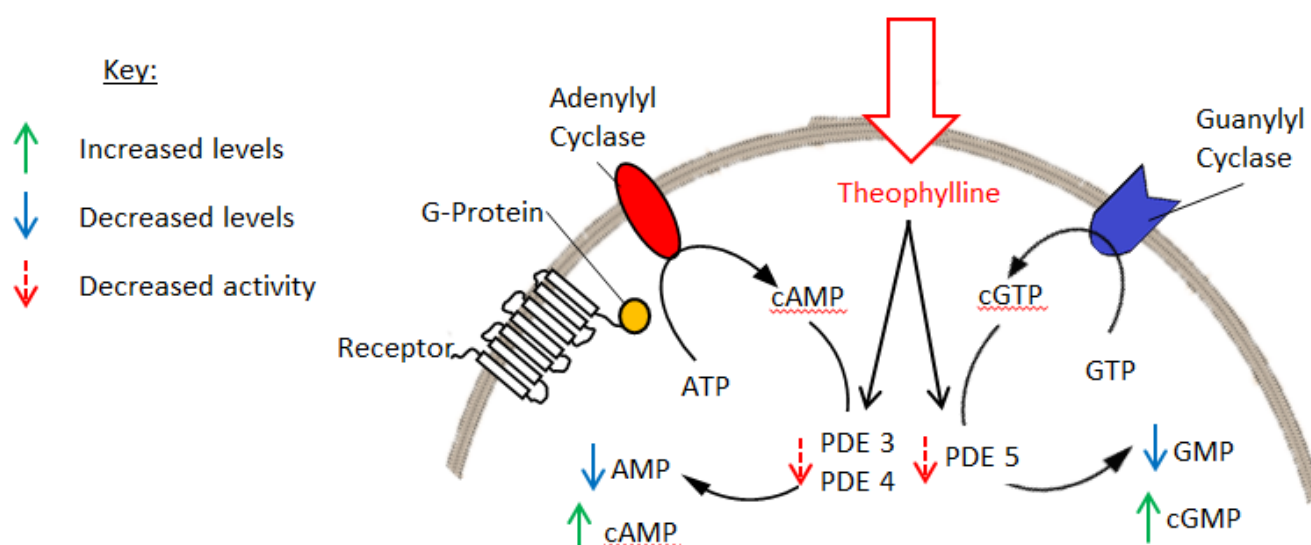


Figure 5.1.2.1.1: Mechanism of action – Aminophylline (i)

The mechanism of action for the effect of theophylline as a PDE3, 4 and 5 inhibitors causing an increase in the amount of cAMP and cGMP in the cell by reducing their hydrolysis into AMP and GMP respectively.

In addition to being a PDE inhibitor, the literature suggests AP in small dosages can also alter histone deacetylase activation (HDAC2) [339]. Phosphoinositide-3-kinase- δ (PI3K δ) metabolism is initially inhibited by theophylline and is important in producing lipids which regulate a number of intracellular signalling pathways [340, 341]. Theophylline increases sensitivity to corticosteroids by increasing HDAC2 activity (an enzyme resulting in diminished function attributed by oxidative stress in patients with COPD) [341, 342]. The activation of the enzymes causes additional deacetylation of essential histones which would normally be acetylated by the histone acetyltransferase enzyme (HATs). HATs co-activation with cAMP response element binding proteins (CREB) proteins regulates gene expression [343], but because of the increase in deacetylation from HDAC2 activity, suppression of NF κ B induced

inflammatory gene production occurs [327]. This mechanism of action depicted in Figure 5.1.2.1.2 is believed to be independent from the PDE inhibition. Corticosteroids act on GR and target HDAC2 activity in a very similar manner to theophylline [344]. The GR and PR receptors are both structurally and functionally very similar and P4 has been shown to act via the GR receptor to decrease pro-inflammatory cytokine production such as IL-1 β in myometrial cells [345, 346].

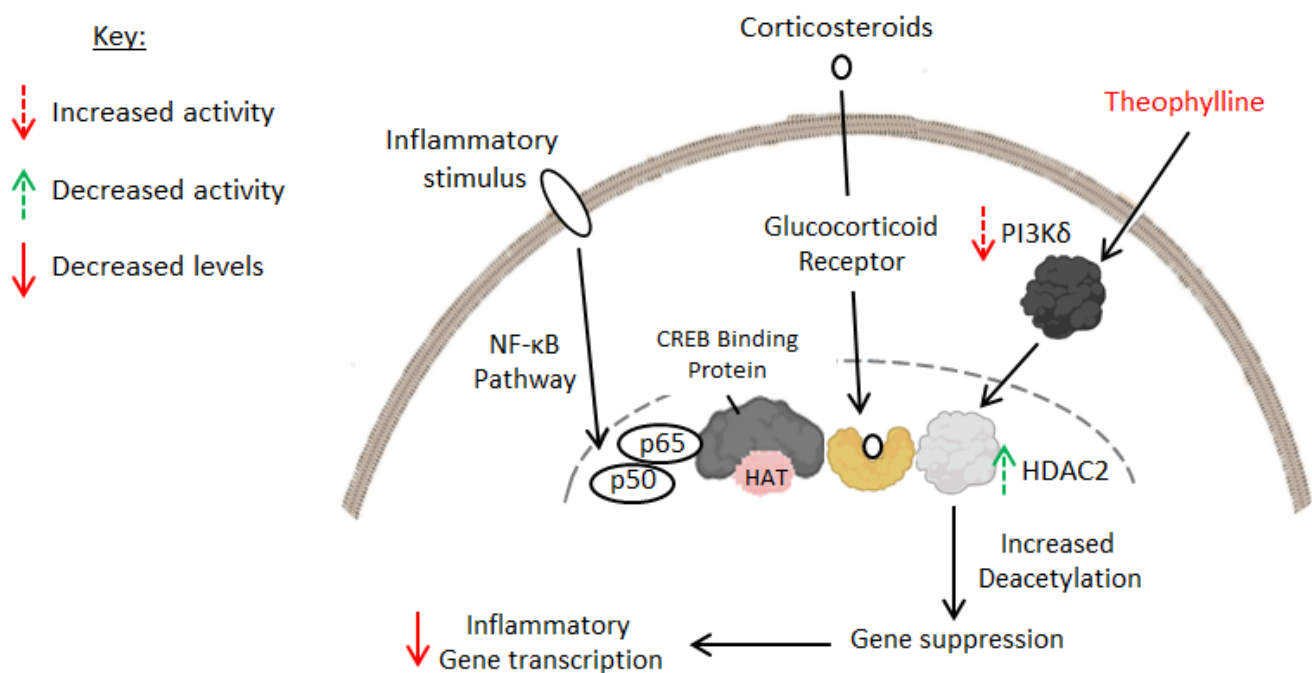


Figure 5.1.2.1.2: Mechanism of action – Aminophylline (ii)

The decreased activity of PI3K δ induced by theophylline resulting in an increase in HDAC2 activity, similar to the effect of using corticosteroids but through an independent mechanism. The increased activation of HDAC2 increases deacetylation of histones causing pro-inflammatory gene transcription to be suppressed.

5.1.2.2 The Effect of Aminophylline on Acquired Immune Cells

As mentioned, aminophylline is a PDE-4 inhibitor [327]. PDE-4 is present on immune cells such as monocytes, T cell and DC [347] and its inhibition has been the target of

reducing inflammatory diseases such as asthma and arthritis [348, 349]. In fact, the majority of work done on theophylline involves its effect on asthma and COPD [344].

Early literature has demonstrated that theophylline *in vitro* inhibits phytohemagglutinin (PHA) induced INF- γ and IL-2 cytokine production [350]. More recently, theophylline was found to decrease the number of activated CD4⁺ T cells in the sputum of asthmatic patients, but, no alterations in activation was shown in systemic T cells [351]. However, separate studies have found that administration of theophylline to asthmatic patients was shown to increase systemic T suppressor cells after treatment [352, 353]. In the presence of high theophylline concentrations, proliferation of CD4⁺ and CD8⁺ T cells is reduced *in vitro* [354]. Moreover, *in vivo* low doses of theophylline treatment increases peripheral blood CD4⁺ and CD8⁺ T cell counts, but decreases proportions of these cells at sites of inflammation in the airways of asthmatic patients [355]. Theophylline has a similar effect in allergen challenged asthma [356]. Additionally, there is evidence to suggest that cAMP can mediate immune suppression of effector T cell responses. For example, Theophylline has been shown to induce apoptosis in T cells by raising intracellular cAMP [357]. Naturally occurring Tregs have high concentrations of cAMP, which they can transfer to effector T cells to mediate their suppressive function [358]. Sakkaset *al.* demonstrated that PDE-4 inhibition affected TCR induced activation subsequently down regulating cytokine production from Th1 and Th17 cells [359].

5.1.2.3 The Effect of Aminophylline on Innate Immune Cells

The anti-inflammatory effects of theophylline in response to innate cell types have also been well documented in the cases of asthma. For example, theophylline administration decreases respiratory burst activity from human alveolar macrophages

in non-asthmatic patients [360]. Furthermore, theophylline suppresses macrophage IL-13 release, a key inflammatory component found in the airways of asthmatic patients [361, 362]. Theophylline was also shown to suppress TNF- α production from human monocytes when incubated *in vitro* [363].

Interestingly, theophylline has been shown to increase apoptosis in systemic neutrophils and eosinophils by down-regulation of GM-CSF and B-cell lymphoma 2 (bcl-2) which reduces the influx of these cells into the airways of asthmatic patients [364]. Another study also showed theophylline's ability to inhibit eosinophils by inducing apoptosis *in vitro* in the presence of IL-5 [357]. However, the induction of apoptosis in granulocytes is not a consequence of PDE-4 inhibition, but the antagonism of the adenosine receptor [357, 364]. Similarly, in T cells adenosine may regulate TCR priming and suppress T cell migration among other functions [365, 366]. In COPD, theophylline was shown to decrease neutrophil numbers and IL-8 concentrations (a potent chemoattractant for neutrophil migration) in sputum. Implying that theophylline has a greater suppressive effect than corticosteroid usage in these patients [344, 367].

5.2 – Chapter Hypothesis

- i. The addition of AP will help to increase the anti-inflammatory effect of P4 *in-vivo* by reducing the percentage of pro-inflammatory cell types in peripheral blood and cervical mucus in high-risk pregnant women.

5.2.1 – Chapter Aims

- i. The aim of this chapter is to assess the longitudinal changes in the immune phenotype in pregnant women at high-risk of PTB receiving either a combination of P4 and AP or P4 alone to prevent PTB.

5.3 – Processing and Methodology

5.3.1 Recruitment

Table 5.3.1.1 - The inclusion and exclusion criteria used to identify high-risk pregnant women who were suitable to recruit for the PROGRAM study.

PROGRAM	
Inclusion Criteria	Recruitment from Maternal Assessment Suite (MAS) and Antenatal Clinic (AC): 1) Singleton pregnancy with intact membranes at recruitment 2) Age 18-40 at time of consent 3) Obstetric history of at least one of the following: - Previous mid trimester loss between 14-26 weeks - Previous delivery <37 weeks - Women who have had a cervical cerclage - Short cervix (≤ 25 mm) at baseline
Exclusion Criteria	1) Intolerance to P4 2) Intolerance to aminophylline 3) No prescribed medication which could interact or alter the effects of P4 and/or AP 4) Women who smoke, drink alcohol excessively or take recreational drugs 5) Women with PE or severe hypertension 6) Women with sepsis or an undiagnosed infection

All participants were recruited following informed consent either at the Maternal Assessment Suite (MAS) or antenatal clinic (AC) of Chelsea and Westminster Hospital. Informed consent was taken from all participants. Ethics approval was obtained from the Brompton and Harefield research ethics committee (10/H0801/45). A high vaginal swab for culture and fluid analysis were collected into separate tubes. A cervical cytobrush (Rovers Medical Services, Netherlands) was used to collect immune cells from the endocervical canal. 24mL of maternal peripheral blood was collected into 2x10mL Lithium-Heparin tubes and a 1x4mL EDTA tube. Pregnant

women were identified as high-risk based on the inclusion criteria listed in Table 5.3.1.1.

5.3.2 Sample Collection

Sample collection following the baseline visit occurred between 13-20⁺⁵ weeks of gestation. These women were randomised to receive either P4 (400mg as a vaginal pessary once daily) or a combined P4 and AP (225mg twice daily) treatment up to week 34 of pregnancy. The first treatment was administered at 18 weeks or at the gestation recruited if the participant was already past 18 weeks. Screening visits occurred at 22, 26, 30 and 34 weeks of pregnancy (+/- 2-day window) and samples were collected and processed fresh and ran on the flow cytometer on the same day. A total of 11 women (6 from the P4 cohort; 5 from the combination treatment) had no onset of labour and underwent a Caesarean section. However, all women who underwent Caesarean section were at term gestation when delivering. Myometrial biopsies were taken from these women and stored at -80°C. Delivery samples and a 6-week postnatal sample was collected Table 5.3.2.1

Table 5.3.2.1 - Shows the time points of when each sample will be taken for the PROGRAM study.

PROGRAM Sample Log	Baseline Wk 13-20 ⁺⁵	Wk22	Wk26	Wk30	WK34	Delivery	6 Weeks Postnatal
Maternal blood	✓	✓	✓	✓	✓		✓
Cervical cytobrush	✓	✓	✓	✓	✓		✓
Vaginal swab	✓	✓	✓	✓	✓		✓
Cord blood						✓	
Placenta sample						✓	
* Myometrial sample						✓	

* Myometrium was only taken from women who underwent Caesarean section with no signs of labour.

5.3.3 Blood Processing

Maternal immune cells from peripheral blood collected in lithium-heparin tubes were isolated using the methods described in chapter 3: Section 3.2 for flow cytometric analysis using the panels outlined in chapter 3: Tables 3.10.1.1 – 3.10.1.5. Maternal plasma was aspirated off and stored at -80°C for later analysis and 1×10^7 PBMC were cryopreserved using the methods described in Chapter 3 Section 3.6.

5.3.4 Swab and Cytobrush Processing

Maternal immune cells collected from the cervical cytobrush samples were isolated using the methods described in chapter 3 Section 3.9 for flow cytometric analysis using the panel outlined in chapter 3: Table 3.10.1.5. Excess mucosa collected from the brush was diluted in RPMI-1640 sup and stored at -80°C. Excess fluid taken from the fFN test was also transferred to an eppendorf and stored at -80°C.

5.3.6 Perfetto Method

Using the protocol set out by Perfetto *et al.* The optimisation of the Fortessa X-20 BD flow cytometer (Beckton, Dickinson) was used to calibrate the machine to reduce mechanical variation between sample collections [151]. Using this protocol as a guideline, I was able to track the stability of the PMT, optimise compensation, increase signal the background ratio of each channel, identify suitable voltage ranges for each laser and optimise target MFI values for each channel. Daily tracking ensured standardisation of longitudinal sample acquisition. Further details have been explained in Chapter 3; Section 3.10.1.

5.3.7 Demographic Data for PROGRAM Recruited Women

Table 5.3.7.1 displays the maternal demographic, obstetric and pregnancy outcomes data of both cohorts: administration of a combination therapy (N= 12) and women given just P4 (N= 11).

Table 5.3.7.1– Maternal demographic, obstetric and pregnancy outcomes data for recruited for the PROGRAM study for women.

Demographic and obstetric data	Women randomised to P4 + aminophylline (n= 12)	Women randomised to P4 treatment only (n= 11)
Median maternal age	36 (31-42)	34 (25-41)
Ethnicity, n (%)		
Other	9 (75.0)	7 (63.7)
White	3 (25.0)	4 (36.3)
Median gestational age at recruitment, (weeks)	16 ⁺⁵ (13-20 ⁺⁵)	16 ⁺⁶ (13 ⁺³ -20 ⁺⁵)
Cervical Length, n (%)		
< 25mm	5 (41.7)	1 (9.1)
≥ 25mm	7 (58.3)	10 (90.9)
Mid-term loss, n (%)		
0	4 (33.3)	4 (36.3)
1	8 (66.7)	7 (63.7)
Median BMI at recruitment (kg/m ²)	25 (19-33)	25 (20-37)
Consumables, n (%)		
Smoking	0 (0.0)	1 (9.1)
Caffeine	3 (25.0)	10 (90.9)
Previous PTB, n (%)		
0	4 (33.3)	5 (45.5)
≥1	8 (66.7)	6 (54.5)
Pre-eclampsia, n (%)		
Yes	0 (0.0)	0 (0.0)
No	12 (100.0)	11 (100.0)
Gestational diabetes, n (%)		
Yes	1 (8.3)	1 (9.1)
No	11 (91.7)	10 (90.9)
Pregnancy Outcomes		
Onset of Labour, n (%)		
Spontaneous	6 (50.0)	6 (54.5)
Induced	0 (0.0)	0 (0.0)
No labour	6 (50.0)	5 (45.5)
Unknown	0 (0.0)	0 (0.0)
PTB, n (%)		
Yes	2 (16.7)	3 (27.2)
No	10 (83.3)	8 (72.8)
Median gestational age at delivery, (weeks)	38 ⁺¹ (33 ⁺⁶ -40 ⁺²)	38 ⁺¹ (35-41 ⁺²)

Medians (m) followed by their corresponding (min to max) range, whereas, numbers (n) followed by (percentages).

5.3.8 Statistical Analysis

The initial sample size of 70 women (35 per arm) was based on the number of patients who attended the Prematurity clinic at Chelsea and Westminster Hospital and who required P4 treatment over a six-month period. Microsoft Excel for Windows 2010 (Microsoft, Washington USA) was used to collate patient and sample data. GraphPad Prism version 8.4.3 (San Diego, USA) was used to calculate all statistical analyses. For the PROGRAM study, the Shapiro-Wilk's test was firstly used to test normality of data distribution. For normally distributed data, an ordinary 2-way ANOVA and a Tukey's multiple comparison test was applied to each set of data. Additionally, a Mann-Whitney test was used to compare pregnant participants randomised to either the combination therapy or P4 only for each time point. All flow cytometry data was collected on BD FACS Diva version 8.0 (San Jose, California, USA) and analysed performed using FlowJo version 10.7.0 (Oregon, USA) expressed as % of positive cells taken from leukocyte populations. Normally distributed data was displayed as mean and standard deviation. All statistical tests were two-tailed, and significance was defined as $P < 0.05$.

The longitudinal nature of the study meant that follow up visits were not met by all study participants. In fact, at baseline, 22 weeks and 26 weeks the peripheral blood data shows that the number of participants for the P4 only and P4/AP cohorts was 11 and 12 respectively, this number dropped to an N of 10 and 11 for week 30 respectively. Then dropped further by week 34 to an N of 7 for both cohorts. The 6-week postnatal visit data, shows an N of 2 and 3 respectively. This diminishing sample size as gestation progresses decreases the reliability of the statistical data and

therefore must be taken consideration when being analysed however, it is an issue which could not be avoided.

5.4 Results

5.4.1 Treg Analysis

The fluorochrome-conjugated antibodies used to surface stain peripheral Treg populations and gating strategy used to phenotype Tregs is described in Chapter 4 Figure 4.4.1.1

The percentage of Tregs observed from baseline to the 6-week postnatal follow up did not show any significant change between the combination treatment when compared to P4 only. Additionally, HLA-DR activation was also associated with no change between study groups throughout gestation (Figure 5.4.1.1).

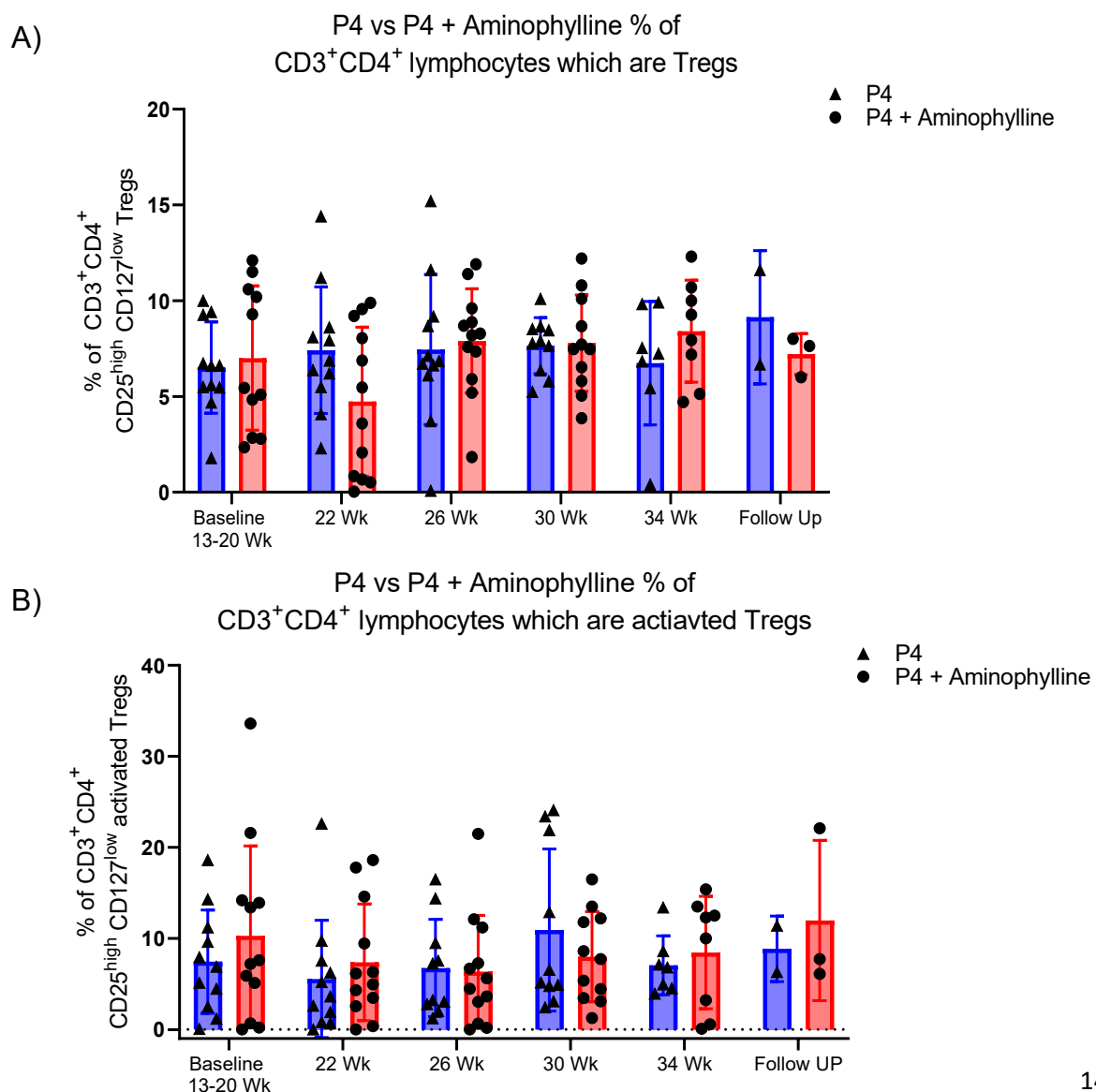


Figure 5.4.1.1: PROGRAM CD3⁺ CD4⁺ lymphocytes which are CD25^{high} CD127^{low}Tregs and HLA-DR activation frequencies.

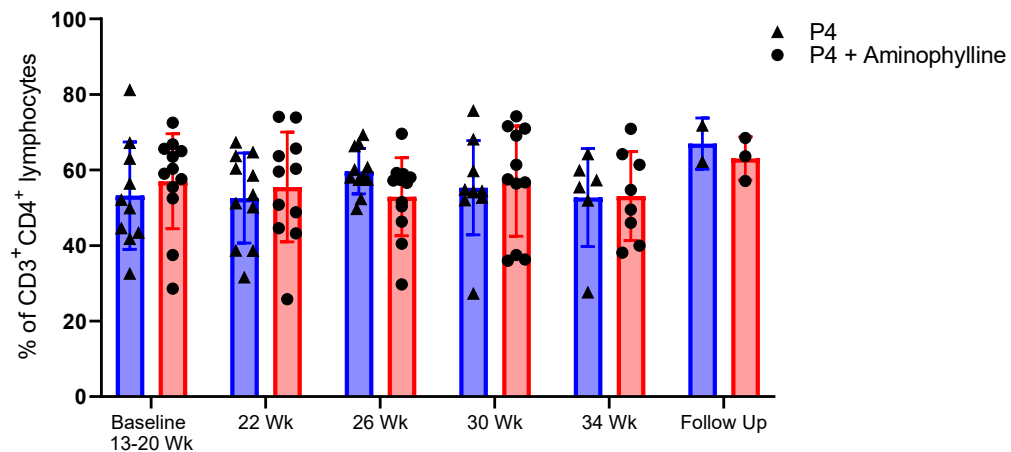
These graphs compare CD3⁺CD4⁺ lymphocytes which are CD25^{high} CD127^{low} Tregs (A) and Treg activation (B) between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 and AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied, significance was defined <0.05.

5.4.2 Effector Cell Analysis

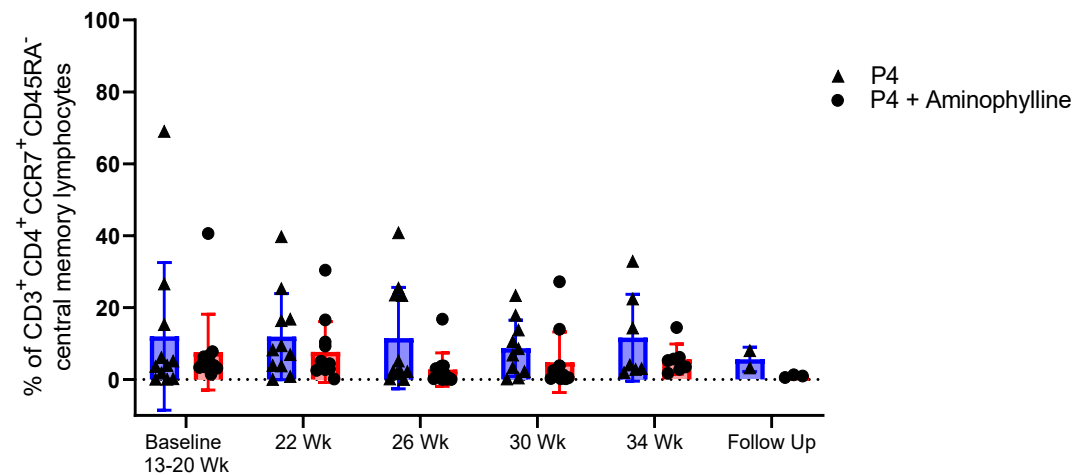
The fluorochrome-conjugated antibodies used to surface stain peripheral T cell populations and gating strategy used to phenotype T cells is described in Chapter 4 Figure 4.4.2.1

Percentage of CD4 and CTL remained consistent throughout gestation in both cohorts (Figures 5.4.2.1 and 5.4.2.2 respectively). No significant changes were observed in naive, transitioning or memory cells from both subsets of T cells. Frequency of cm CD4 cells was no different in the combination group when compared to the P4 cohort. Em for both CD4 and CTL did not show any change throughout pregnancy. Functionally distinct populations of effector cells 1-4 expressed by CTL also showed no significant change throughout gestation (Figure 5.4.2.3) nor did terminally differentiated effector memory RA (TEMRA) cell populations change for either CD4 or CTL.

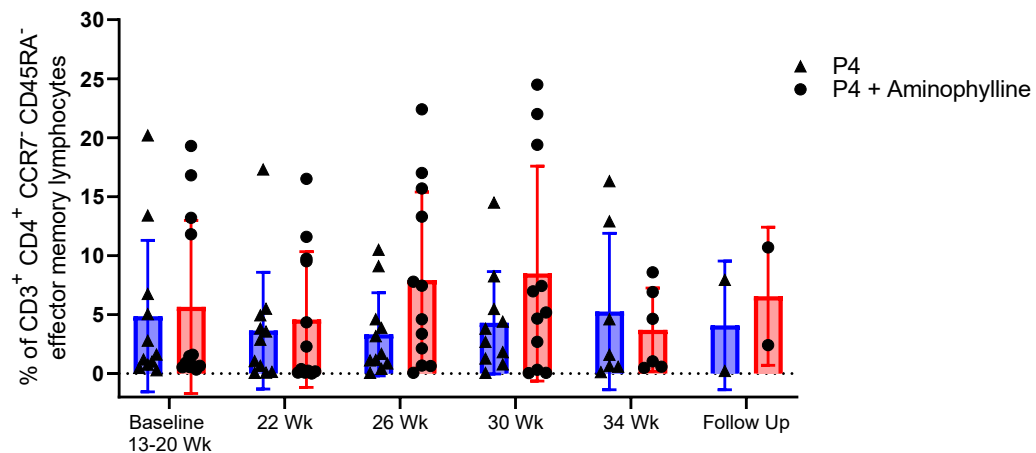
A) P4 vs P4 + Aminophylline % of CD3⁺CD4⁺ lymphocytes



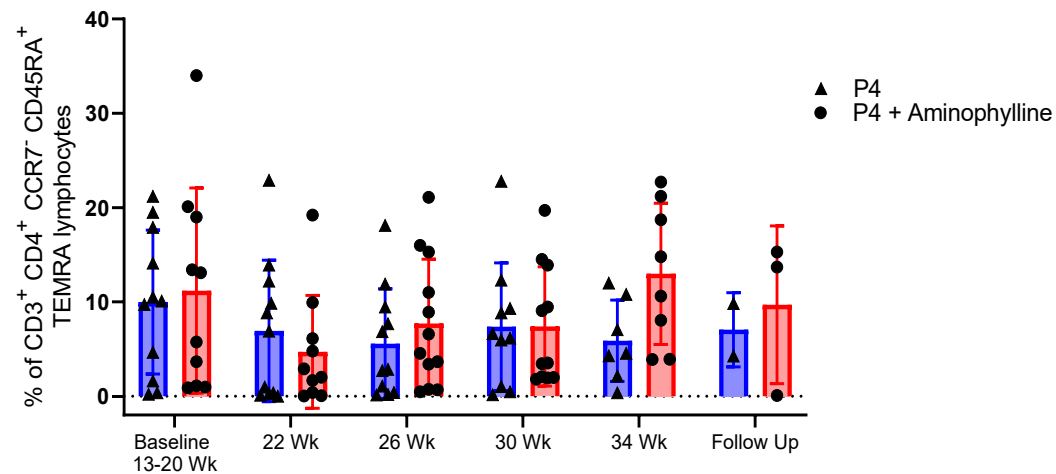
B) P4 vs P4 + Aminophylline % of CD3⁺CD4⁺ CCR7⁺CD45RA⁻ central memory lymphocytes



C) P4 vs P4 + Aminophylline % of CD3⁺CD4⁺ CCR7⁻CD45RA⁻ effector memory lymphocytes



D) P4 vs P4 + Aminophylline % of CD3⁺CD4⁺ CCR7⁻CD45RA⁺ TEMRA lymphocytes



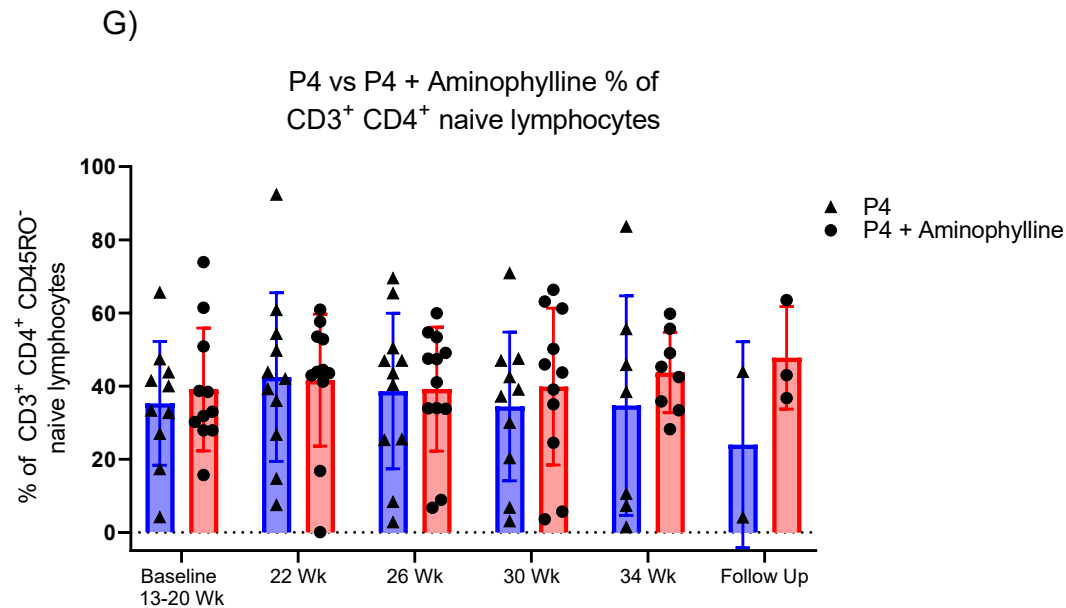
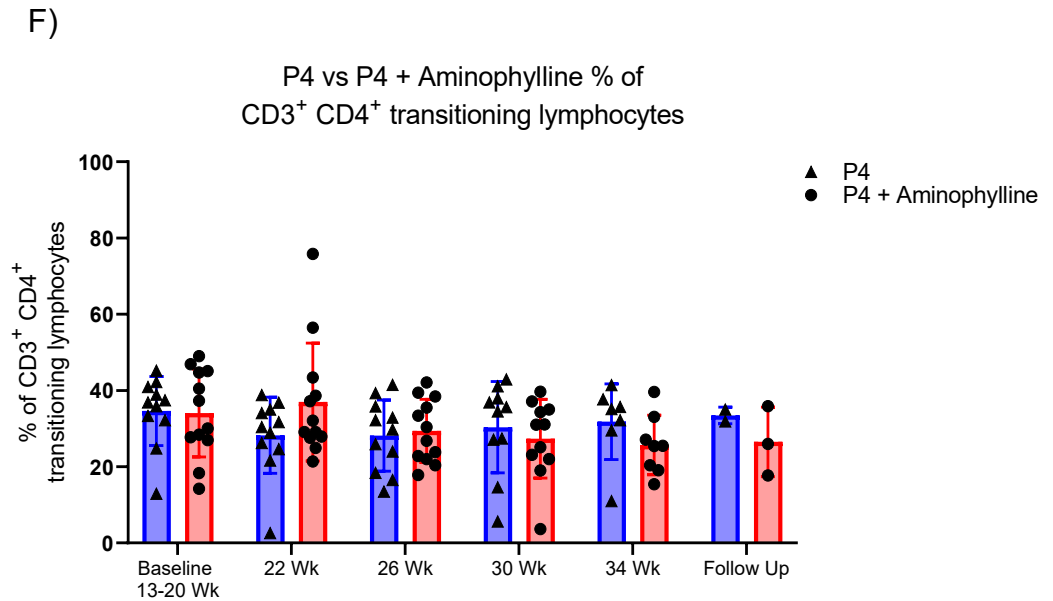
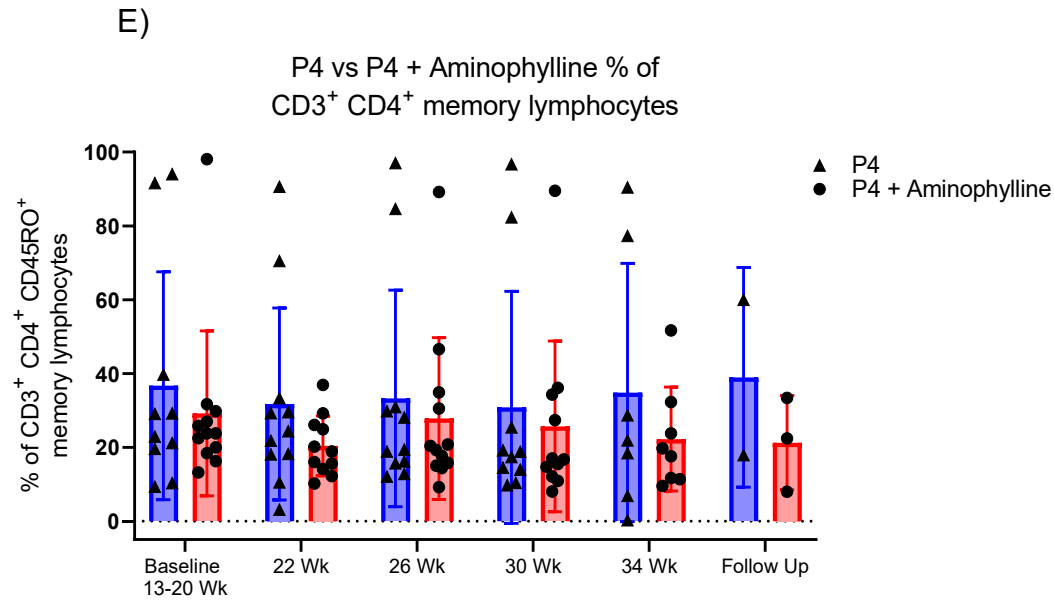
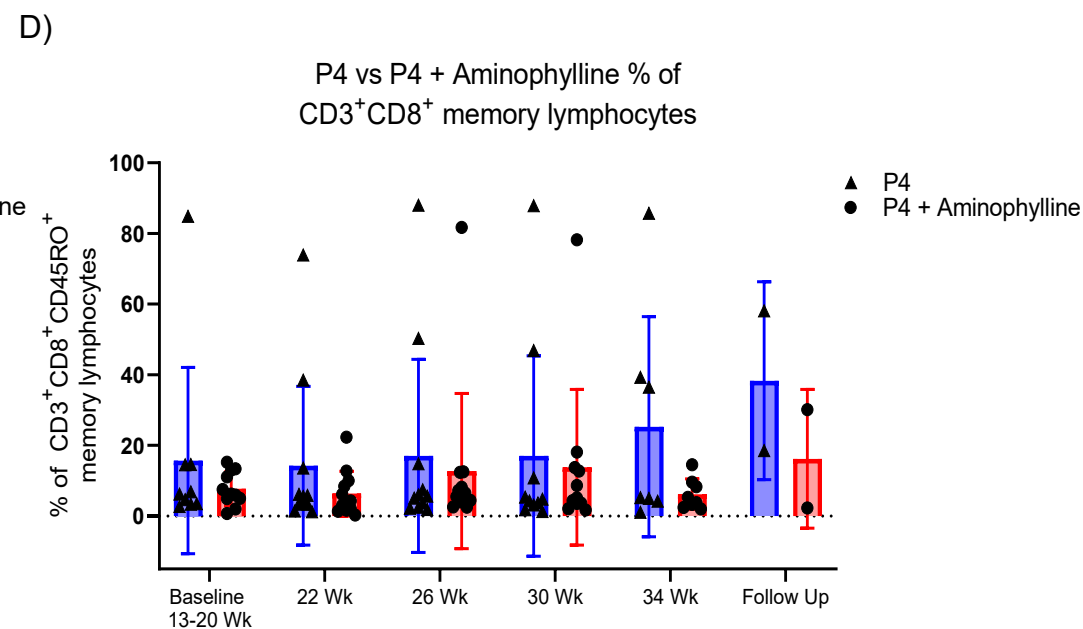
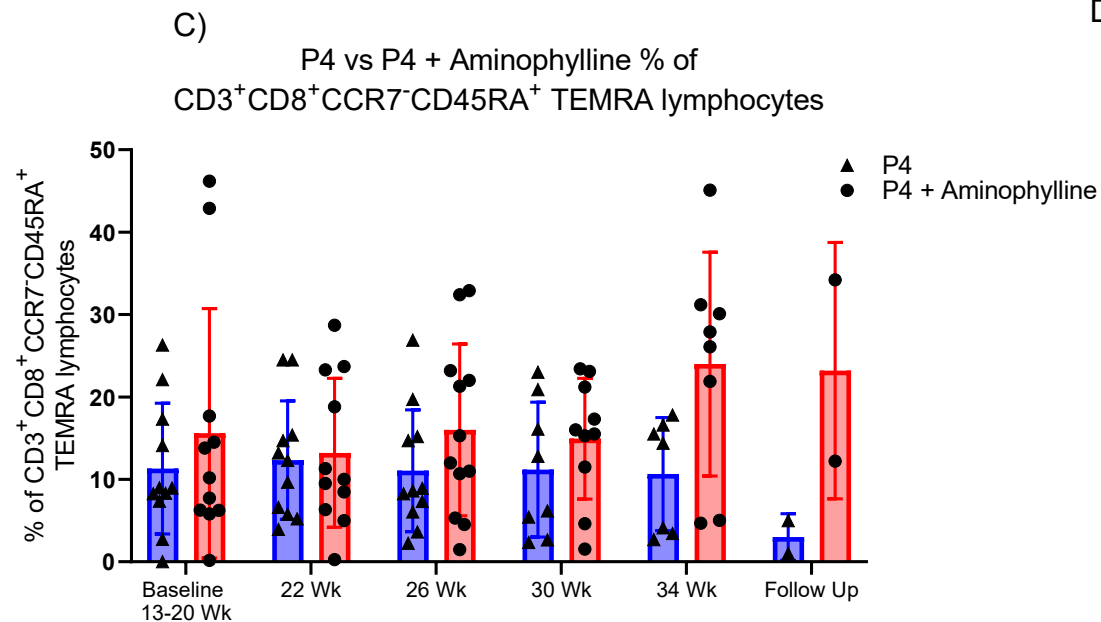
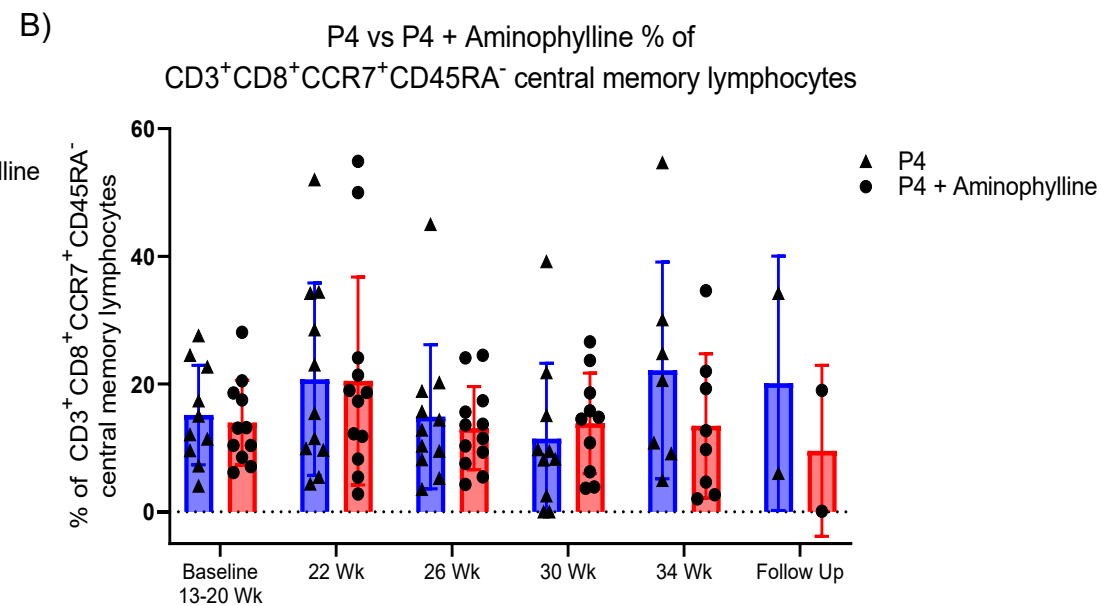
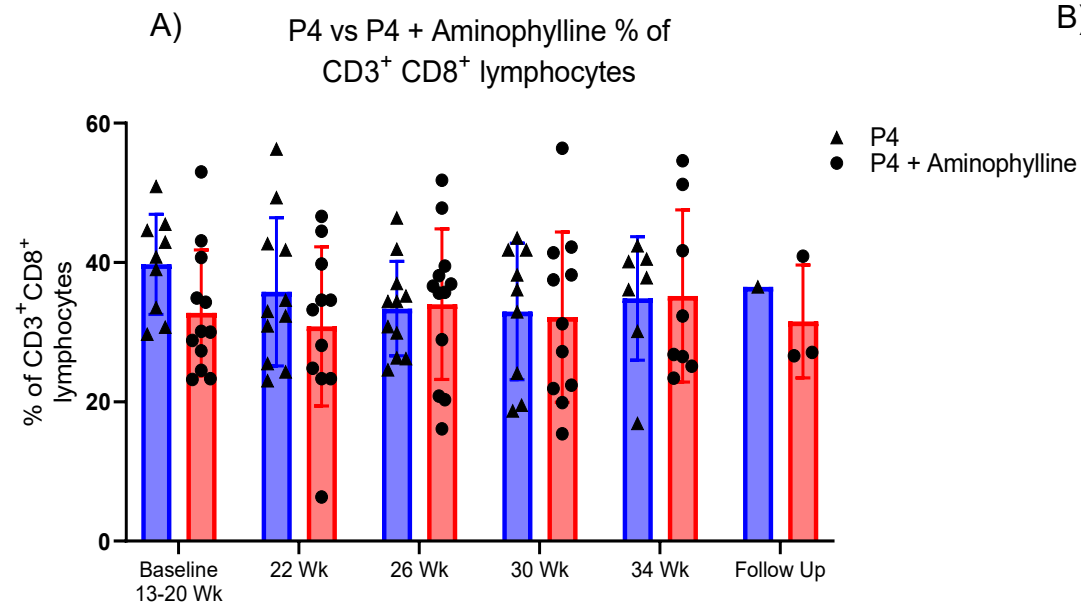


Figure 4.4.2.1: Memory subsets of CD3⁺ CD4⁺ T cell lymphocytes.

These graphs show frequencies of CD3⁺ CD4⁺ T cell lymphocytes in peripheral blood (A); Cm (B); Em (C); TEMRA (D); CD45RO⁺ memory (E); transitioning (F) and naïve (G) T cells between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 and AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively).. Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P <0.05.



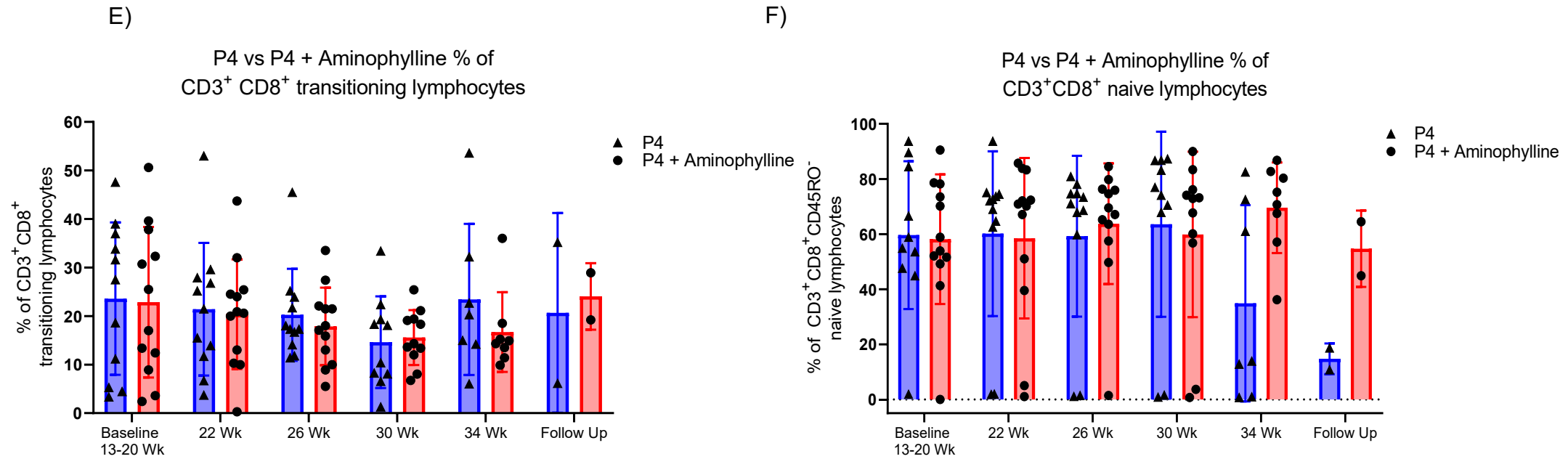


Figure 5.4.2.1: Memory subsets of the CD3⁺ CD8⁺ T cell lymphocytes.

These graphs show frequencies of CD3⁺ CD8⁺ T cell lymphocytes in peripheral blood (A); Cm (B); TEMRA (C); CD45RO⁺ memory (D); transitioning (E) and naïve (F) T cells between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 and AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P < 0.05.

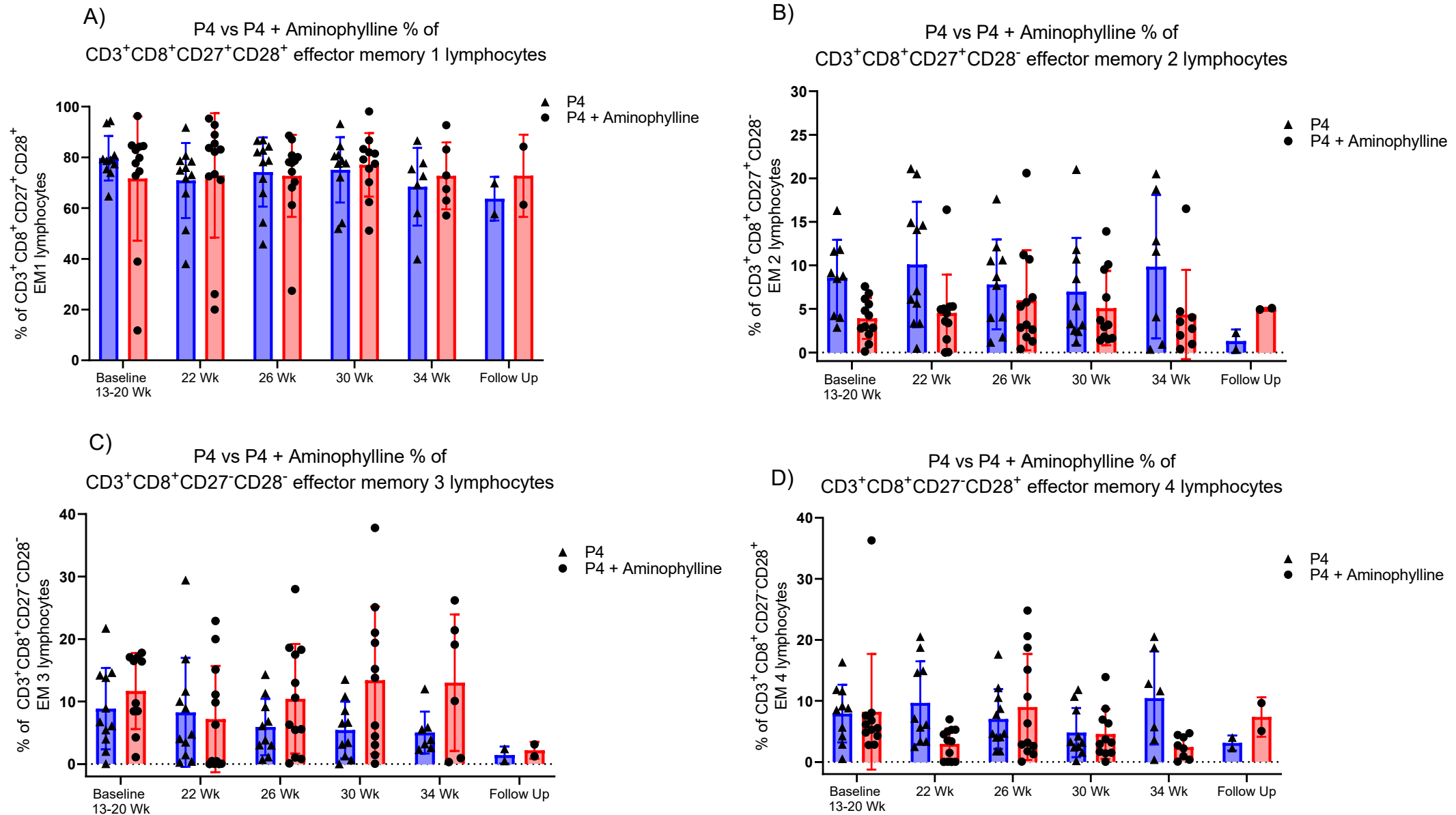


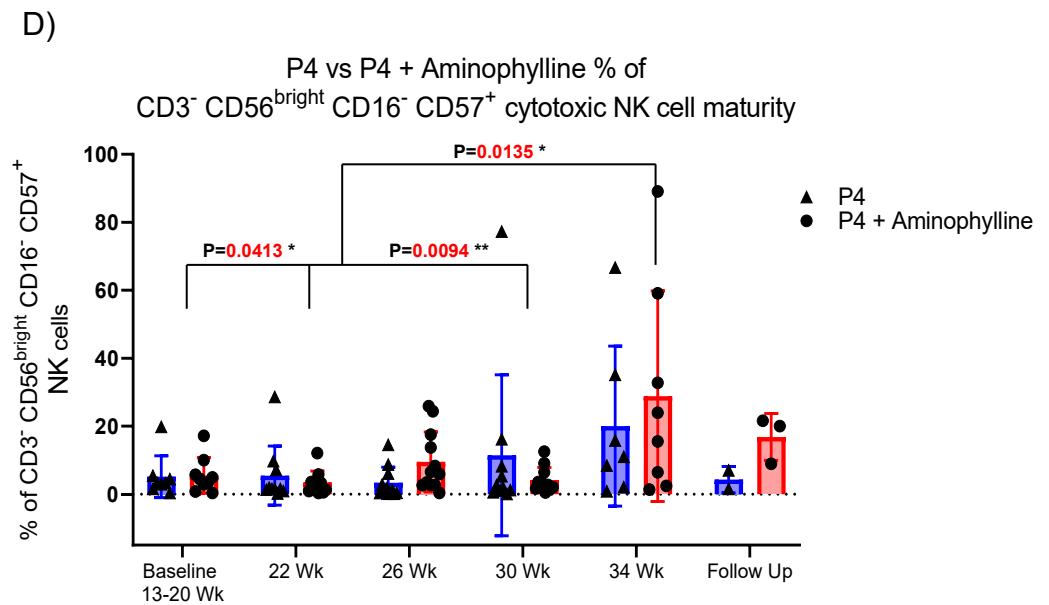
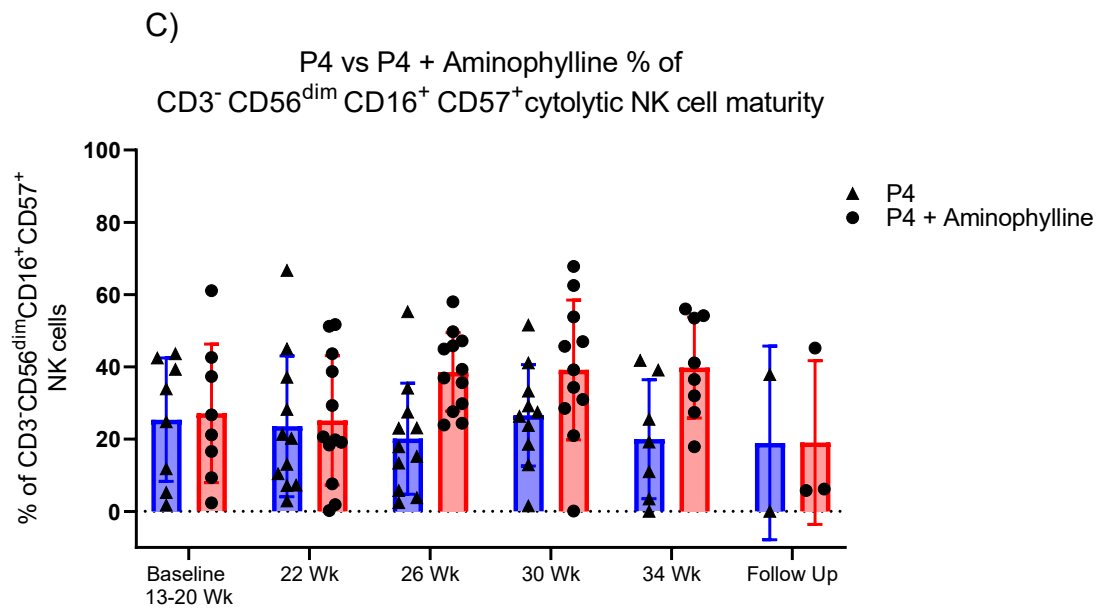
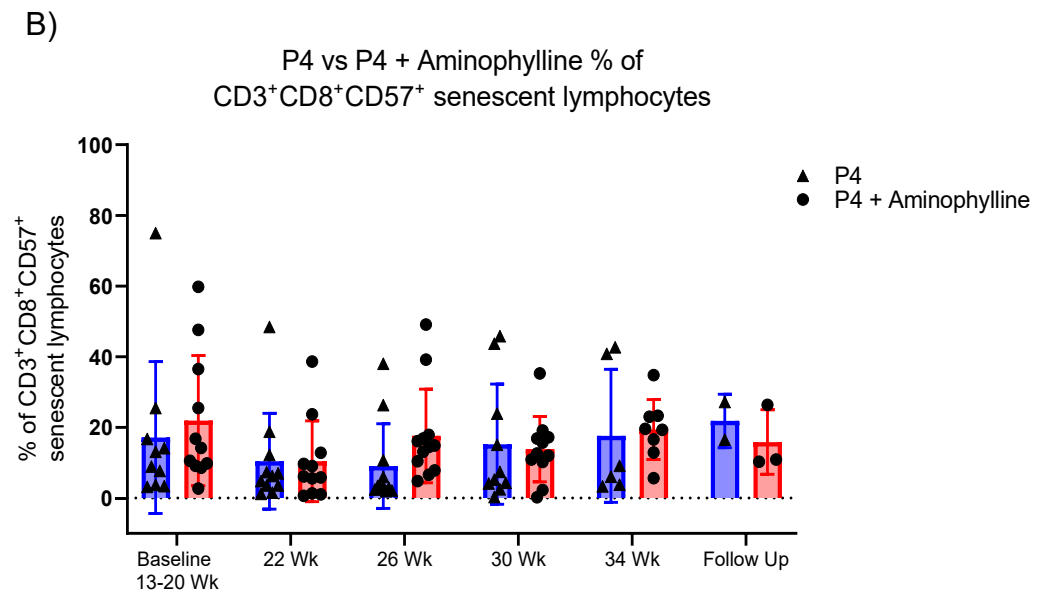
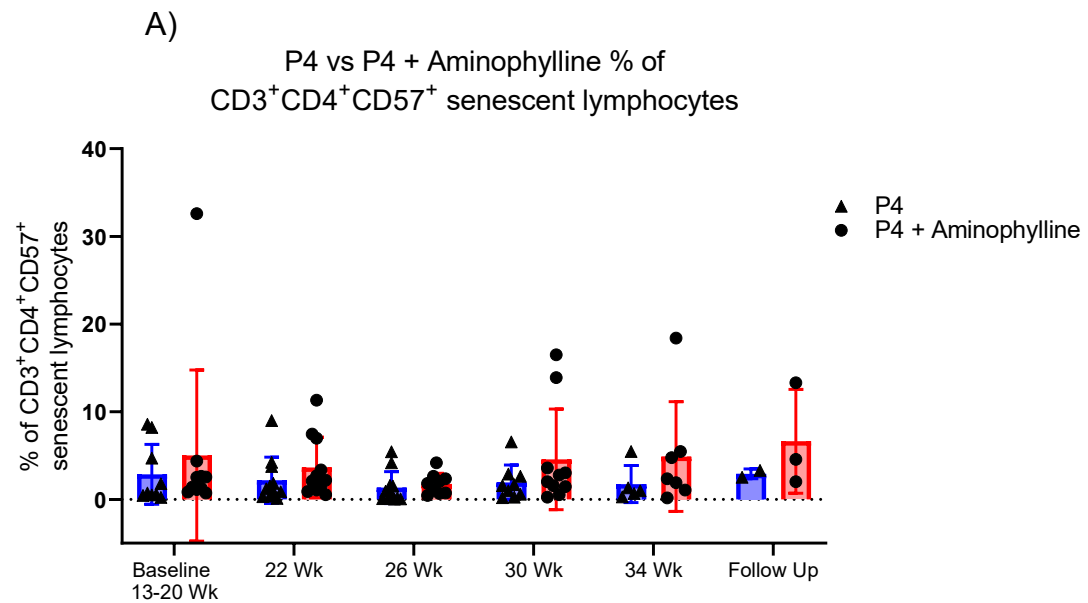
Figure 5.4.2.2: CD27 CD28 Effector memory subsets of CD3⁺ CD8⁺ T cell lymphocytes.

These graphs show frequencies of CD3⁺ CD8⁺ Em1 (A); Em 2 (B); Em 3 (C) and Em 4 (D) T cell lymphocytes between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 and AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as $P < 0.05$.

5.4.3 Exhaustion and Senescence Analysis

The fluorochrome-conjugated antibodies used to surface stain exhaustion, maturity and senescent frequencies on T cells and NK cells. The gating strategy used to phenotype these markers are described in Chapter 4 Figure 4.4.3.1

No significant changes were observed in exhaustion, senescence or PD-1 expression from both CD4 and CTL in either cohort throughout pregnancy (Figure 5.4.3.1). The only significant change was observed in CD57⁺ cytotoxic NK cells (CD56^{bright} CD16⁻) where a significant increase in the combination therapy was observed at the 5th time point of pregnancy (34 wk) when compared to baseline, the 2nd (22 wk) and 4th (30 wk) time points. The p values from these increasing trends were as follows: (Baseline – 34 wk; $p=0.0413$), (22 wk – 34 wk; $p=0.0094$) and (30 wk – 34 wk; $p=0.0135$). The same significant increase in NK cell maturity across gestation was not observed in the P4 only cohort.



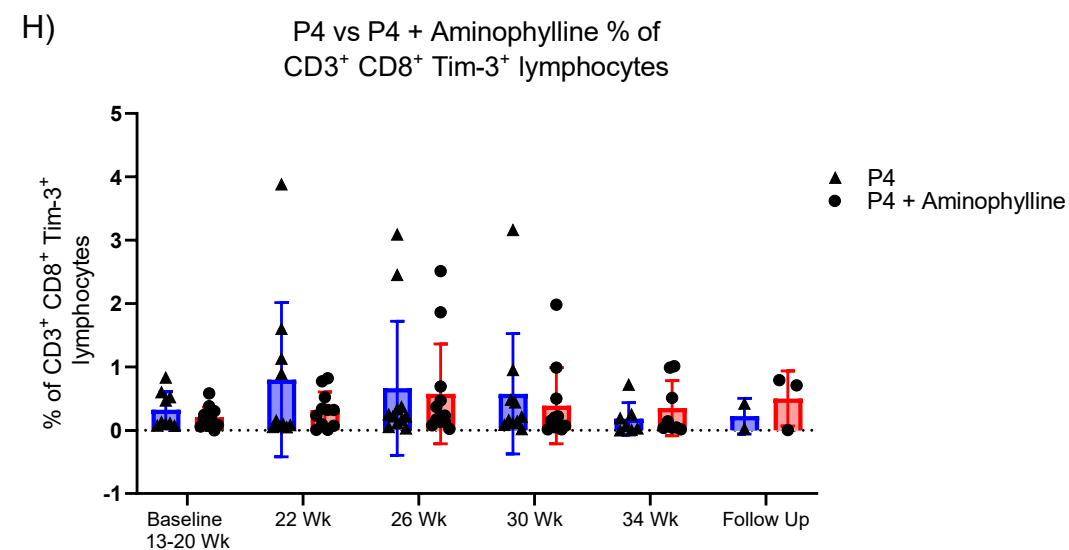
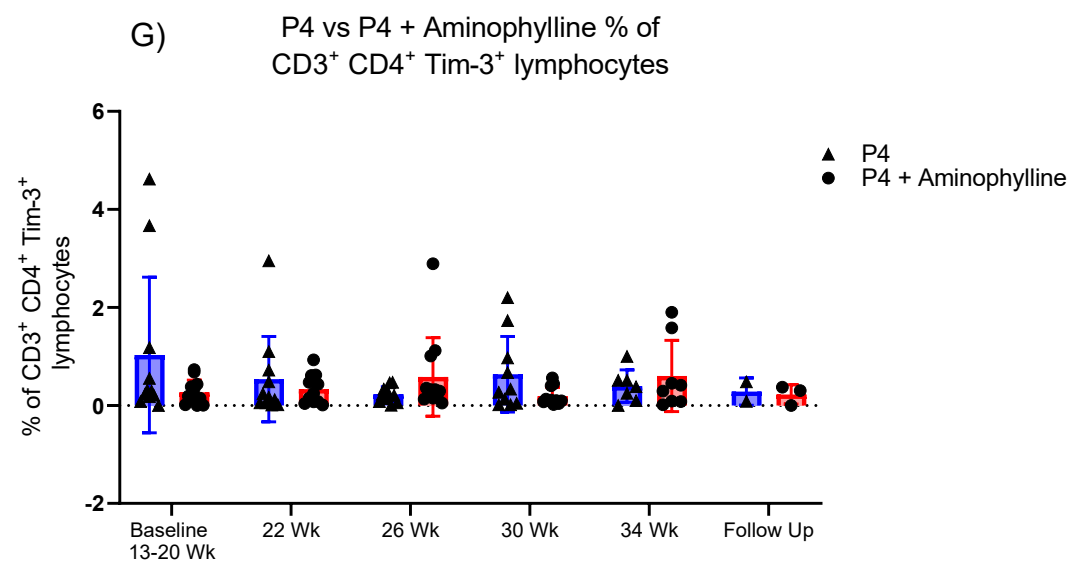
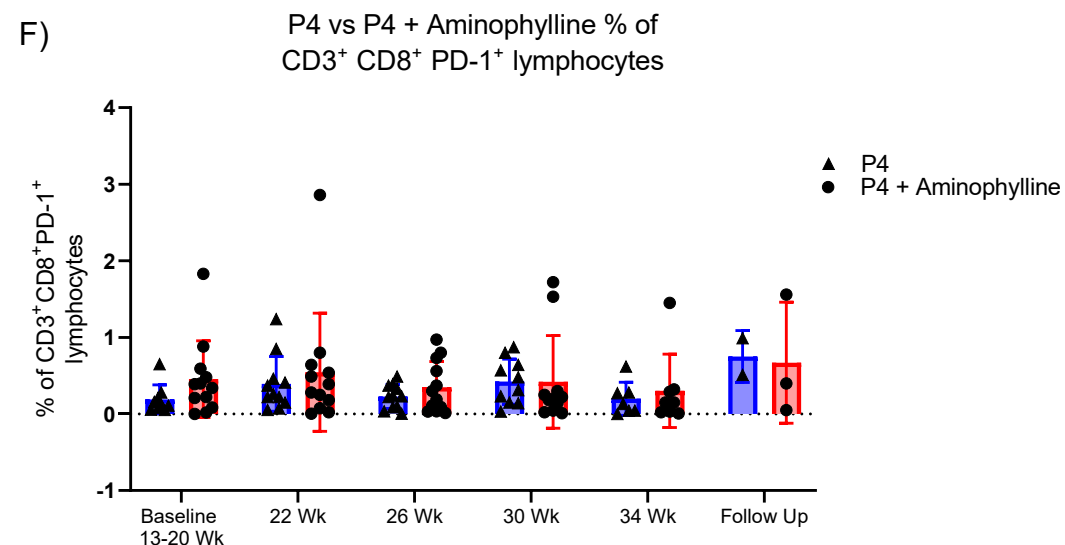
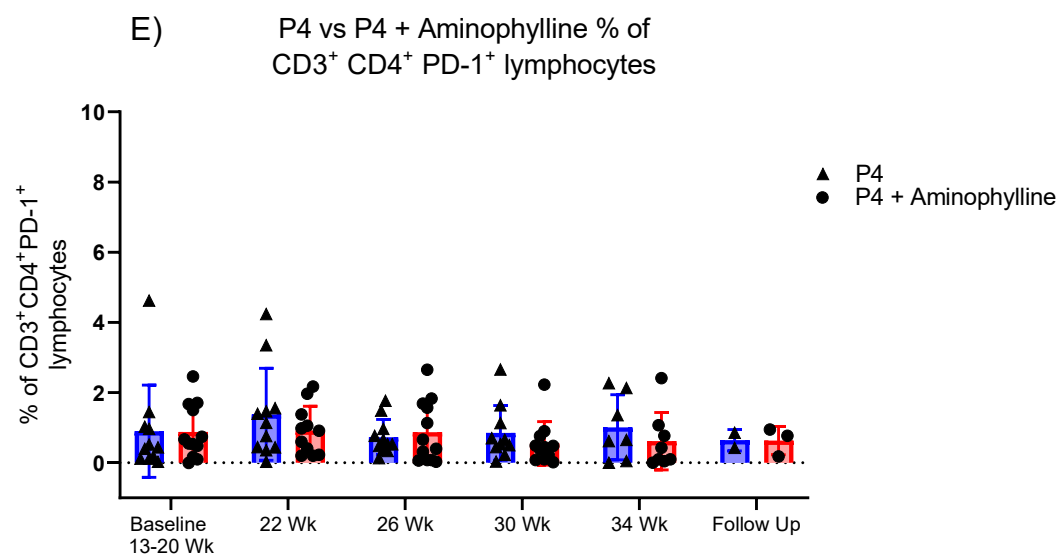


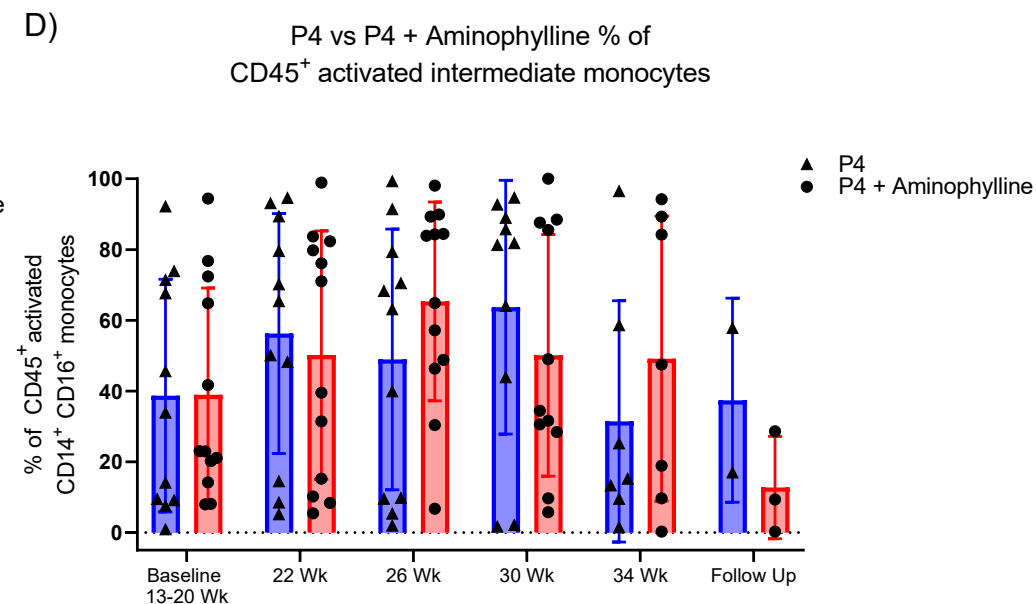
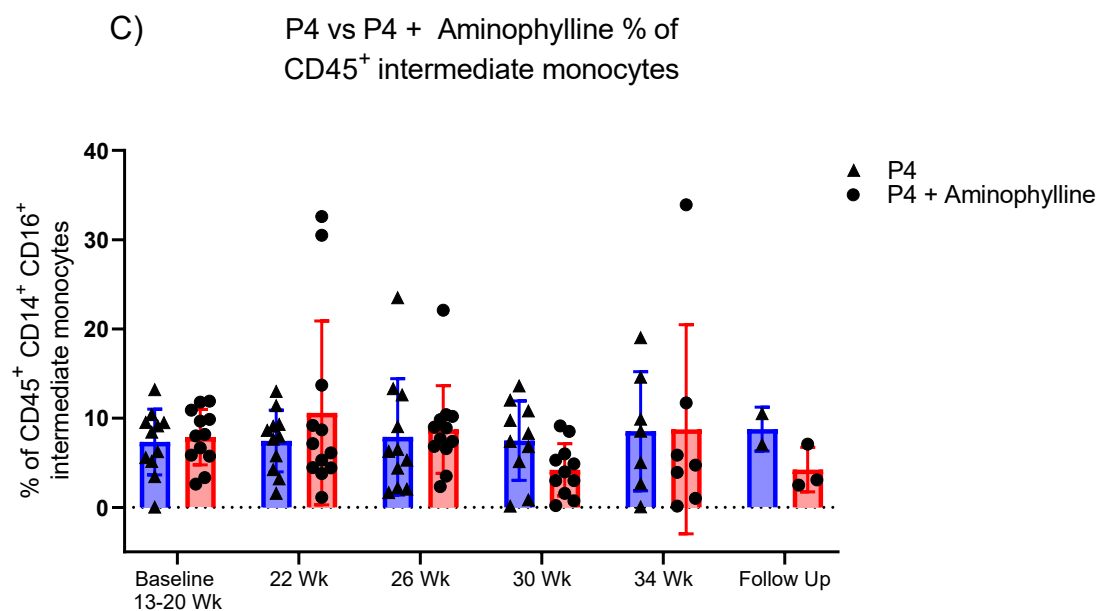
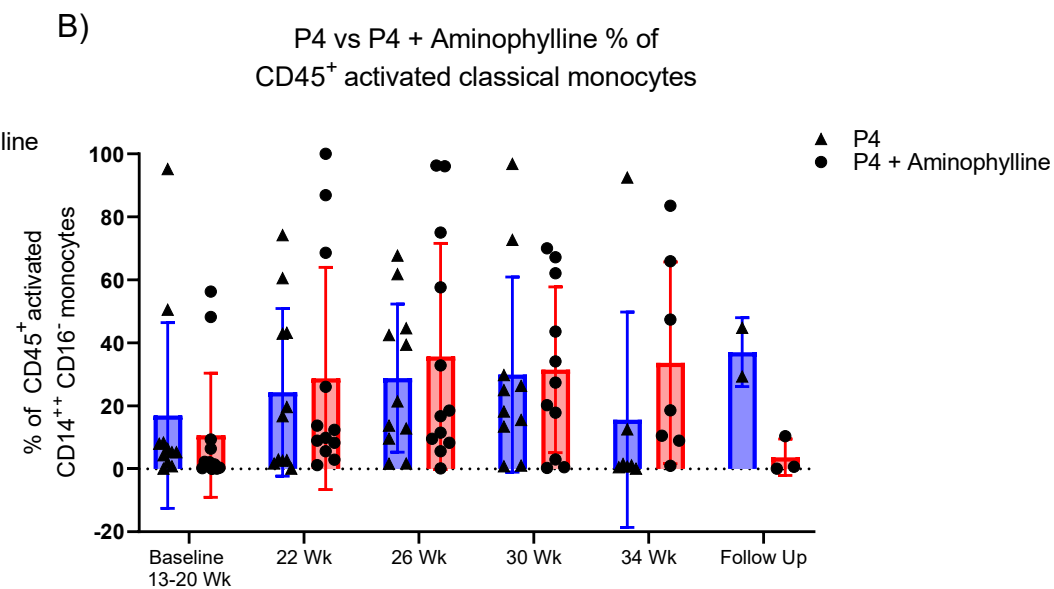
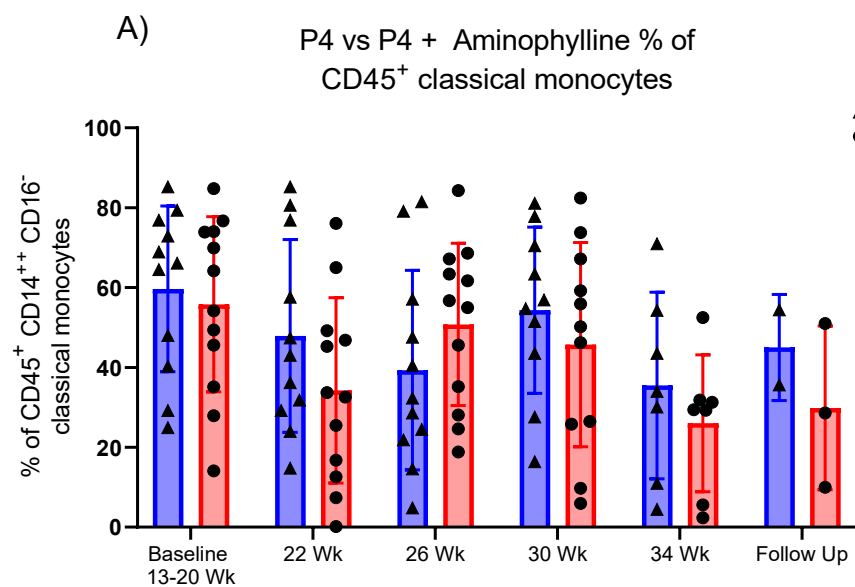
Figure 5.4.3.1: PROGRAM CD3⁺ CD4⁺ and CD8⁺ T cell lymphocyte and NK cell exhaustion, senescent and maturity associated marker expressions.

These graphs show the expression of CD57 expression on CD3⁺ CD4⁺ T cell lymphocytes (A); CD8⁺ T cell lymphocytes (B); CD3⁺ CD56^{dim} NK cells (C); CD3⁺ CD56^{bright} NK cells (D). PD-1 and Tim-3 expression was also measured on CD3⁺ CD4⁺ T cell lymphocytes (E, G) and CD3⁺ CD8⁺ T cell lymphocytes (F, H) between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 and AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P <0.05.

5.4.4 Peripheral Monocyte Analysis

The fluorochrome-conjugated antibodies used to surface stain peripheral monocyte populations and gating strategy used to phenotype monocytes is described in Chapter 4 Figure 4.4.4.1

The combination therapy showed no change in the number of classical monocytes (CD14⁺⁺CD16⁻) throughout pregnancy (Figure 5.4.4.1). Added to this, no significant change in HLA-DR expression on classical monocytes was also observed as pregnancy progressed. However, non-classical monocyte (CD14⁺CD16⁺⁺) populations showed a significant increase in the combination treated women from baseline to the 5th time point (34 wk) (p=0.0329), this significant increase was also observed when comparing the 2nd time point (22 wk) to the 5th time point (34 wk) (p=0.0428). However, HLA-DR activation of these non-classical monocytes was increased significantly from baseline to the 4th time point (30 wk) in the P4 only cohort which was also the highest frequency of non-classical monocyte activation during pregnancy (p=0.0097). This change in activation was not seen in the combination therapy. Furthermore, no differences in intermediate monocyte (CD14⁺CD16⁺) levels throughout gestation or markers of activation were observed.



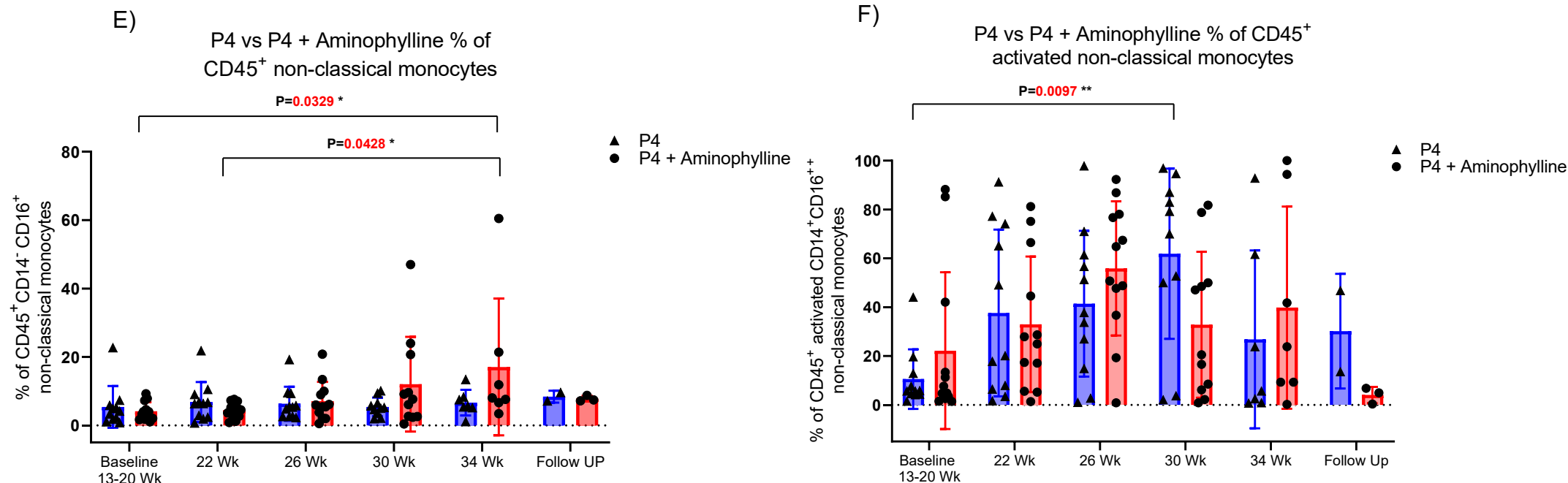


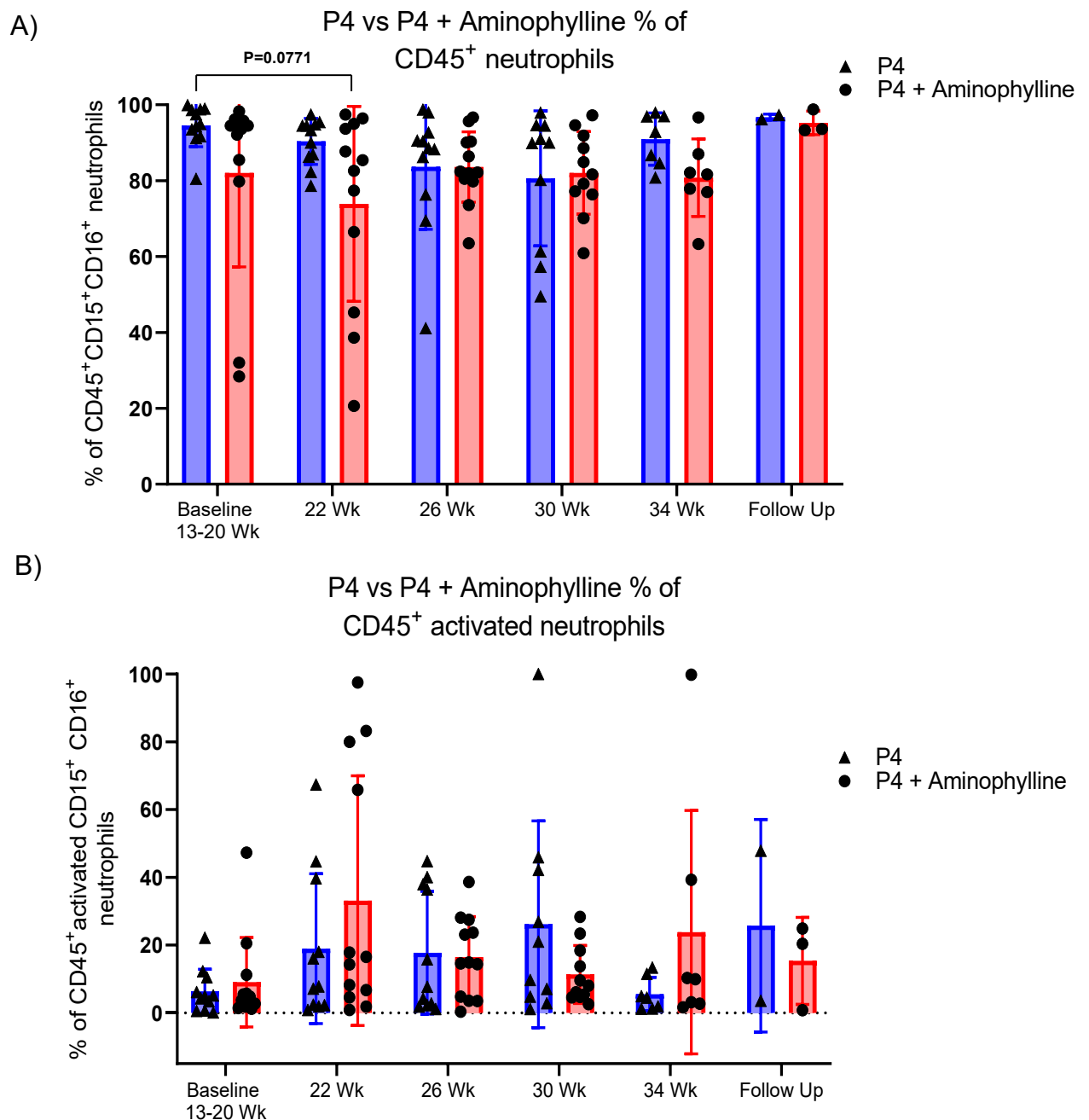
Figure 5.4.4.1: PROGRAM Peripheral CD45⁺ CD14⁺ CD16⁺ monocytes subsets and HLA-DR activation.

These graphs show levels of CD14⁺⁺ CD16⁻ classical monocytes (A); classical monocyte activation (B); CD14⁺ CD16⁺ intermediate monocytes (C); intermediate monocyte activation (D); CD14⁺ CD16⁺⁺ non-classical monocytes (E) and non-classical monocyte activation (F) between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 and AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as $P < 0.05$.

5.4.5 Peripheral Granulocyte Analysis

The fluorochrome-conjugated antibodies used to surface stain peripheral granulocyte populations and gating strategy used to phenotype neutrophils and eosinophils is described in Chapter 4 Figure 4.4.5.1

There was no significant effect of either treatment on neutrophil or eosinophil levels or HLA-DR activation (Figure 5.4.5.1).



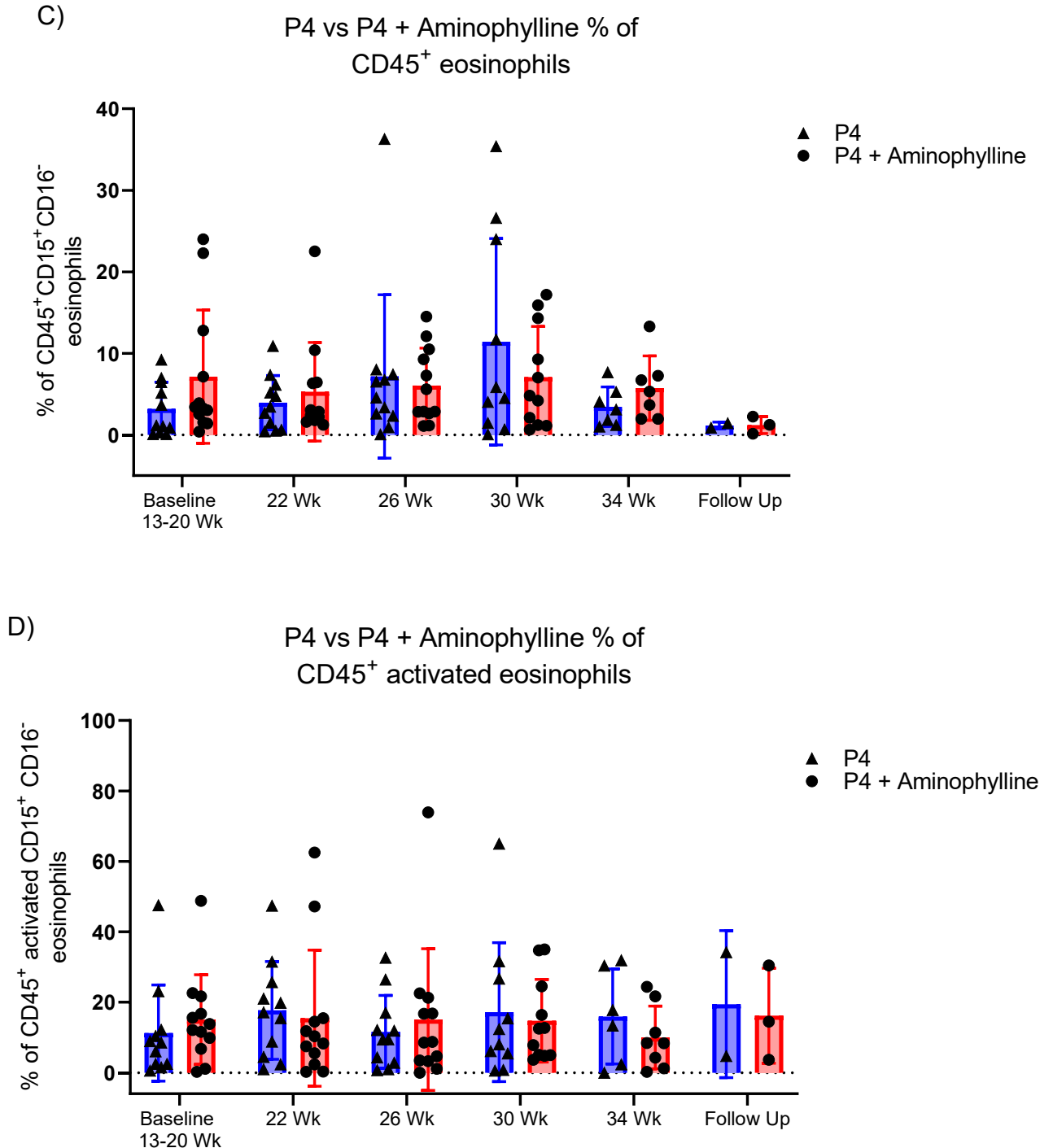


Figure 5.4.5.1: PROGRAM Peripheral CD45⁺ CD15⁺ CD16^{+/-} neutrophil and eosinophil frequencies and HLA-DR activation.

These graphs show the changes in CD45⁺ CD15⁺ CD16^{+/-} neutrophils (A); neutrophil activation (B); eosinophils (C) and eosinophil activation (D) between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 and AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P <0.05.

5.4.6 NK Cell Analysis

The fluorochrome-conjugated antibodies used to surface stain peripheral NK cell populations and gating strategy used to phenotype NK cell subsets and NCR is described in Chapter 4 Figure 4.4.6.1

Neither treatment affected cytotoxic or cytolytic NK cell populations throughout pregnancy (Figures 5.4.6.1 and 5.4.6.2). Activation markers and NCR expression on the NK cell subsets did not change. Furthermore, no differences were observed in the iNKT cell population (Figure 5.4.6.3).

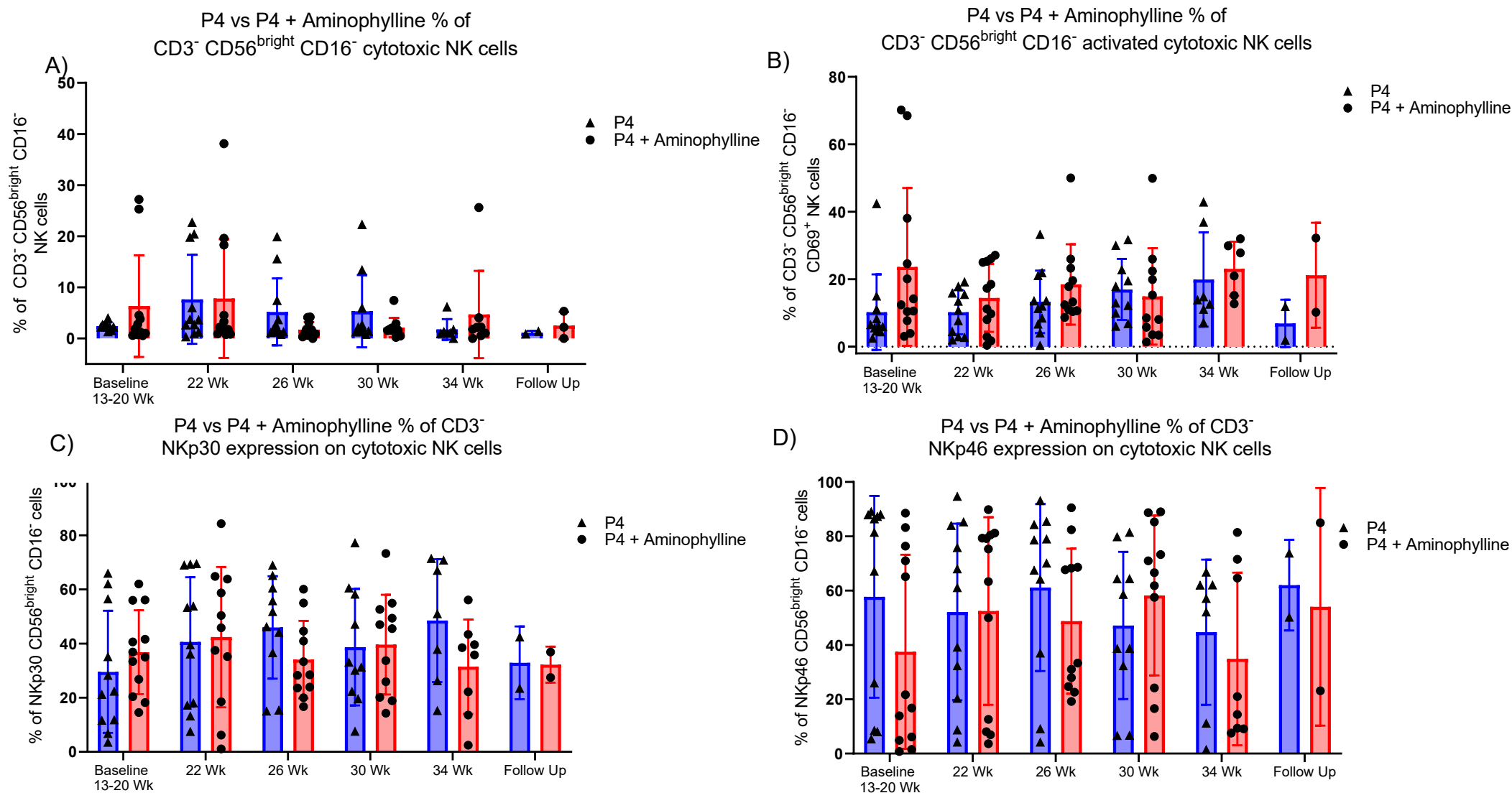


Figure 5.4.6.1: Composition of CD56^{bright} CD16⁻ NK cells, including CD69 Activation and NCR expression on NK cell subsets.

These graphs show frequencies of CD3⁻ CD56^{bright} CD16⁻ (A); Activation (B); NKp30 expression (C) and NKp46 expression (D) on NK cells between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 and AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P < 0.05.

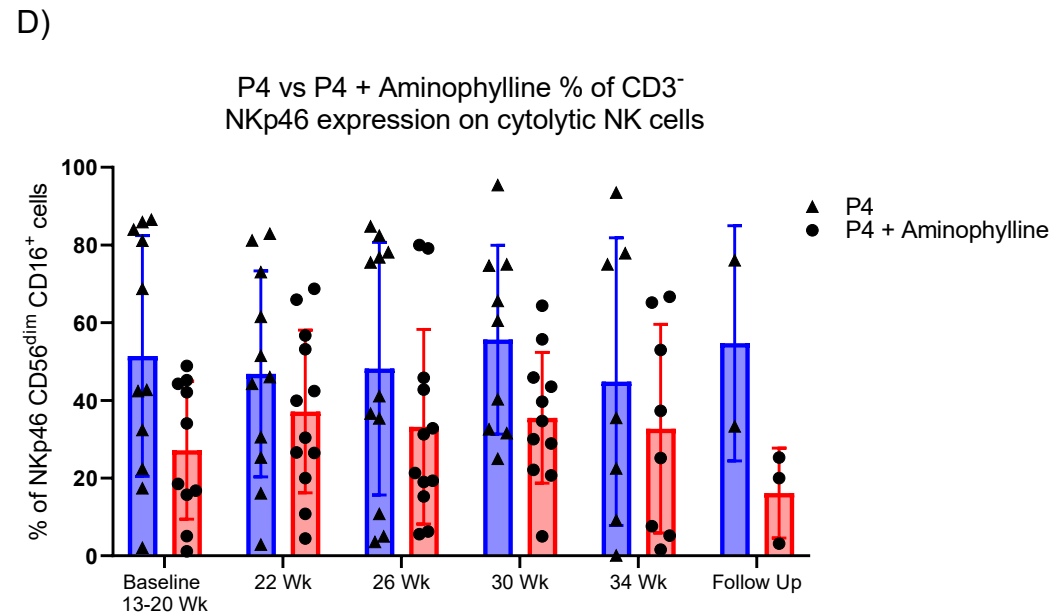
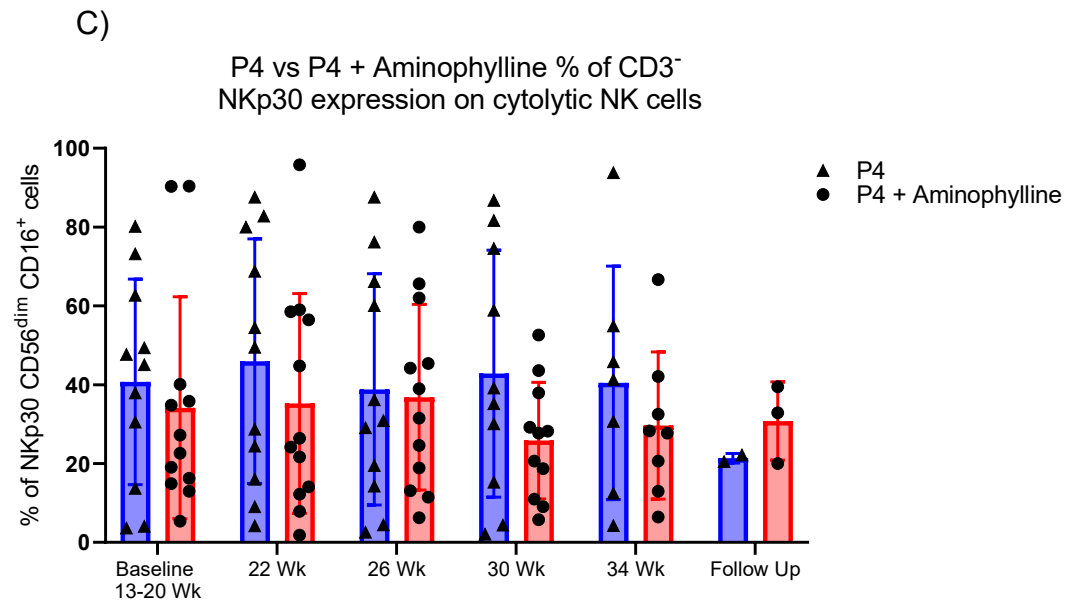
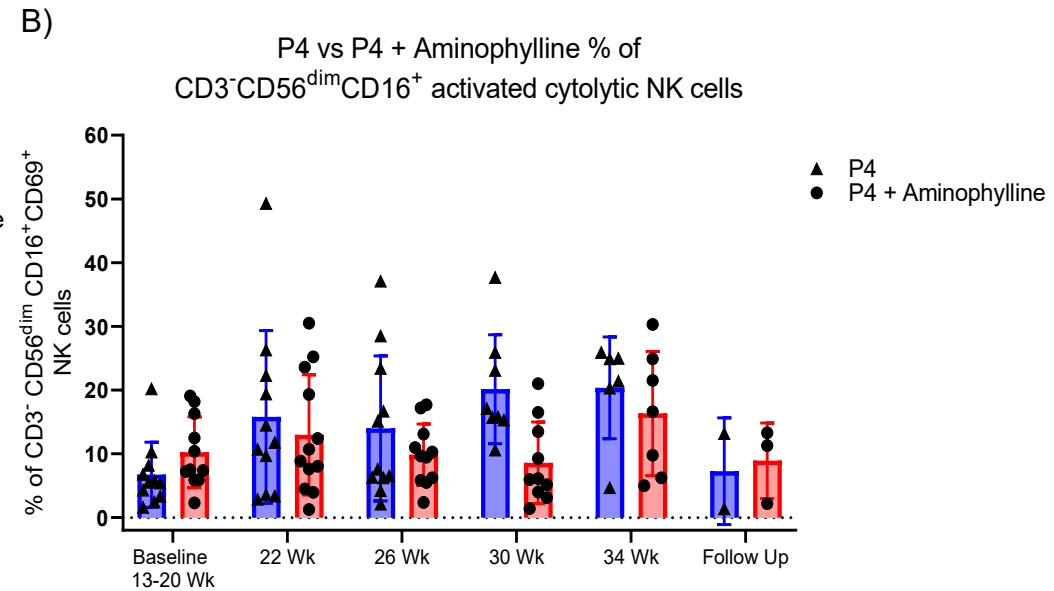
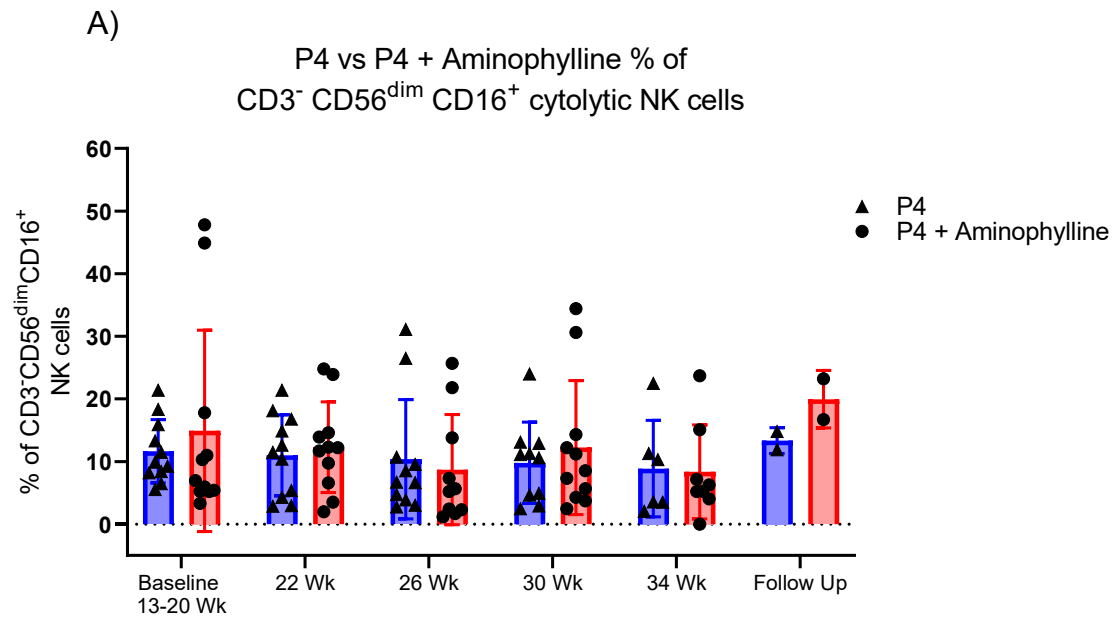


Figure 5.4.6.2: Composition of CD56^{dim} CD16⁺ NK cells, including CD69 activation and NCR expression on NK cell subsets.

These graphs show frequencies of CD3⁻ CD56^{dim} CD16⁺ (A); Activation (B); NKp30 expression (C) and NKp46 expression (D) on NK cells between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 + AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P < 0.05.

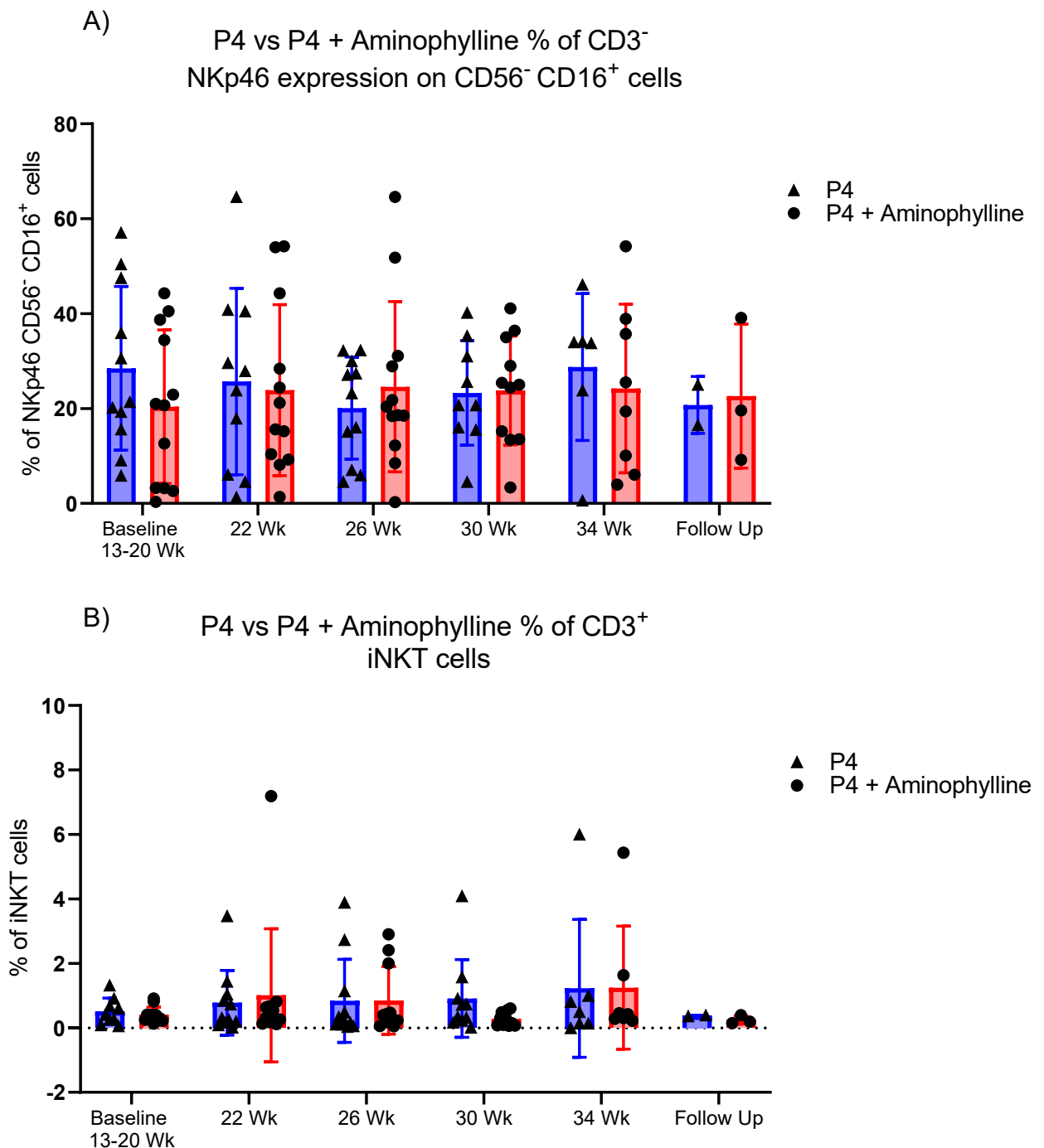


Figure 5.4.6.3: NKp46 expression on CD3⁻ CD56⁻ CD16⁺ cells and CD3⁺ iNKT cell frequency

These graphs show frequencies of NKp46 expression on CD3⁻ CD56⁻ CD16⁺ cells (A) and frequency of CD3⁺ iNKT (B) cells between P4 (Baseline, 22wk, 24wk; N=11; 30wk; N=10; 34wk; N=7) and P4 and AP (Baseline, 22wk, 24wk; N=12; 30wk; N=11; 34wk; N=7) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P <0.05.

5.4.7 Cervical Swab Analysis

The fluorochrome-conjugated antibodies used to surface stain cervical immune cell populations and the gating strategy used to phenotype these cells is described in Chapter 4 Figure 4.4.7.1

From the limited number of swabs able to be analysed, the percentage of cervical eosinophils and neutrophils did not seem to change throughout pregnancy in either treatment. Markers of activation of these immune cells did not change either (Figure 5.4.7.1). Composition of cervical monocytes did not show to change throughout gestation in the cervix in the combination study group. Furthermore, HLA-DR activation expression on cervical monocytes did not see an increase (Figure 5.4.7.2).

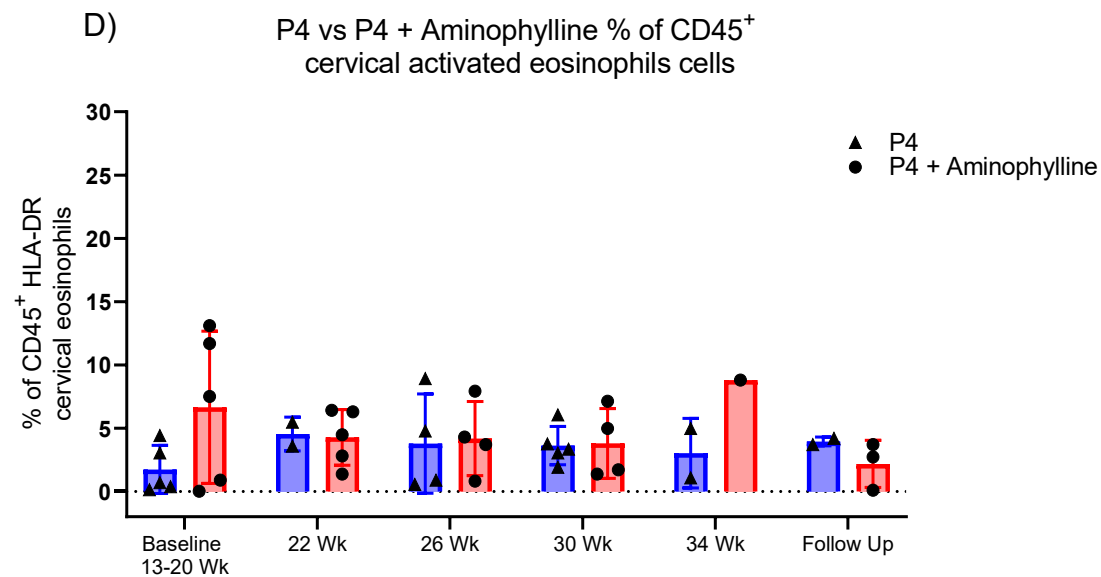
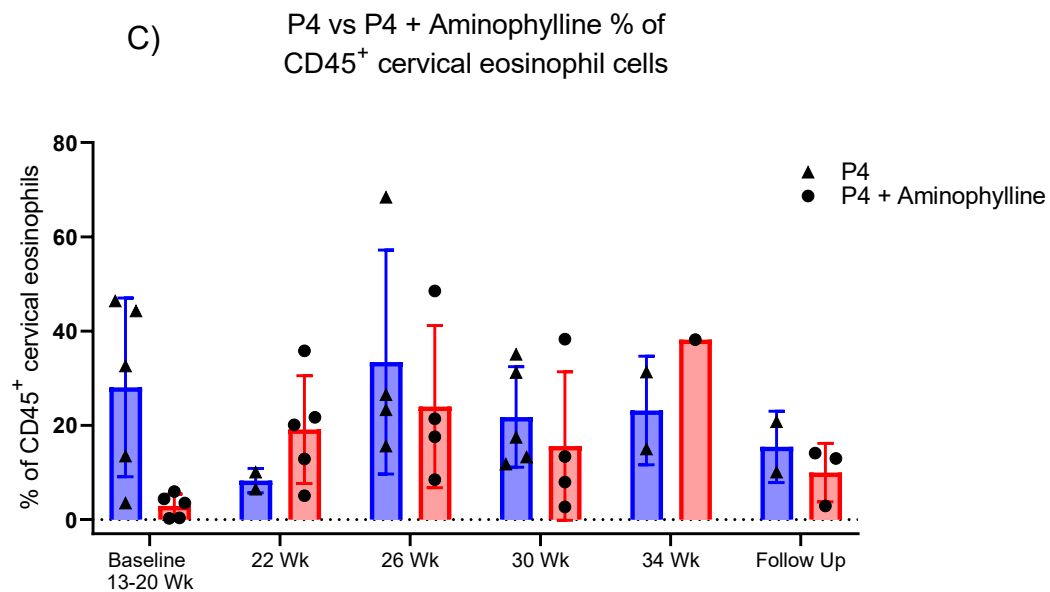
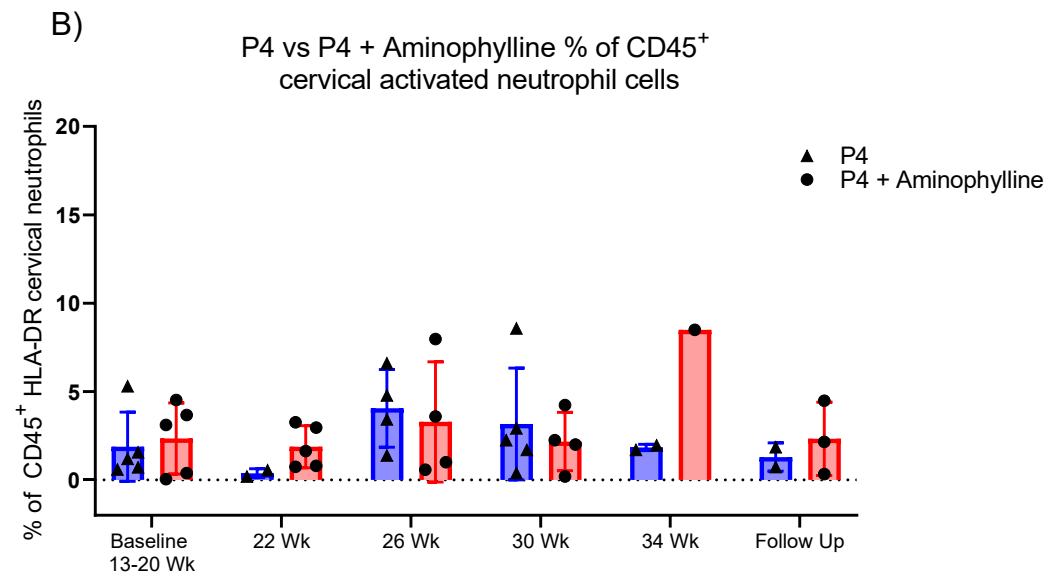
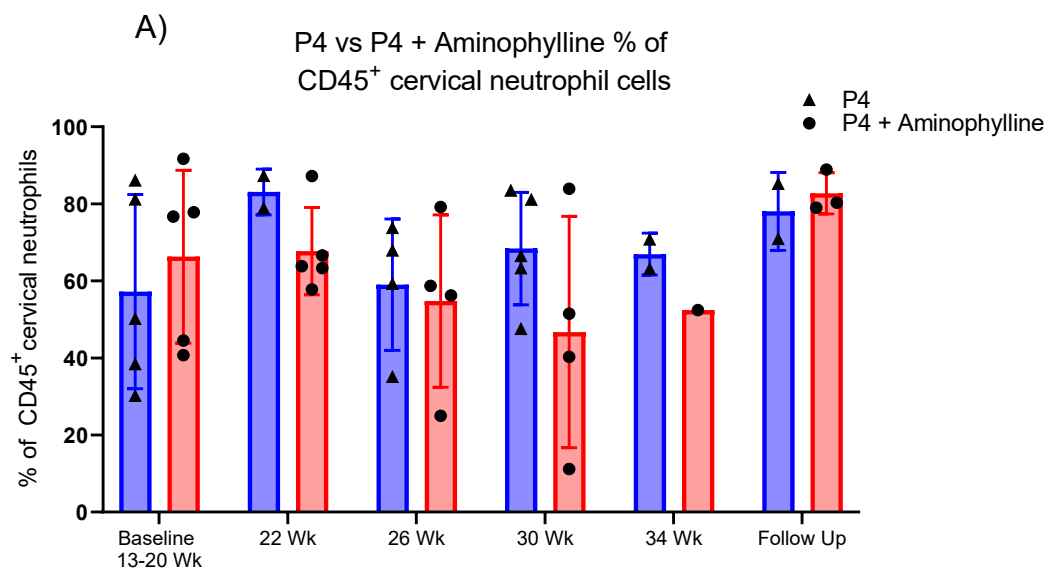


Figure 5.4.7.1: CD45⁺ cervical granulocyte frequencies and HLA-DR expression.

These graphs show frequencies of CD45⁺ cervical neutrophils (A); neutrophil activation (B); cervical eosinophils (C) and eosinophil activation (D) between P4 (Baseline; N=5; 22wk; N=2; 24wk; N=4; 30wk; N=5; 34wk; N=2) and P4 and AP (Baseline, 22wk, 24wk; N=5; 30wk; N=4; 34wk; N=1) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P <0.05.

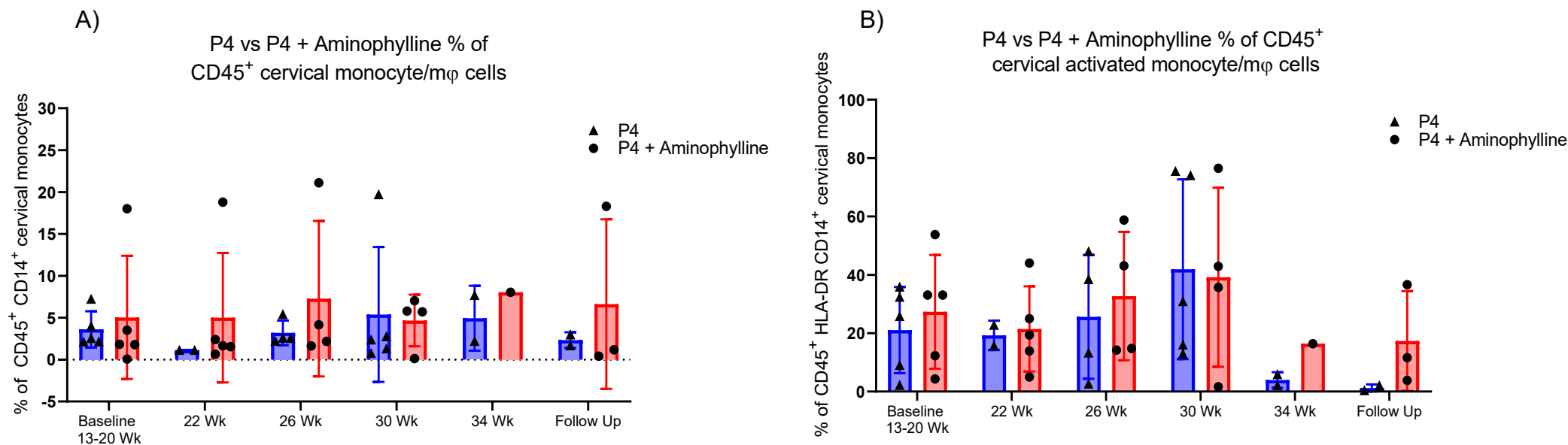


Figure 5.4.7.2: CD45⁺ Cervical monocytes/mφ frequencies and HLA-DR expression.

These graphs show frequencies of CD45⁺ cervical monocytes/mφ (A) and monocyte/mφ activation (B) between P4 (Baseline; N=5; 22wk; N=2; 24wk; N=4; 30wk; N=5; 34wk; N=2) and P4 and AP (Baseline, 22wk, 24wk; N=5; 30wk; N=4; 34wk; N=1) across all 5 time points during pregnancy and the 6 week post-natal follow up (N=2 and N=3 respectively). Statistical analysis was undertaken using a 2-way ANOVA and a Tukey's multiple comparison test was applied. Significance was defined as P <0.05.

5.5 Discussion

This chapter explored the impact of administering a combination of AP and P4 compared to P4 alone to high-risk pregnant women for the prevention of PTL by observing the longitudinal changes in their systemic and local immune systems throughout pregnancy. Previous work has documented that pregnancy is associated with an increase in white blood cell counts [368]. However, there is no current literature which establishes the immunological changes that occur throughout pregnancy in women who are at risk of going into PTL receiving preventative treatment. To add to this, the use of fresh cells with a routinely calibrated flow cytometer using the Perfetto method gives a better representation of what is happening *in vivo* [151]. Here, we highlight the variations that occur in systemic T cells, NK cells, granulocytes and effector cells, but also assess the immune environment in the cervix.

5.5.1 The Combination Therapy on Acquired Immune Cells

Tregs: Systemic and decidual Treg frequency has been shown to increase as pregnancy progresses in both mice and humans [287, 369, 370], this has been suggested to be a result of the increase in P4 and other hormonal changes associated with pregnancy [287]. However, Treg levels stayed the same as gestation progressed in both study groups. With no comparison to non-pregnant controls, it is difficult to verify whether Treg levels increase as a consequence of gestation, nevertheless, it appears that treatment with AP had no circulatory effect on Treg frequency. Additionally, HLA-DR activation did not change as gestation progressed. Surprisingly, Mao *et al.* found that P4 up-regulated IL-10 producing Tregs which had increased suppressive capacity at midterm pregnancy both *in vivo* and *in vitro* [287]. However, in

the current study the same observation was not made. It is important to note that the Mao study was performed in mice so that may account for the difference seen. In human pregnancy, Shah *et al.* demonstrated that P4 supplementation is associated with a decline in Treg numbers as gestation progressed [146]. *In vitro* work showed that CD4⁺ CD25^{high} FoxP3⁺ Tregs stimulated with P4 and 17β- oestradiol had a reduced suppressive ability [371]. Unfortunately, no assessment of the suppressive ability of peripheral Tregs was performed in the current study. These data imply that AP may enhance the anti-inflammatory effect of P4 but through other immune cell types and not specifically Tregs.

T cells: No change in systemic CD4⁺ and CTL were observed throughout pregnancy in contrast to the current literature, which reports that P4 decreases the frequency of pro-inflammatory CTL responses and increases naïve T cell differentiation into CD4⁺ T cells whilst suppressing the proliferation of CTL [146, 147, 292]. Furthermore, theophylline has been shown to down-regulate IL-12 (a key cytokine influencing CTL maturation from naïve T cells) production in cord blood mononuclear cells [372]. The literature suggests that both AP and P4 repress inflammation by controlling the proliferation of cytotoxic immune cell types; however, this was not obvious from the data collected in the current study. That being said, no functional work was performed on T cell subsets so the impact on their cytokine production is unknown.

Memory responses observed in the combination cohort were no different to that of the P4 cohort. It has been shown that P4 added exogenously to memory T cells reduces antigen sensitivity to aid in tolerance [146]. In the decidua, CD4⁺ and CD8⁺ Em T cells respond greater to mitogen stimulation when compared to peripheral blood Em cells. However, P4 action on these decidual Em cells inhibits INF-γ and TNF-α production whilst increasing IL-4 production. This may suggest that P4 modulates the immune

environment in gestational tissue at the fetal-maternal interface to promote tolerance [373, 374]. Therefore, in terms of systemic memory T cells, it doesn't appear that the addition of AP changes the memory mediated phenotype of the T cell populations. Since decreased Cm and Em on CD4⁺ and CD8⁺ T cells are associated with higher susceptibility of infection [375, 376], the lack of this observation in the data collected may imply the acquired immune system is polarised to maintain tolerance in both study groups.

There is evidence showing that Tim-3 expression on CTL decreases during the second trimester but then returns to normal in the third [108]. Tim-3 when bound to the galactin-9 ligand induces apoptosis of Th1 cells [295, 377], but neither CTL numbers nor Tim-3 expression on these cells changed throughout gestation in either study group. This may suggest that AP and P4 supplementation may not directly inhibit Tim-3 expression on these cells, or these CTL have a diminished cytokine response not necessary for termination to maintain peripheral tolerance. Tim-3 expression is important in healthy pregnancy, as it promotes the proliferation of Th2 cells required for the immunosuppressive environment in the decidua needed to maintain pregnancy [378]. Adding to this, Meggyes and colleagues showed the Tim-3 expression in circulatory cells altered NK cell and CD8⁺ T cell cytotoxicity during normal pregnancy [379], implying that Tim-3 has immunomodulatory effects which promote tolerance. PD-1 along with Tim-3 has also been shown to be a mediator of specific T cell subset proliferation in pregnancy [378] and functions to inhibit chronic inflammation and autoimmune responses on T cells [380]. No differences were observed between study groups in CD4⁺ and CTL PD-1 expression. In the decidua, elevated levels of PD-1 are presented on CD4⁺ Em T cells in healthy pregnancies, but not elevated in peripheral blood [381], meaning that AP and P4 may not have an effect on systemic PD-1

expression. CD57⁺ expression on the cell surface of CD4⁺ and CTL showed no differences between the combination therapy and P4 cohort which supports the findings found in literature. Shah *et al.* demonstrated that CD57 expression during the 2nd and 3rd trimesters of healthy pregnant women was the same when compared to non-pregnant healthy controls on both CD4⁺ and CTL [108]. On the other hand, decidual tissue of PTL women, higher surface density of CD57⁺ Tim-3⁺ and PD-1⁺ expressions are identified on memory CD4⁺ and CTL [382]. This may suggest that in healthy pregnancy, regulation of systemic CD57 on T cells is not modulated by AP or P4 but perhaps locally.

5.5.2 The Combination Therapy on Innate Immune Cells

Peripheral Monocytes: Normal pregnancy is associated with the increase of peripheral monocyte numbers after the first trimester [233], specifically, elevation of the non-classical subset and its activation is important in healthy pregnancy as these cells differentiate into M2 macrophages which invade into the decidua and are key mediators of infection control and promote spiral artery remodelling [115]. Interestingly, the combination therapy saw a significant increase in non-classical monocyte frequency at 34 weeks of gestation when compared to baseline, 18 week and 22 week levels. Whereas the P4 group did not show any increase across pregnancy. However, theophylline has been shown to down regulate monocyte activity due to the inhibition of PDE-4 [383]. Furthermore, Spatafora and colleagues demonstrated that theophylline reduces TNF- α production from systemic monocytes [363]. Therefore, the significant increase in non-classical monocytes in the combination therapy cohort from baseline, maybe a result of gestation changes and not a result of AP treatment. Following this, activation in the combination group did not

show the same significance, instead the P4 group showed a significant increase from baseline to 34 weeks in non-classical monocyte activation. P4 has been shown to mediate TLR-4 activation on macrophages by down regulation of nitric oxide, IL-12 production and to reduce TNF- α production by LPS-induced macrophages by NF κ B inhibition [285, 384]. Theophylline has also been shown to induce an anti-inflammatory effect on monocytes, by reducing TNF- α and IL-6 production after LPS stimulation [385]. Therefore, the combination treatment could be potentiating the anti-inflammatory effect of P4 by reducing the amount of activation in non-classical monocyte in response to infection.

Intermediate and classical monocyte frequencies and activation did not show any change in the combination group throughout pregnancy. Normal pregnancy is associated with the decrease of phagocytic classical monocytes and increase of intermediate monocytes which function as both phagocytic and inflammatory responders to infection [232, 233, 264]. However, this was not observed in either study group. This may imply that supplementation of both AP and additional P4 induce suppression by reducing innate cell production and activation either by PDE-4 inhibition or by inhibiting the NF κ B signalling pathway.

Cervical Monocytes/ m_{ϕ} : In the previous chapter, low cervical monocyte counts were observed in true PTL women ($n=3$). However, due to the low number of samples analysed this data cannot be relied upon, but was comparable to the findings in literature, where the failure to detect cervical macrophages in the early to late second trimester was found in women who subsequently went into PTL [140]. Although no difference was observed in the composition or activation status of cervical monocytes/ m_{ϕ} in women treated with AP, both study groups did show a consistent frequency throughout pregnancy. *In vitro*, theophylline decreases oxidative burst in

macrophages derived from human alveoli inhibiting their function and enhancing suppression [360, 386]. Likewise, P4 has been shown in mice models to increase the production of M2 anti-inflammatory macrophages which also promotes uterine remodelling and angiogenesis [387]. However, the presence and activation of these cells do not change from baseline which may imply that *in vivo* the local effects of P4 supplementation and the added oral consumption of AP did not affect the cervical milieu as expected, at least with the pilot numbers associated with this study.

Peripheral Neutrophils: Neutrophil levels have shown to increase during pregnancy [388]. However, in this study neutrophil frequency had a tendency to decrease at the second time point following treatment with AP, however, levels then returned to normal after. In addition, no change in activation was shown in these neutrophils. AP in high dosages has been shown to decrease chemotaxis of neutrophils and reduce the number of inflammatory neutrophils in the lungs of patients with emphysema [389]. In contrast, low doses, which are unable to meet the therapeutic range, have been shown to increase peripheral neutrophils in preterm infants [390]. However, both these findings were *in vitro* [389, 390]. An *in vivo* study demonstrated that theophylline improved pulmonary function by decreasing neutrophil activation in allergen induced asthma, however, it failed to decrease the duration and severity of the late asthma response in these patients [391]. This implies that AP supplementation in pregnancy may temporarily attenuate pro-inflammatory peripheral blood immune activation as shown by reduced proportions of neutrophils, but may not be sufficient to have prolonged effect on the immune system.

Cervical Neutrophils: Surprisingly, no change in frequency or activation from baseline was observed in cervical neutrophils despite the use of a 400mg vaginal pessary in both cohorts of women. AP has been shown to have an anti-inflammatory effect in the

circulation by decreasing neutrophil counts [392]. Added to this, a combination of P4 and oestradiol has been shown to inhibit neutrophil responses *in vitro*, however, this effect was only temporary [316]. There is no current literature on the effects of AP or P4 in relation to cervical mucus, and with the absence of change in this population it is difficult to draw conclusions as to what effect, if any has occurred with the use of treatment.

Peripheral Eosinophils: Neither gestational progression nor treatment with AP showed any change in systemic eosinophil frequency or activation. Theophylline has been shown to deplete inflammatory eosinophils in asthmatic patients [393] and has been shown to reduce degranulation [394]. Mechanistically, theophylline acts as an adenosine receptor antagonist which modulates granulocyte functions [395]. Although theophylline is a derivative of AP [320], the effect on eosinophils is not the same. For example, a study found that intravenous administration of AP did not have any effect on reducing peripheral eosinophil counts [396]. It is therefore unlikely that oral administration given to these pregnant women would be potent enough to induce a change in eosinophil levels.

Cervical Eosinophils: No differences were observed in cervical eosinophil frequency or activation as gestation progressed. Although baseline levels of eosinophils were lower in the combination group than the P4 cohort, levels became similar following treatment. With that being said, theophylline has been shown to reduce eosinophil numbers in bronchial tissue, lavage and sputum even at low dosages [397, 398]. Added to this, P4 has shown to reduce degranulation in circulating eosinophils [399]. Collectively, these data suggest that AP and P4 supplementation did not affect cervical eosinophil levels.

NK cells: No alteration in CD56^{bright} or CD56^{dim} NK cells was observed throughout gestation in either study group. Peripheral NK cells express surface PR and their function and apoptosis are modulated by P4 [400], specifically, P4 reduces the cytotoxicity of these NK cells by releasing PIBF [285]. On top of this, Theophylline has been shown to inhibit NK cell activity *in vivo* after high oral dosages (400-500mg twice daily) in asthmatic patients [401]. Theoretically, since increased cytotoxicity of CD56^{bright} NK cells has been associated with recurrent miscarriage [402], any reduction in their activity may be of benefit. Markers of activation were constant throughout pregnancy in both the study groups and since increased CD69 expression on NK cells has been observed in pregnant women who experience spontaneous abortion [220], the addition of AP and P4 in these women may help to prevent excessive activation of both major NK cell subsets and CD69 activation by inhibiting any excessive cytotoxicity to maintain tolerance.

Neither NKp30 nor NKp46 expression on both CD56^{bright} and CD56^{dim} NK cell subsets were altered during pregnancy in either study group. There is no evidence to suggest that AP or P4 regulates NCR expression. However, these NCR are an important cross-link to the adaptive immune system in response to infection [215]. Therefore, the absence of a diminishing expression of these NCR may imply that these women were able to respond adequately to pathogenic stimuli. Additionally, no change in iNKT cell frequency was observed, however, iNKT cells make up a very small percentage of PBMC (as low as 0.01%) in a healthy individuals [403]. Therefore, any systemic changes that AP will have are unlikely to be seen in such a small population.

Maturity of CD56^{bright} NK cells was shown to increase significantly in the combination therapy by week 34 from baseline. Interestingly, NK cells expressing higher CD57 have an increased cytotoxic potential, but are also less susceptible to excessive

activation in response to inflammatory stimuli [253]. NK cells with higher expression of CD57⁺ produce less pro-inflammatory cytokines, in particular INF- γ [253] which is important to maintain the immunosuppressive environment of pregnancy as it promotes a cytokine positive feedback loop which recruits other innate cells to response to infection [404, 405]. Therefore, the addition of AP may help to potentiate the anti-inflammatory effect of P4 by increasing maturity of the cytotoxic NK cells by modulating their response towards infection.

5.5.3 Chapter Conclusion

The addition of AP appeared to have little effect in altering the immune profile of high-risk pregnant women to that of a more anti-inflammatory environment. The changes observed shown in Figure 5.5.3.1 were gestation associated increases in maturity of cytotoxic NK cells and markers of non-classical monocyte activation. These observations may suggest that AP is having an antagonistic effect on P4 action, by increasing the levels of a pro-inflammatory cytokine producing immune cells. However, it is uncertain that the change in non-classical monocyte levels and activation was due to AP as it is likely that the effects of AP are subtle. Additionally, no non-pregnant controls were recruited in this study which means the changes observed could be gestation related rather than a consequence of treatment.

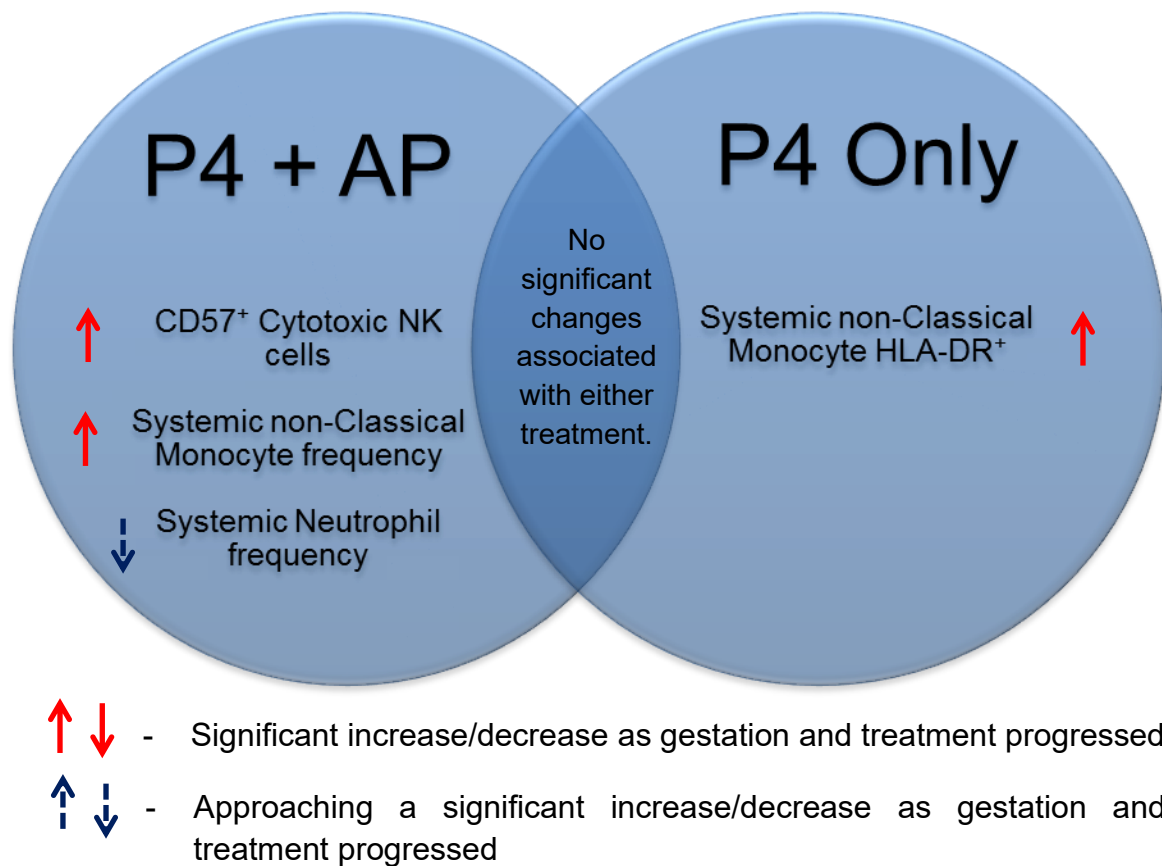


Figure 5.5.3.1: Venn diagram illustrating phenotypic changes in immune cell types observed throughout pregnancy.

This Figure illustrates the changes associated with the treatment of AP + P4 (left) when compared to the changes seen with P4 treatment only (right). Changes associated with both study groups are shown in the middle.

6. The Effect of Aminophylline in Response to Monocyte/Macrophage Function

6.1 – Introduction

In the previous chapter, the effects of the combination of AP and P4 on the maternal immune system were compared to the effects of P4 alone. The efficacy of AP is likely to be subtle as only a small proportion of cells are affected (NK cell maturity and non-classical monocytes) during the longitudinal analysis, so looking directly at AP response against immune cell function may help to clarify whether the observed changes were a result of AP or of increasing gestation. Interestingly, at later gestations, the combination therapy showed monocyte frequency significantly higher but with lower activation in the non-classical, more pro-inflammatory subset [67] when compared to baseline pre-treated women. AP and its derivatives have been shown to increase the anti-inflammatory effect of P4 [149, 406, 407], questioning why the combination of P4/AP increased a more pro-inflammatory monocyte subset when compared to P4 alone. In this chapter, the impact of AP on monocyte function will be investigated defining its impact on monocyte function during high-risk pregnancy.

Novel PDE-4 inhibitors have been shown to exert an anti-inflammatory response in monocytes/ m_{ϕ} [408, 409]. Since AP is a non-specific PDE inhibitor, it might be expected to have an anti-inflammatory effect on monocyte function too. Here, monocyte numbers and cytokine production were investigated in samples obtained from pregnant women taking AP to investigate whether AP exerts an anti-inflammatory effect via a diminution of pro-inflammatory cytokine production or by decreasing the numbers of these cells. If no differences were found in the cytokine production in P4 treated women verses those taking P4/AP, then further studies would investigate what

concentration of AP would reduce monocyte cytokine production in response to the TLR-4 ligand LPS, which is known to evoke a marked monocyte cytokine response [410].

6.1.1 Lipopolysaccharide

LPS is a well-known pathogen associated molecular pattern (PAMP) and consists of small glycolipid molecules found in the outer cell membrane of Gram negative bacteria [411]. LPS has been used in many studies as a potent endotoxin to assess the function of innate cell immunity due to their high affinity toward pattern recognition receptors (PRR), in particular TLR-4. *Escherichia coli* (E.Coli) and *Salmonella* are model systems commonly used in LPS scientific research due to their availability [412].

6.1.2 Mechanism of Lipopolysaccharide action

LPS-binding proteins are found in the circulation and attach themselves to LPS monomers. They then traffic these monomers to either soluble CD14 or glycosylphosphatidylinositol anchors found on the cell surface of monocytes/ $m\phi$ [413]. CD14 transports the LPS monomer to the ectodomain of TLR-4 where an accessory protein myeloid differentiation 2 (MD-2) induces homo-dimerisation of TLR-4 [414, 415]. This change on the cell surface initiates cytoplasmic toll IL-1 receptors (TIR) which recruit adapter proteins such as MyD88 to be activated [413, 416]. The activation of NF κ B activates a cascade of intracellular signalling events which culminates in cytokine production [413].

6.1.3 Pro-inflammatory Cytokine Influence on Preterm Labour

In response to inflammatory stimuli, monocytes and macrophages produce TNF- α , IL-8 and IL-12 [417]. These cytokines has been linked to adverse pregnancy outcomes in the literature, for example, a high expression of TNF- α has been shown to induce miscarriage in mice models [418]. TNF- α is a potent inducer of cellular apoptosis [419]. Mechanistically, TNF- α signals through both TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) [420]. Fortunato and colleagues found that increased activation of TNFR1 increases MMP-9 concentration in the amniochorionic membranes which is associated with rupture of membranes [421]. A more recent study showed that TNF- α expression in fetal membranes was higher in women who had premature rupture of membranes when compared to women who had term rupture of membranes [422]. These data suggests that the increase of TNF- α may be critical to the progression and onset of labour.

A study which looked at cytokine level production in both neonatal and maternal plasma from healthy women undergoing vaginal delivery (VD) was compared to a cohort of women undergoing an elective caesarean Section (ECS). After delivery, increased: IL-6, INF- γ , IL-1 β and higher respiratory burst expression measured by enzyme linked immunosorbent assay (ELISA), was observed in maternal plasma of healthy women who had a VD compared to women who had ECS [423]. Added to this, IL-6 and respiratory burst levels in cord blood plasma was higher in the VD cohort when compared to ECS [423]. These findings imply that inflammatory cytokines not only play a key role in labour, but also have an important role in neonatal host defence.

Interestingly, Olsen and colleagues found that prostaglandins act together with IL-8 to induce cervical ripening [424]. IL-8 is a potent chemoattractant for neutrophil migration

[425]. This indicates that higher concentration of IL-8 in uterine tissue will attract neutrophils to that tissue. In the myometrium, increased mRNA expression of IL-8/CXCL8 is higher in term labouring women than non-labouring women [173]. In maternal serum, elevated levels of IL-8 was found in preterm labouring women compared to preterm non-labouring women [426]. Furthermore, increased IL-8 concentrations in both amniotic fluid and cord blood were found in women with intra-amniotic *Ureaplasma urealyticum* infection who delivered preterm when compared to women who delivered preterm but with no infection [427]. With the aforementioned studies, they suggest that the increased expression of IL-8 plays a key role in infection-induced PTB but also sPTB.

6.1.4 Anti-Inflammatory Cytokine Influence on Preterm Labour

Peripheral blood intermediate monocytes (CD14⁺⁺ CD16⁺), tolerogenic DC and Tregs are the main producers of IL-10, a protective anti-inflammatory cytokine which enables the suppression of inflammatory stimuli [264, 428, 429]. Interestingly, IL-10 has been shown to have a key role in human and mice pregnancies. Bennett and colleagues found that human trophoblasts isolated from term placenta biopsies expressed IL-10 [430]. It was also demonstrated that IL-10 concentration is increased in the amniotic fluid at parturition, regardless of a term, preterm or infection-induced delivery [431]. Furthermore, studies have shown that IL-10 inhibits the inflammatory response initiated when macrophages are exposed to LPS [432, 433]. In IL-10 deficient mice, fetal growth restrictions were shown with smaller doses of LPS administration when compared to the controls which were able to produce endogenous IL-10 [434]. Additionally, increased mRNA concentrations of TNF- α and IL-6 were found in the uterus and placenta of these IL-10 deficient mice [434]. The same study also found

that exogenous IL-10 reduced fetal loss [434], which suggests that IL-10 modulates pro-inflammatory stimuli in gestational tissue to maintain tolerance during pregnancy. In human pregnancy, IL-10 is an important immunosuppressive cytokine and its deficiency has been associated with adverse pregnancy outcomes [435-437]. Huck *et al.* showed that incubation of systemic immature and mature DC with P4 and estradiol increased their production of IL-10 *in vitro* [438]. Another study showed that monocyte derived DC isolated from the decidua increased IL-10 and IL-27 production when incubated with P4 and estradiol *in vitro* [439]. This evidence suggests that IL-10 production may be mediated by the effects of pregnancy related hormones such as P4 and estradiol on immune cell function to maintain a tolerogenic state during pregnancy.

6.1.5 Theophylline's Response to LPS

No current literature has looked into the effect of AP in response to LPS-induced monocytes; however, a few studies have looked at theophylline. For example, Ito and colleagues demonstrated that LPS can act by down regulating HDAC enzyme activity, which results in an increase in pro-inflammatory gene expression but, low doses of theophylline can reverse this effect [440]. Furthermore, Spatafora *et al.* performed an *in vitro* dose response of theophylline on human and mice LPS-treated alveolar macrophages and found that TNF- α production was suppressed at a concentration of 50 μ M [441]. Another study, using the same 50 μ M concentration of theophylline in combination with dexamethasone (0.1 μ M), significantly decreased TNF- α and IL-6 production after 24 hours from purified monocytes stimulated with LPS [442]. Low doses of theophylline are also shown to attenuate the production of IL-6 and IL-8 in lung fibroblasts from patients with COPD [443]. In addition, theophylline (100 μ mol/L)

inhibited LPS-induced TNF- α production in human peripheral monocytes [444]. However, another study found that although theophylline did not reduce monocyte cytokine release, it did reduce the monocyte respiratory burst induced by LPS [445]. Interestingly, the addition of intra-amniotic LPS induced alveolarisation arrest in neonatal pups, similar to that observed with chorioamnionitis, but these changes were reversed by theophylline treatment in association with increased TGF- β 1,2 and 3 and reduced TNF- α and macrophage inflammatory protein (MIP) 1 and 2 levels [446].

6.1.6 Progesterone's Response to LPS

Like AP, P4, along with other hormones associated with pregnancy such as estradiol, cortisol and prolactin has been shown to modulate LPS-induced monocyte and macrophage function. A study by Chávez *et al* used ELISA and flow cytometry based techniques to demonstrate that pregnancy hormones such as the above mentioned, modulate pro-inflammatory responses from LPS-induced monocytes by increasing IL-10 production whilst, synergistically reducing IL-6, IL-8 and IL-1 β production *in vitro* [447]. Suggesting that the addition of P4 is retained by monocytes which may help to modulate their function during cell culture. However, there is conflicting literature to suggest that P4 alone does not diminish TNF- α and IL-1 β cytokine production from LPS-induced human monocytes [448]. This may imply that the immunomodulation effects of P4 are not mediated via inflammatory cells as they do not possess a PR. Thongngarm and colleagues demonstrated that both P4 and oestrogen influence apoptosis in LPS-stimulated monocytes in order to mediate immune suppression during pregnancy [449]. Interestingly, in non-pregnant mice models, P4 supplementation has been shown to reduce mRNA and protein levels of TNF- α in LPS activated macrophages [450]. However, there is evidence to suggest, at least in

human pregnancy, that PR expression is altered in leukocytes during gestation which can change their responsiveness [451].

6.1.7 Progesterone Levels in Non-Pregnant Women

Unlike AP, P4 is a steroid hormone, which is essential for the progression of reproduction [452]. The corpus luteum produces P4 in early pregnancy, with the major site of production shifting to the placenta after 7-8 weeks of pregnancy [279]. In non-pregnant women, the levels of P4 vary depending on the menstrual cycle phase. During the follicular phase in non-pregnant women, levels of P4 are at their lowest ranging between 1-2nM and rise after ovulation to a peak of around 50nM in the mid luteal phase, declining in the absence of pregnancy in the late luteal phase before the onset of menstruation [279]. These concentrations of P4 can be raised by using oral contraception [453] or a vaginal pessary. Two papers in the 1980s showed that administration of a 400mg vaginal pessary increased P4 serum levels in non-pregnant women with maximal absorption between 2-4 hours. The first paper published in 1985, gave 5 non-pregnant participants during the follicular phase of their cycle a 200mg and 400mg vaginal pessary every 12 hours for 5 days. After excluding participants who were on oral contraception, the increase of P4 in serum from baseline was calculated to be 31nM and 32nM after the first 4 hours of administration for the 200mg and 400mg pessaries respectively. Each subsequent dose raised P4 serum levels, but not as significantly as the initial dosage [454]. The second paper published in 1987, gave 8 participants a 400mg vaginal pessary during the luteal phase of their cycle, P4 blood serum levels were checked before treatment and then every 2 hours after the administration of the pessary. The increase in serum P4 from baseline was 31nM after the 4 hours of pessary administration [455].

6.1.8 Progesterone Levels in Pregnant Women

Circulating P4 levels have been well documented throughout pregnancy. *Tan et al*, measured serum P4 levels in healthy pregnant women during their first, second and third trimesters and the average serum P4 concentrations were calculated to be 67nM, 184nM and 296nM respectively [456]. Another study looked at the change in P4 serum levels in healthy pregnant women who delivered at term at each trimester. Overall serum P4 concentration was calculated to be 81nM, 153nM and 413nM between weeks 10-12, 20-22 and 35-37 weeks respectively [457]. P4 serum levels peak at term gestation with James *et al* reporting that the average serum P4 concentration was 584nM at 40 weeks of gestation in 60 women [458]. Studies have also been conducted on changes in serum P4 after supplementation. Many clinicians prescribe P4 supplementation to reduce the risk of PTL usually in the form of a vaginal pessary studies summarised in Section 4.1.0. The vaginal pessary dose ranges from 100mg to 800mg. Caritis *et al* measured systemic serum P4 in healthy pregnant women at a mean gestational age of 23 weeks. Baseline P4 levels were calculated to be 149nM and 12 hours after a 200mg vaginal pessary was administered, serum P4 levels was increased to 171nM [459]. In a pilot study, P4 serum levels taken at 14-18 week of gestation in pregnant women who had a history of sPTB, found no differences in systemic P4 concentration between women who had a short cervix (<25mm) when compared to those who did not [460].

6.1.9 Efficacy of Aminophylline

Although AP can be administered either intravenously or by an oral dosage, Intravenous injections are only used rarely, when a patient has severe airway obstruction. However, Nair and colleagues found that intravenous AP does not

increase bronchodilation when compared to using inhaled beta-(2)-agonists in patients with asthma [461]. In pregnancy intravenous AP is not recommended [462], but oral use is safe. Reviewing the literature over the past few decades, serum levels of AP have been assessed in several studies after oral administration in pregnant women. The first paper, published in 1979, administered 4 different derivatives of theophylline at a dose of 240mg to 8 healthy patients. The maximum serum concentrations ranged from 5.7µg/ml-6.3µg/ml and peaked at 2 hours after administration, with the authors concluding that 35µM was the maximal oral absorption [463]. In a second paper published in 2000, 3 different derivatives of AP and 1 of theophylline were given to 6 healthy non-pregnant patients at a dose of 200mg in tablet form. The maximum serum concentration ranged from 6.0µg/ml-7.0µg/ml which again peaked at 2 hours after administration, with the authors concluding that 25µM was the maximum absorption [464]. The half-life of AP was established to be 8 hours from both papers, which matches the average half-life of 4-8 hours in young adults [465] found in theophylline pharmacokinetics literature, based on this, the concentration at the IC₅₀ was calculated from both papers to be 24µM and 12µM respectively [463, 464]. From these two papers, it is estimated that approximately 15% of an oral dosage of theophylline/AP is absorbed into the blood serum. *In vitro* work investigating the effects of AP on the myometrium has also been performed, Lai and colleagues demonstrated a 50% reduction at 250µM and a 85% reduction in spontaneous myometrial contractions at 750µM of AP [466]. It is important to note that at these concentrations, AP is toxic and would be harmful to patients. A study which looked at the toxicity of theophylline and its potential risks associated with use above the therapeutic range included: arrhythmia, hyperglycaemia and seizures. It is postulated that theophylline increases

the expression of catecholamine (a hormone produced by the adrenal gland) by stimulating beta-1 and beta-2 receptors [467].

6.2 – Chapter Hypotheses

The hypotheses of this chapter are:

- i. The addition of AP will help to increase the anti-inflammatory effect of P4 *in vivo* by reducing the percentage of pro-inflammatory cell types in peripheral blood monocytes.
- ii. AP cultured with LPS-induced peripheral monocytes will modulate a reduction in pro-inflammatory cytokine production *in vitro*.

6.2.1 – Chapter Aims

The aims of this chapter are:

- i. To compare the impact of P4 and AP versus P4 alone on LPS-induced cytokine production by CD14⁺ monocytes.
- ii. To perform a dose response study of the ability of AP to reduce CD14⁺ monocyte LPS-induced cytokine release.

6.3 – Processing and Methodology

6.3.1 Recruitment – LPS Stimulation

To achieve the first aim of the study, PBMCs from pregnant women enrolled in the PROGRAM study were used in an *in vitro* stimulation assay. The samples were obtained at baseline, before randomisation to either treatment arm of the study, and subsequently at time points 1 and 2 after treatment initiation.

6.3.1.1 Recruitment – Aminophylline Dose Response

In the second part of the study, healthy pregnant women at between 18-20 weeks of gestation and non-pregnant controls were recruited.

6.3.2 Blood Processing

Maternal immune cells were cryopreserved following isolation from peripheral blood collected in the lithium-heparin tubes using the methods described in chapter 3: Section 3.2 for the LPS-stimulation assay. Intra-cellular cytokine staining and flow cytometric analysis outlined in chapter 3: Table 3.10.1.6 was used to describe key inflammatory markers produced from stimulated CD14⁺ cells.

6.3.3 Frozen Cell Viability

A revival step was implemented to increase frozen cell viability. The method used for the revival step is described in Chapter 3 Section 3.7.1. Once PBMC were isolated, trypan blue was used to check cell viability. Samples with less than 40% viability were not used. Unfortunately, 5 sets of patient samples had to be excluded from the LPS-

stimulation assay due to poor cell viability. These cells were not stored in liquid nitrogen, but at -80°C for between 18-24 months which may have led to this.

6.3.4 LPS Titration

LPS titration was required in order to stimulate monocytes. The methods used for LPS titration is described in Chapter 3 Section 3.10.17.

6.3.5 LPS Incubation

Cell viability decreases with prolonged exposure to LPS; therefore, an optimal stimulation time of 6 hours was identified. The methods used for LPS incubation is described in Chapter 3 Section 3.10.18.

6.3.6 Aminophylline Dose Responses

The assessment of AP in response to LPS-induced monocyte function was compared to P4 response. The rationale for the concentrations of both P4 and AP used in this experiment are described in Sections 6.1.7, 6.1.8 and 6.1.9. The method used for the aminophylline dose response is described in Chapter 3 Section 3.10.19.

6.3.7 Demographic Data – Aminophylline Dose Response

Table 6.3.7.1 displays the maternal demographic, obstetric and pregnancy data collected at the point of recruitment to the study. Patients were recruited from the antenatal clinic on the 1st floor at Chelsea and Westminster Hospital and were considered as healthy if they had no history of PTB and delivered at term. The non-

pregnant controls recruited to the study were healthy women not taking an oral contraceptive.

Table 6.3.7.1 – Demographic and obstetric data for women recruited to the aminophylline dose response experiment.

The data are expressed as median (range) and numerical value (percentage).

Demographic and obstetric data	Healthy pregnant women (HP) (n=3)	Non-pregnant controls (NP) (n=3)
Median age	30 (23-36)	28 (25-30)
Ethnicity, n (%)		
Other	0 (0.0)	3 (100.0)
White	3 (100.0)	0 (0.0)
Median gestational age at recruitment, (weeks)	20 ⁺¹ (19 ⁺⁵ -20 ⁺⁵)	n/a
Median BMI at recruitment (kg/m ²)	25 (21-30)	21.6 (19-25)
Parity, n (%)		
0	2 (66.6)	2 (66.6)
≥1	1 (33.3)	1 (33.3)
Previous PTB, n (%)		
0	3 (100.0)	3 (100.0)
≥1	0 (0.0)	0 (0.0)
Pre-eclampsia, n (%)		
Yes	0 (0.0)	n/a
No	3 (100.0)	
Gestational diabetes, n (%)		
Yes	0 (0.0)	n/a
No	3 (100.0)	
Median gestational age at delivery, (weeks)	40 ⁺⁵ (40 ⁺⁴ -40 ⁺⁶)	n/a

6.3.8 Statistical Analysis

GraphPad Prism version 8.4.3 (San Diego, USA) was used for the statistical analyses.

The Shapiro-Wilk's test was used to assume normality of data distribution. For the LPS-stimulation assay a non-parametric Spearman's rank-order correlation test was used to assess the strength of the association between gestation and cytokine production from CD14⁺ monocytes. For both the LPS stimulation and AP dose response assays the changes in cytokine production between LPS-induced monocytes was measured using a Mann-Whitney non-parametric test for intergroup

statistical analysis. All flow cytometry data were collected on BD FACS Diva version 8.0 (San Jose, California, USA) and analysed performed using FlowJo version 10.7.0 (Oregon, USA) expressed as % of positive cells taken from leukocyte populations. All data is displayed as mean and standard deviation. All statistical tests were two-tailed, and a p value of less than 0.05 was considered to be statistically significant.

6.4 Results

6.4.1 LPS Stimulation Cytokine Production – PROGRAM

Fluorochrome-conjugated antibodies were used for surface and intracellular staining of peripheral monocyte populations. The gating strategy used to phenotype monocytes is described in Figure 6.4.1.1. An example flow cytometric plot of each cytokine at each time point showing the comparison between control and LPS-stimulated cells is illustrated in Figure 6.4.1.1.2.

Cytokine production from LPS-stimulated monocytes between women treated with the combination of AP and P4 were compared to women treated with P4 only. Because baseline gestation varied between patients recruited for the PROGRAM study (91-145 days), relationships between gestation and cytokine production were not expected to be linear; therefore, a Spearman's correlation was applied. IL-8, TNF- α and IL-10 production showed no correlation to gestational age (Figure 6.4.1.1,3).

All patients at baseline had no prior treatment with P4 or Am to begin with. Peripheral monocytes were treated with LPS at all 3 time points. Treatment of P4 and Am was used to determine whether pro-inflammatory cytokine production would change from baseline. At the 1st (22 weeks) and 2nd (26 weeks) time points after treatment with either exogenous P4 and/or Am, the percentage change of IL-8, TNF- α and IL-10 cytokine production from CD14⁺ cells at baseline was compared between the two groups. No significant changes in positive cells were observed in any of the three cytokines measured at either time point (Figure 6.4.1.1.4).

6.4.1.1 Intracellular Cytokine Staining – LPS Stimulated Cells

Channel	FITC	BV421	BV510	APC-Cy7	PE	PE-Cy7
Antibody	TNF α	IL-10	Live/Dead	CD16	IL-8	CD14
Concentration	1/20	1/20	1/200	1/40	1/160	1/40

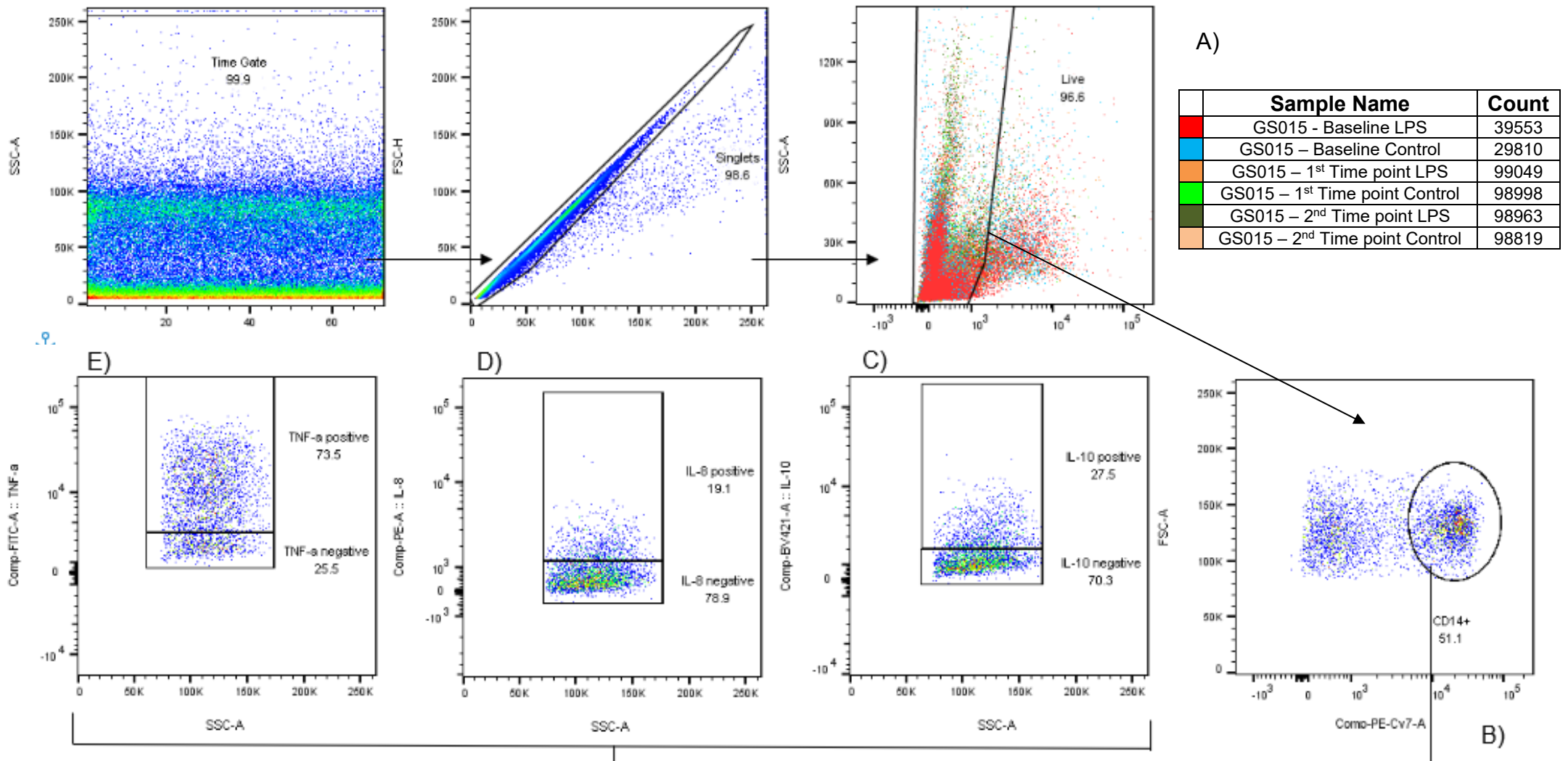


Figure 6.4.1.1.1: An example of ICCS gating from frozen monocytes. Cells were stimulated for 6 hours at 37°C. Live cells were gated using a viability dye and an example of cell counts were collected (A) Due to low numbers CD14⁺ FSC against SSC was not used to gate on monocytes, instead CD14⁺ cells were gated directly (B). IL-10 (C); IL-8 (D) and TNF- α (E) production was measured from CD14⁺ cells.

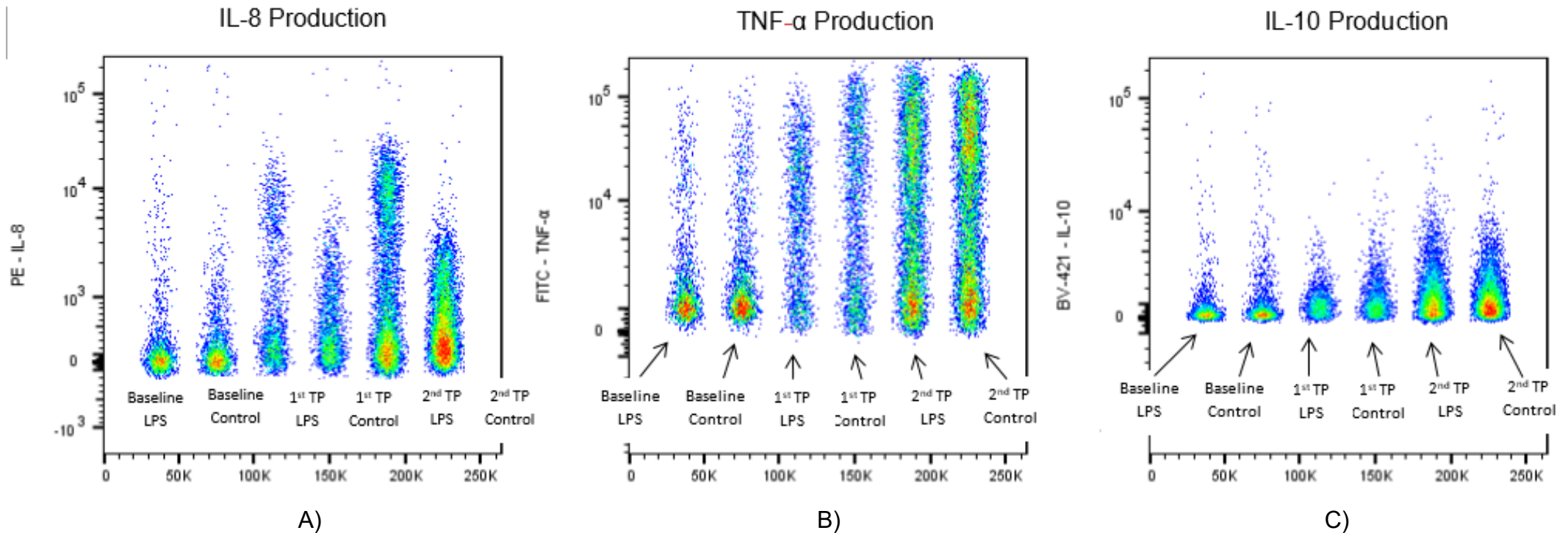
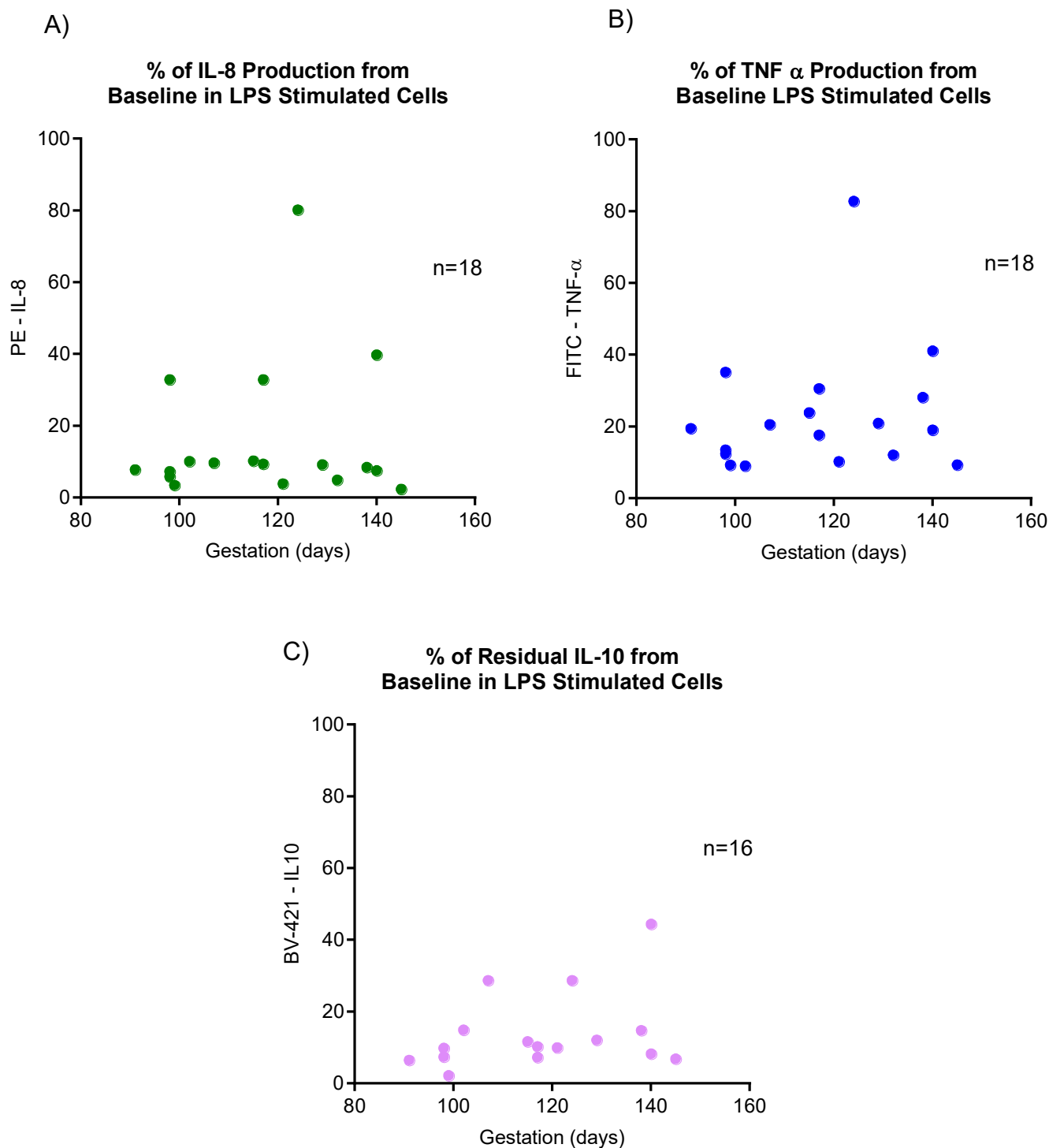
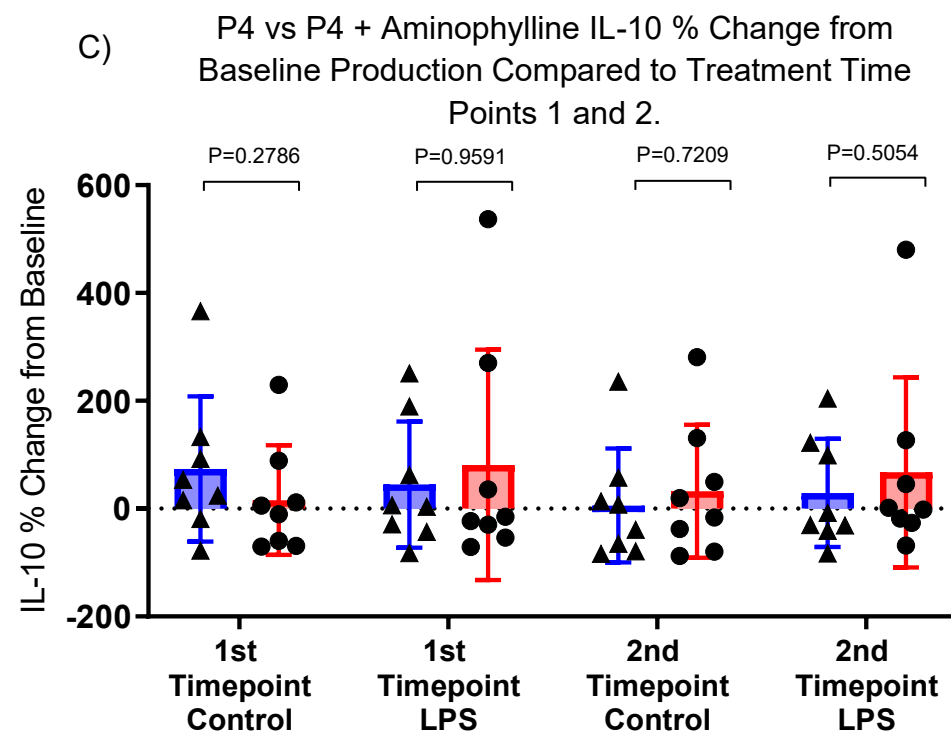
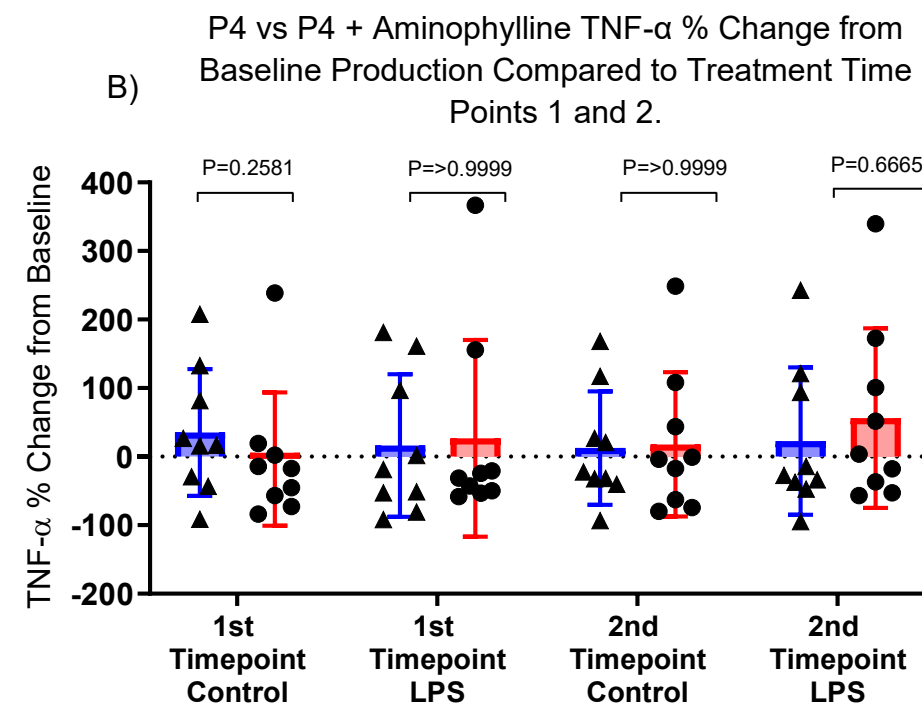
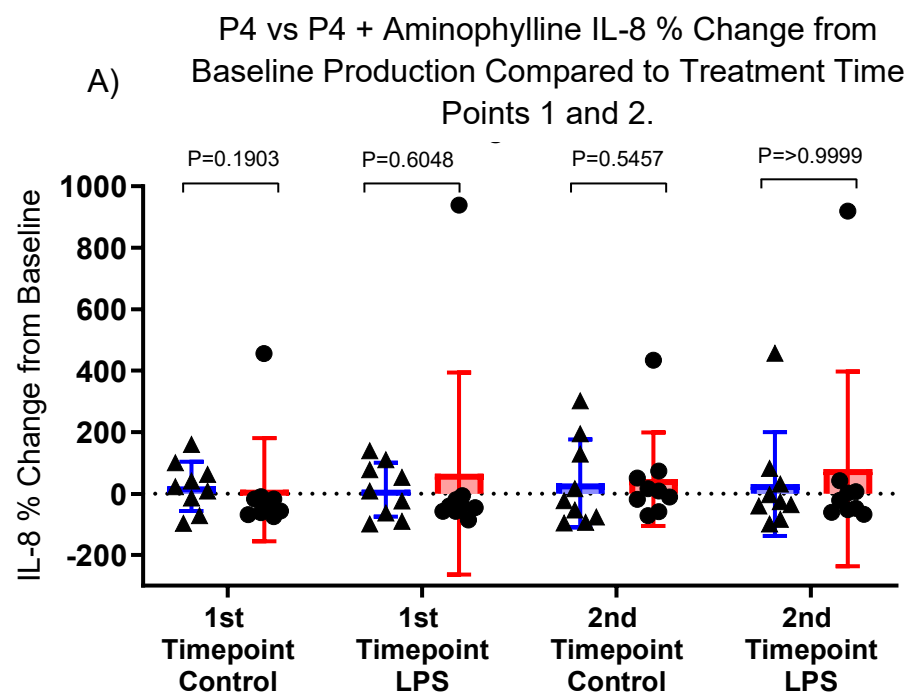


Figure 6.4.1.1.2: An example of cryopreserved PBMC, flow cytometry gating strategy used for the analysis of LPS stimulated, cytokine producing CD14⁺ monocytes. MFI of IL-8 (A); TNF α (B) and IL-10 (C) production for each time point was recorded and compared as a percentage increase/decrease to baseline levels which were also LPS-induced. A stopping gate of 100,000 events was set.



Cytokines	R_s	P Values
IL-8	-0.03573	0.888
TNF α	0.13870	0.583
IL-10	0.31560	0.232

Figure 6.4.1.1.3: These graphs show cytokine percentage changes of IL-8 (A); TNF- α (B) and IL-10 (C) across baseline gestations of LPS stimulated patient monocytes. Statistical analysis was undertaken using a Spearman's correlation where significance was defined as $\alpha = 0.05$.



▲ P4
● P4 + Aminophylline

Figure 6.4.1.1.4

These graphs show the percentage change of positive cells from cytokines: IL-8 (A), TNF- α (B) and IL-10 (C) production from baseline LPS-induced monocytes between P4 (N=9) and P4/AP (N=9) across 2 time points (week 22 and week 26 respectively) after treatment. Statistical analysis was undertaken using a Mann-Whitney non-parametric test, significance was defined as $P < 0.05$ (*).

6.4.2 Aminophylline Dose Responses

The fluorochrome-conjugated antibody panel and gating strategy was the same as described in Figure 6.4.1.1. A concatenated example of IL-8 production for each of the conditions used in the AP dose response from LPS-stimulated cells is illustrated in Figure 6.4.2.1.

It is important to note that NP and HP peripheral monocytes were cultured at different P4 concentrations (Shown in Figures 6.4.2.2, 6.4.2.3 and 6.4.2.4). Condition 1 was meant to simulate the average serum concentration absorption from a 400mg vaginal pessary for both cohorts (30nM). Condition 2 for the NP cohort was meant to simulate the average serum concentration of P4 during the early second trimester (150nM), and for the HP cohort, the theoretical serum concentration of a double dose of a 400mg vaginal pessary (60nM). Finally condition 3 for the NP cohort was meant to simulate the serum concentration of P4 during the third trimester (500nM), whereas the HP cohort, the theoretical serum concentration of a triple dose of a 400mg vaginal pessary (100nM) was used.

No significant differences were observed in cytokine production from LPS-stimulated monocytes between HP and NP controls in all experimental conditions used. IL-8 production is shown in Figure 6.4.2.2; TNF- α production is shown in Figure 6.4.2.3 and IL-10 production is shown in Figure 6.4.2.4.

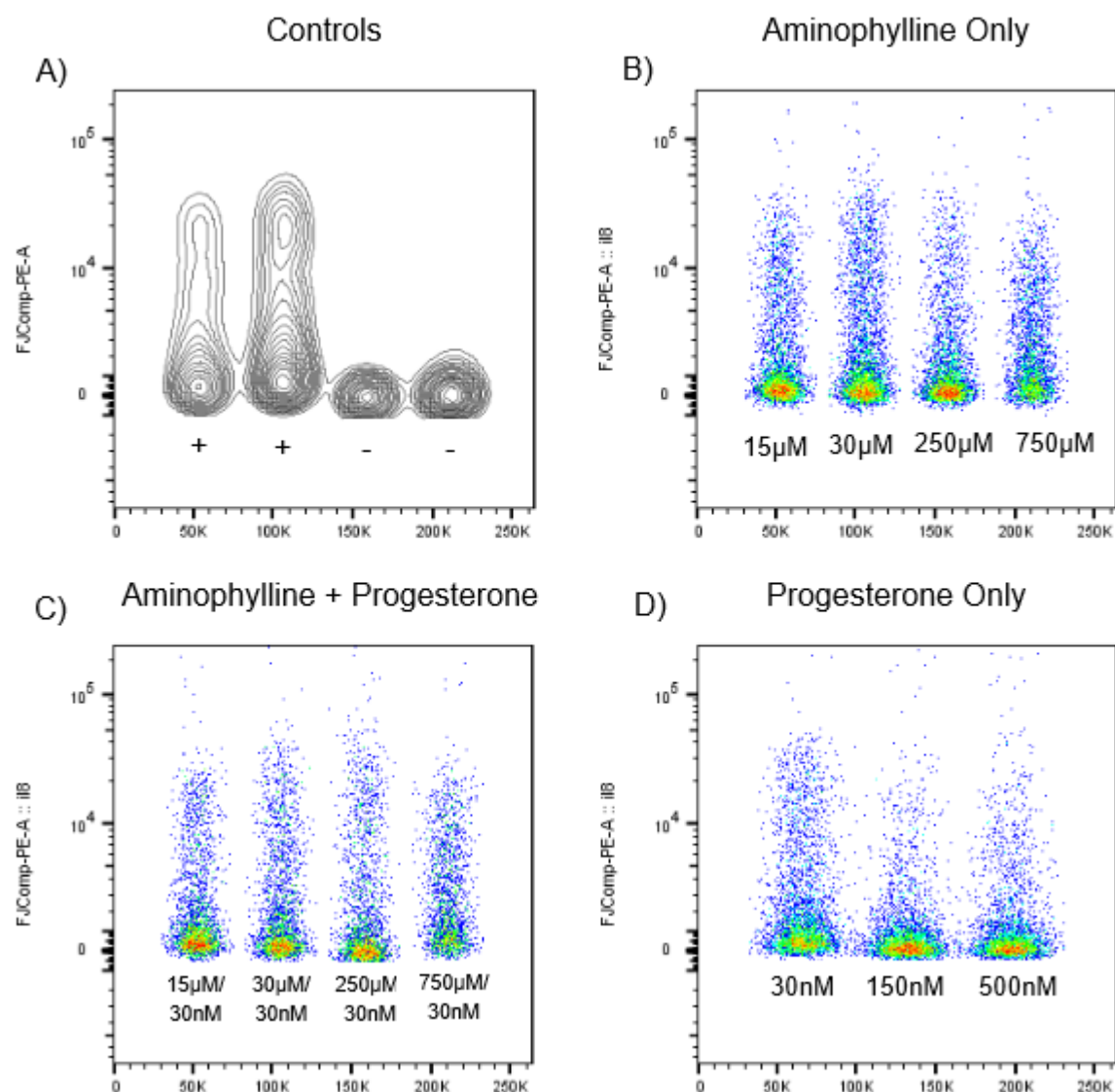


Figure 6.4.2.1: An example of the IL-8 production from CD14⁺ monocytes taken from a non-pregnant control under each condition measured. Positive controls (A) were stimulated with 50ng/mL of LPS and negative controls (A) had no stimulation with LPS. Experimental conditions were stimulated with 0.5ng/ml of LPS. Cytokine production from monocytes cultured with AP only (B); AP/P4 (C) and P4 only (D) was measured.

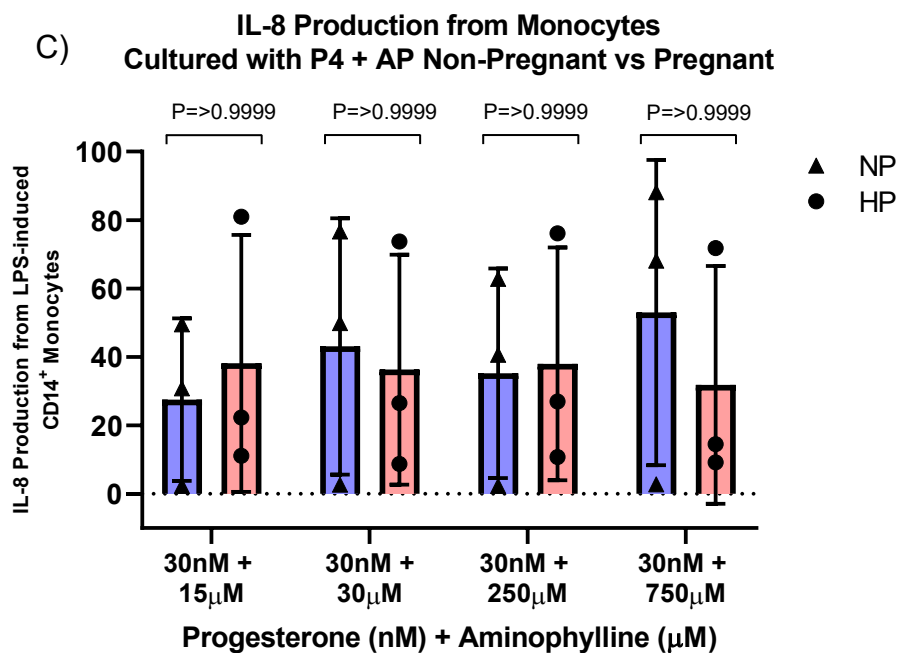
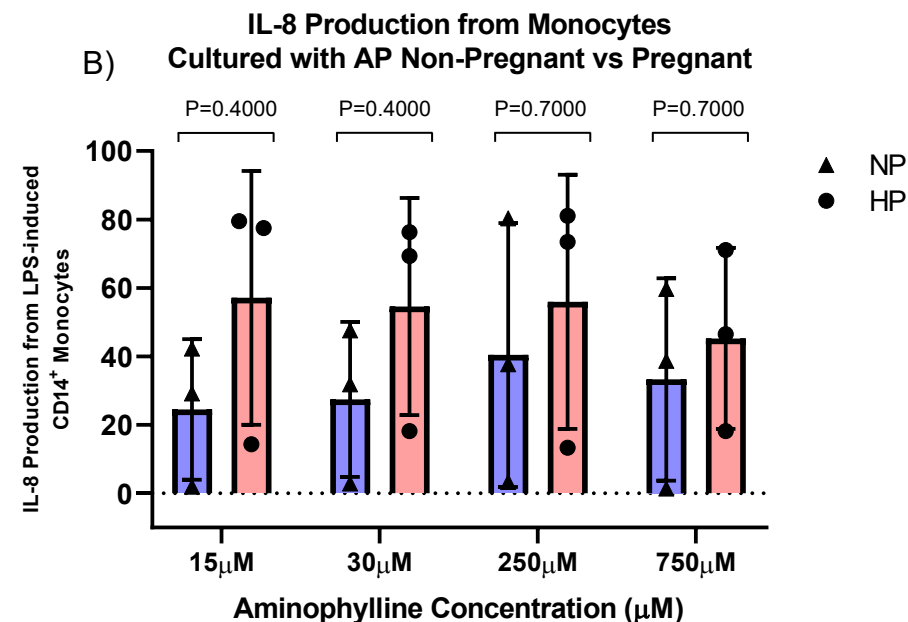
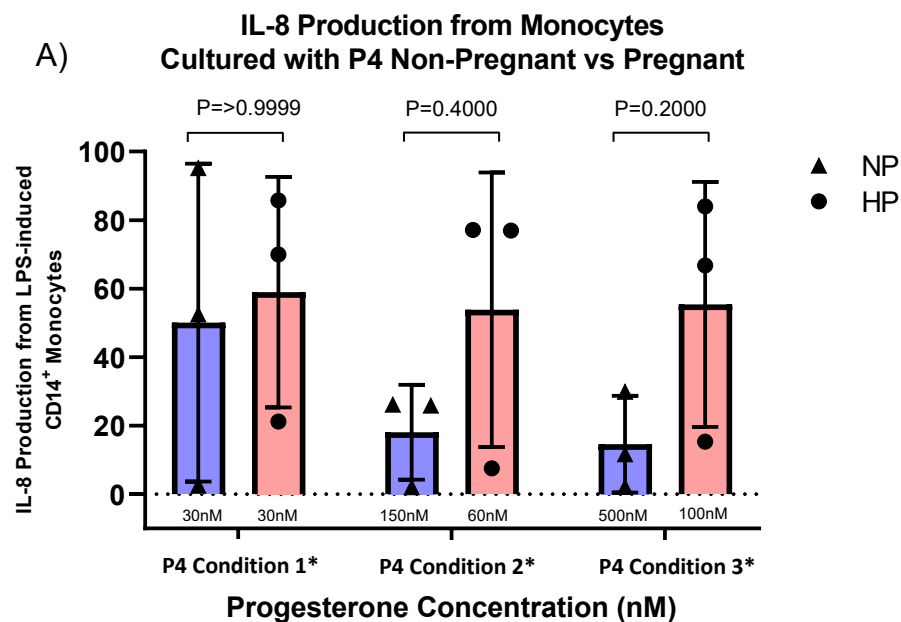


Figure 6.4.2.2

These graphs show IL-8 production from monocytes between non-pregnant (NP; N=3) and healthy pregnant (HP; N=3). A) monocytes cultured with P4 only. B) monocytes cultured with AP only. C) monocytes cultured with P4/AP. Statistical analysis was undertaken using a Mann-Whitney non-parametric test, significance was defined as $P < 0.05$ (*).

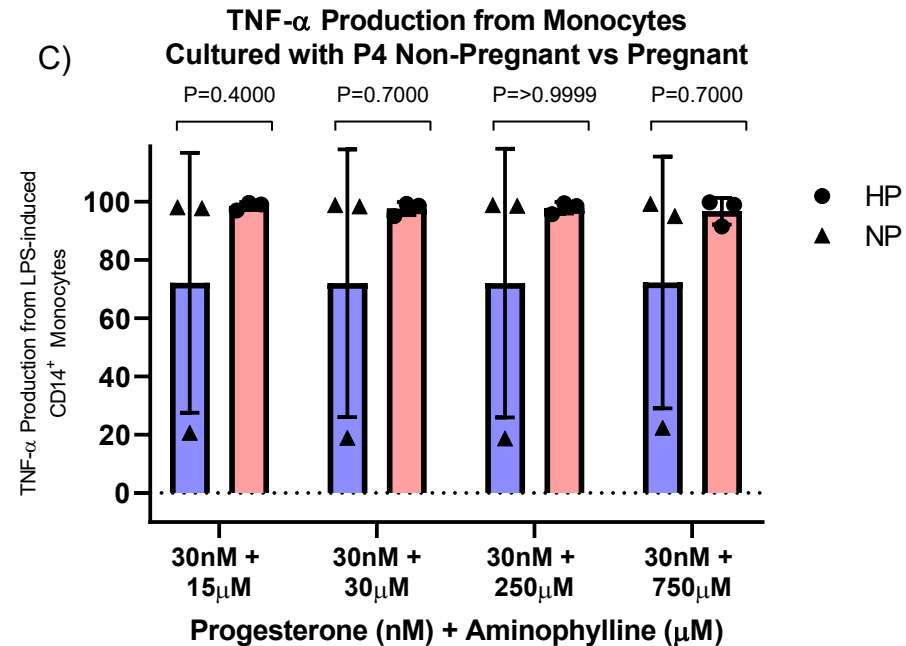
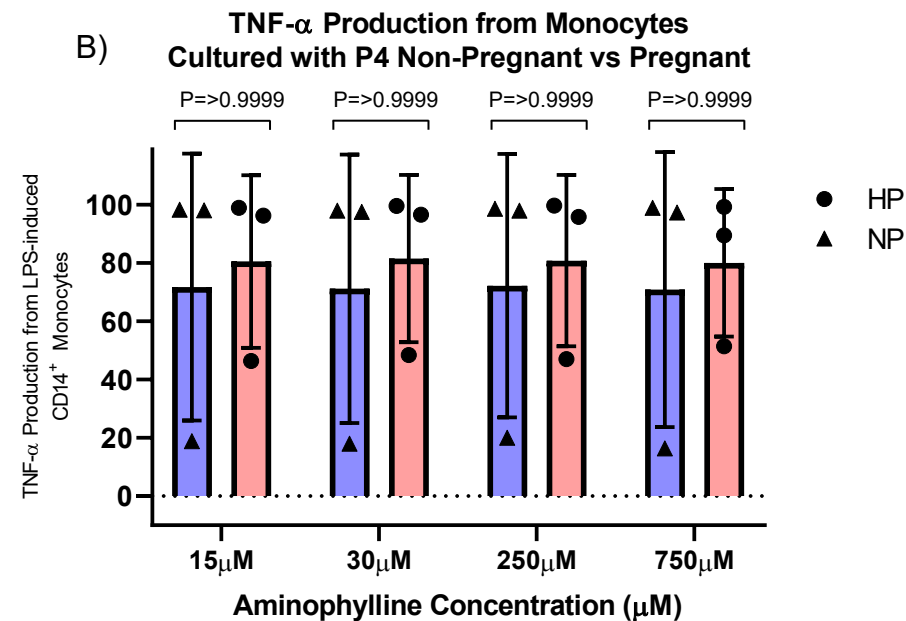
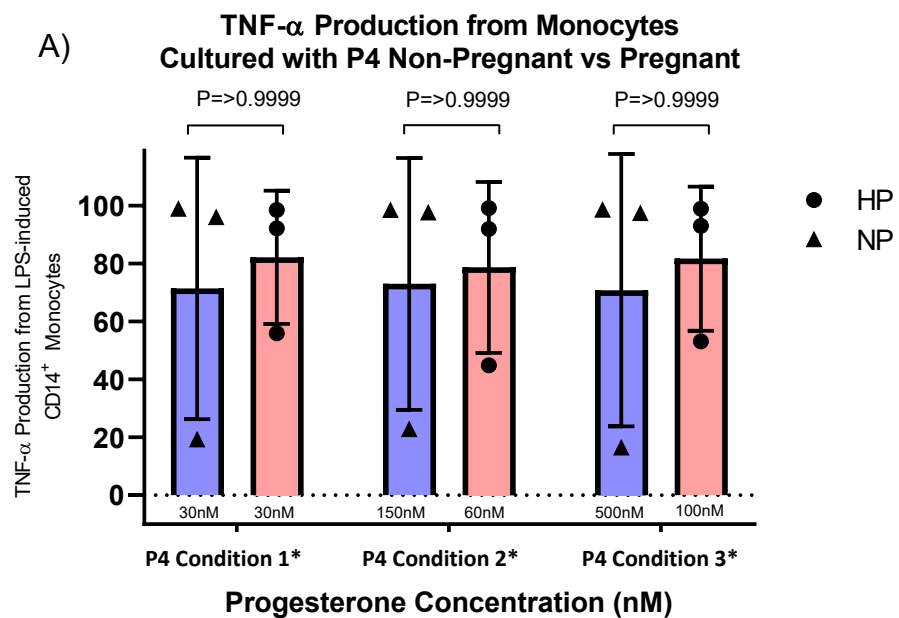


Figure 6.4.2.3

These graphs show TNF α production from monocytes between non-pregnant (NP; N=3) and healthy pregnant (HP; N=3). A) monocytes cultured with P4 only. B) monocytes cultured with AP only. C) monocytes cultured with P4/AP. Statistical analysis was undertaken using a Mann-Whitney non-parametric test, significance was defined as $P < 0.05$ (*).

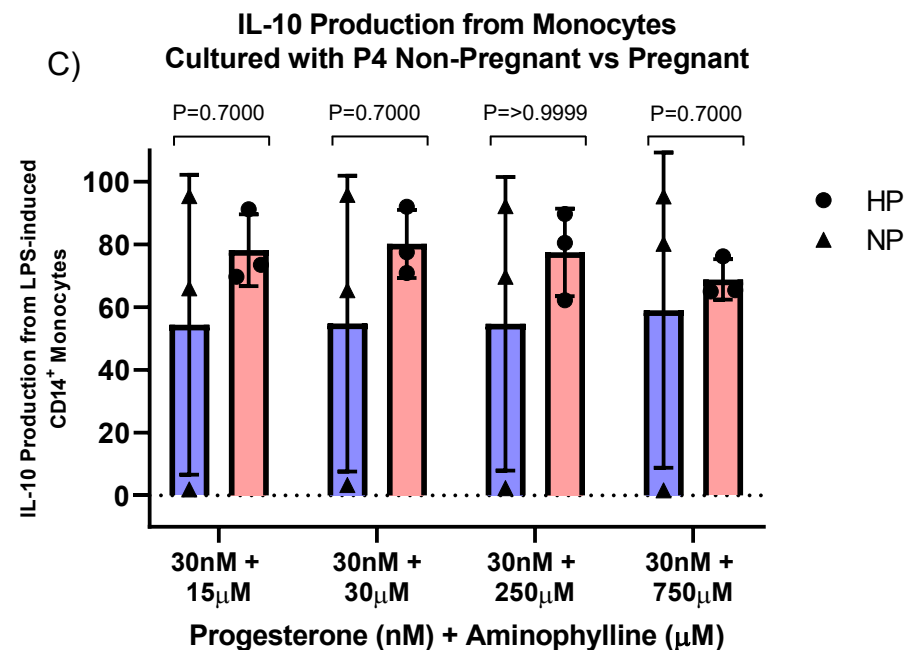
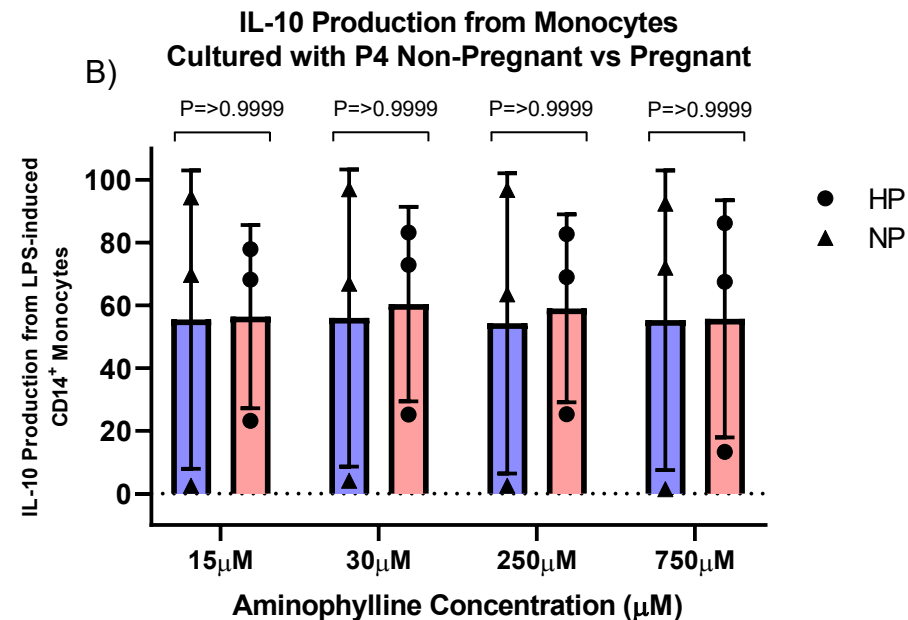
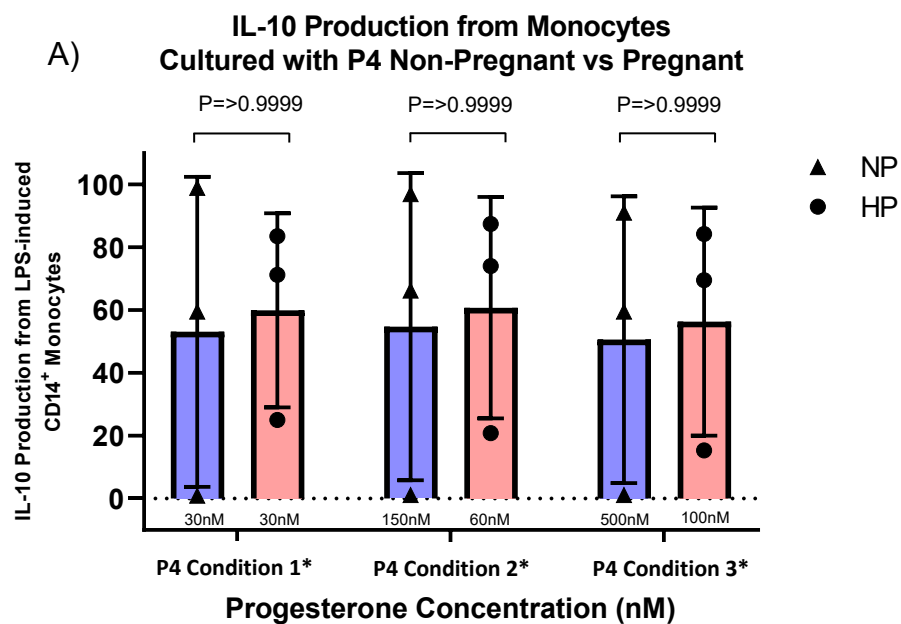


Figure 6.4.2.4

These graphs show IL-10 production from monocytes between non-pregnant (NP; N=3) and healthy pregnant (HP; N=3). A) monocytes cultured with P4 only. B) monocytes cultured with AP only. C) monocytes cultured with P4/AP. Statistical analysis was undertaken using a Mann-Whitney non-parametric test, significance was defined as $P < 0.05$ (*).

* Monocytes derived from HP and NP cohorts were cultured in different concentrations of P4. The rationale regarding these concentrations is mentioned in Section 6.4.2.

6.5 Discussion

In this chapter, how AP affects the cytokine production of LPS-stimulated monocytes isolated from high-risk pregnant women before and after treatment was assessed.

6.5.1 LPS Stimulation – PROGRAM

Since baseline gestation varied, a Spearman's correlation was used to explore the relationship between cytokine production and gestational age before P4 and AP supplementation. Baseline recruitment for PROGRAM occurred at the end of the first trimester up till mid-second trimester (13-20⁺⁵ weeks of gestation). Added to this, obstetric history showed that 61.1% of the PROGRAM participants used for the LPS-stimulation assay had a previous sPTB.

At preconception, increased systemic inflammation has been linked to infertility [468]. A study has shown that elevated levels of high sensitivity C-reactive protein (hsCRP) is associated with chronic, systemic inflammation in women who had a previous pregnancy loss and the administration of daily low dose aspirin (81mg) to these women increased clinical pregnancy outcomes [469]. That being said, during normal pregnancy, the cytokine environment changes as gestation progresses [470]. In the first trimester, an increase in systemic inflammation from preconception levels occurs up until the beginning of the second trimester, this is due to the breakdown of the epithelium surrounding the uterus to allow the invasion of blastocysts for implantation. This invasion of blastocysts causes constant shifting of invading and repairing cells and cellular debris builds up which is subsequently removed by macrophages as they are effective inducers of apoptosis [470-472]. Lye and colleagues analysed the mRNA gene expression in maternal whole blood from women presenting with TPTL at 17-23

and 27-33 weeks of gestation. Interestingly, increased expression in 13 genes associated with sPTB including elevated IL-1 and IL-6 levels at both time points were found in the asymptomatic women who went onto deliver a subsequent sPTB when compared to women who had term deliveries [473]. Suggesting that increased inflammation during pregnancy in the circulation maybe indicative of sPTB in asymptomatic women. Though, in this study, Mann-Whitney analysis showed no differences between the production of inflammatory markers TNF- α and IL-8 between participants who had a sPTB compared to women who did not at each time point in the LPS-stimulation assay.

Spearman's analysis revealed no correlation between gestation and cytokine production by LPS-induced monocytes was observed in the PROGRAM baseline samples. This implies that any changes observed at weeks 22 and 26 are likely to be a consequence of treatment as oppose to gestational changes. However, no significant differences were observed at the 1st and 2nd time points after treatment in the percentage change in cytokine production from baseline. Herbert *et al* demonstrated that in the myometrium of pregnant mice, supplementation of AP enhanced the anti-inflammatory effect of P4 and reduced LPS-induced inflammation [149], however, this was not shown in the current study, where cytokine production by LPS-treated systemic monocytes was unaffected. This suggests a differential response in leukocytes and reproductive tissues to the effects of P4/AP. Peltier *et al* showed that P4 enhances IL-8 production in LPS-stimulated monocytes but down regulated their TNF- α production [474]. However, the minimum concentration that this was achieved was at 3180nM, significantly higher than endogenous levels of P4 in blood serum which have been shown to peak at around 500nM at term [475]. The results of this study suggest that oral AP at 225mg has no effect of the monocyte

response to LPS either alone or in combination with P4. De Waal Malefyt *et al.* demonstrated that LPS-induced human monocytes start to produce IL-10 at around 7 hours after stimulation, with the maximal response occurring at 24-48 hours [476]. However, a more recent study found that IL-10 release from human macrophages occurs only 6 hours after initial stimulation, with the maximal response occurring at 24 hours [477]. Because of the toxicity of LPS towards immune cells, a time-course of cell viability in response to LPS was measured at: 6, 12 and 24 hours after stimulation. Cell viability was found to decrease after 6 hours in frozen cells and therefore was used in this study. However, no data was obtained on the effects of LPS in relation to cell viability on fresh cells. Low levels of IL-10 production were observed from the results, it is likely that the cause of this is the 6 hour stimulation used in this study, if a longer stimulation period were to be used it is possible that IL-10 production would increase, but cell viability would decrease. Another possibility is that the IL-10 observed is residual. De Waal Malefyt and colleagues found that endogenous IL-10 in systemic human monocytes suppressed the production of TNF- α and GM-CSF. This was shown by using LPS activated monocytes incubated with neutralising IL-10 monoclonal antibody which blocked IL-10 production [432].

Interestingly, the sample GS015 had a considerably higher cytokine response to LPS, such variations have been reported in the literature previously [478, 479] and may reflect an altered susceptibility to infection secondary to an inherited trait.

6.5.2 Aminophylline Dose Response

To further explore whether LPS-induced monocytes cultured *in vitro* with AP isolated from HP and NP groups modulates pro-inflammatory cytokine production, a Mann-Whitney non-parametric test was used. Three experimental conditions were

implemented, these included patient monocytes cultured with increasing concentrations of P4 only, AP only and a combination of P4/AP.

With increased concentrations of AP, no change in IL-8, TNF- α or IL-10 cytokine production was observed in both NP and HP controls at each of the experimental conditions used. Surprisingly, theophylline has been shown to inhibit LPS-induced cytokine release from monocytes and macrophages obtained from patients with COPD and asthma, but never in the context of pregnancy [442, 480]. In addition, Zhang *et al.* showed that human lung fibroblasts produced inflammatory IL-6 and IL-8 in patients with COPD, but with the administration of theophylline at 5 μ g/mL, production of these cytokines produced by fibroblasts was lowered [443]. It is possible that since the samples were collected in the late second trimester, that the monocytes were maximally repressed and that AP/P4 either alone or in combination could exert no additional effect.

P4 has been shown to reduce pro-inflammatory cytokine production to maintain maternal tolerance [314, 481]. However, no change in TNF- α or IL-8 cytokine production was observed in HP and NP groups at any concentration of P4 added. P4 supplementation has been shown to prevent embryonic reabsorption of LPS in pregnant mice by increasing the anti-inflammatory environment of the maternal uterus, this resulted in a decreased rate of pregnancy loss [482]. Another study demonstrating the anti-inflammatory effects of P4, has shown that it can inhibit activation of LPS-induced macrophages *in vitro* decreasing their mRNA gene expression of IL-12 and nitric oxide synthase 2 (NOS2) [315]. Flores-Espinosa and colleagues found that amniotic epithelium explants treated with 1000ng/mL of LPS increased TNF- α production by 30-fold, however co-stimulation with P4 at a concentration of 1.0 μ M

decreased the levels of TNF- α by 2.8-fold [483]. The contradicting findings that P4 did not have any effect on reducing LPS-induced cytokine production from systemic monocytes in this study, maybe due to the low sample size of HP and NP groups collected (N=3) preventing any reliability to the experimental findings of the AP dose response assay. A bigger sample size would provide more statically relevant conclusions to be made.

6.5.3 Chapter Conclusion

in vitro analysis of LPS-stimulated monocytes in response to the combination of AP and P4 did not show any down regulation in inflammatory cytokine production. Added to this, even at toxic concentrations of AP (250 μ M and 750 μ M) no attenuation of inflammatory cytokine production was observed. This may suggest that the anti-inflammatory effects of AP are either not mediated by monocytes but perhaps a different immune cell type or even by gestational tissue, or that the concentration of AP is too low to elicit a response to stimuli derived from infection. However, the pilot nature of the AP dose response assay limits the power of the results due a low sample size. With increased N numbers the power and reliability of the results may give us a better picture as to the effect AP is having on peripheral monocyte function.

7. Final Summary and Discussion

This study aimed to test the overarching hypothesis that high-risk pregnancy is associated with a lack of immunological reinforcement necessary for supporting pregnancy, with the aims: to characterise the phenotype of acquired and innate immune cells in the cervical mucus and the circulation between women who present with TPTL and experience a PTB verses those who do not, to investigate the longitudinal effects on the immune system of high-risk pregnant women taking preventative treatment for delaying the onset of PTL, whilst still keeping the mother and the baby healthy, and to assess the anti-inflammatory properties of AP towards maternal immune cells *in vivo* and *in vitro*. The activation, maturity, senescence and function of local and systemic immune cells have not been explored previously throughout high-risk pregnancy. Therefore, it was unknown as to how immunological variations in these women would be different when compared to healthy pregnancy.

7.1 Summary of Results

The results from the first chapter showed a number of immunological changes between women presenting with TPTL who experienced PTB compared to those who gave birth at term (Figure 7.1.1). From the peripheral blood data, lower CD4⁺ TEMRA cells were observed in the sPTB group when compared to the TB group. sPTB women exhibited a lower proportion of cytotoxic CD56^{bright} NK cells when compared to the HP group. Furthermore, CD69 expression of cytolytic CD56^{dim} NK cells was higher in the sPTB compared to the HP group, though this was approaching significance, while NKp46 expression was higher in the sPTB group compared to the HP group. A reduced frequency of CD14⁺ CD16⁺⁺ non-classical monocytes was observed in the

sPTB group when compared to the HP, whereas a reduced frequency of CD14⁺⁺ CD16⁻ classical monocytes was observed in the sPTB group when compared to the HP group. However, this was at the margin of statistical significance. HLA-DR activation was found to be lower in the sPTB and HP groups when compared to the TB group in each subset of monocyte. Both C_m and PD-1 expression was lower in CD4⁺ and CD8⁺ T cells in the TB group when compared to the HP group. From the cervical data, higher proportions of neutrophils with increased HLA-DR activation was observed in the sPTB group when compared to the HP group, furthermore, lower frequency and HLA-DR activation of monocytes was observed in the sPTB group when compared to the HP group. Although, the cervical data cannot be relied upon due to low sample sizes analysed.

In the results from the second chapter, changes in the immune phenotype were observed between high-risk pregnant women receiving either a combination of P4 and AP or P4 alone (Figure 7.1.1). From the peripheral blood data, higher CD57 expression from baseline up to week 34 was observed on cytotoxic CD56^{bright} NK cells in the P4/AP group. Throughout pregnancy, increased frequency of CD14⁺ CD16⁺⁺ non-classical monocytes from week 22 up to week 34 was observed in the P4/AP group, whereas non-classical monocyte HLA-DR expression increased after baseline up to week 30 in the P4 only group. Neutrophil frequency decreased from baseline to week 22 in the P4/AP group. However, this was approaching significance and levels resumes after week 22. In the cervical mucus, a comparison between neutrophils, eosinophils and monocytes revealed no differences between the two cohorts in frequency or activation.

The first part of the third results chapter looked at the effects of AP *in vitro* on LPS-induced cytokine production of frozen, systemic monocytes isolated from participants

recruited for the PROGRAM study. No alterations were observed from the first or second time points after treatment when compared to baseline cytokine production of: TNF- α , IL-10 and IL-8 between women receiving a combination a P4/AP verses P4 only.

The second part of the third results chapter further explored whether LPS-induced fresh, systemic monocytes cultured *in vitro* with AP isolated from HP and NP women modulates TNF- α , IL-10 and IL-8 cytokine production. No diminished inflammatory response was observed with increasing AP, even to physiologically toxic dosages.

From these results, it is found that high-risk pregnancy is associated with a number of adaptations which differ from healthy pregnancy possibly disrupting immune tolerance. On the other hand, the effect of AP supplementation given to help prevent PTB was inconclusive, as AP appeared to have limited anti-inflammatory properties both *in vivo* and *in vitro*.

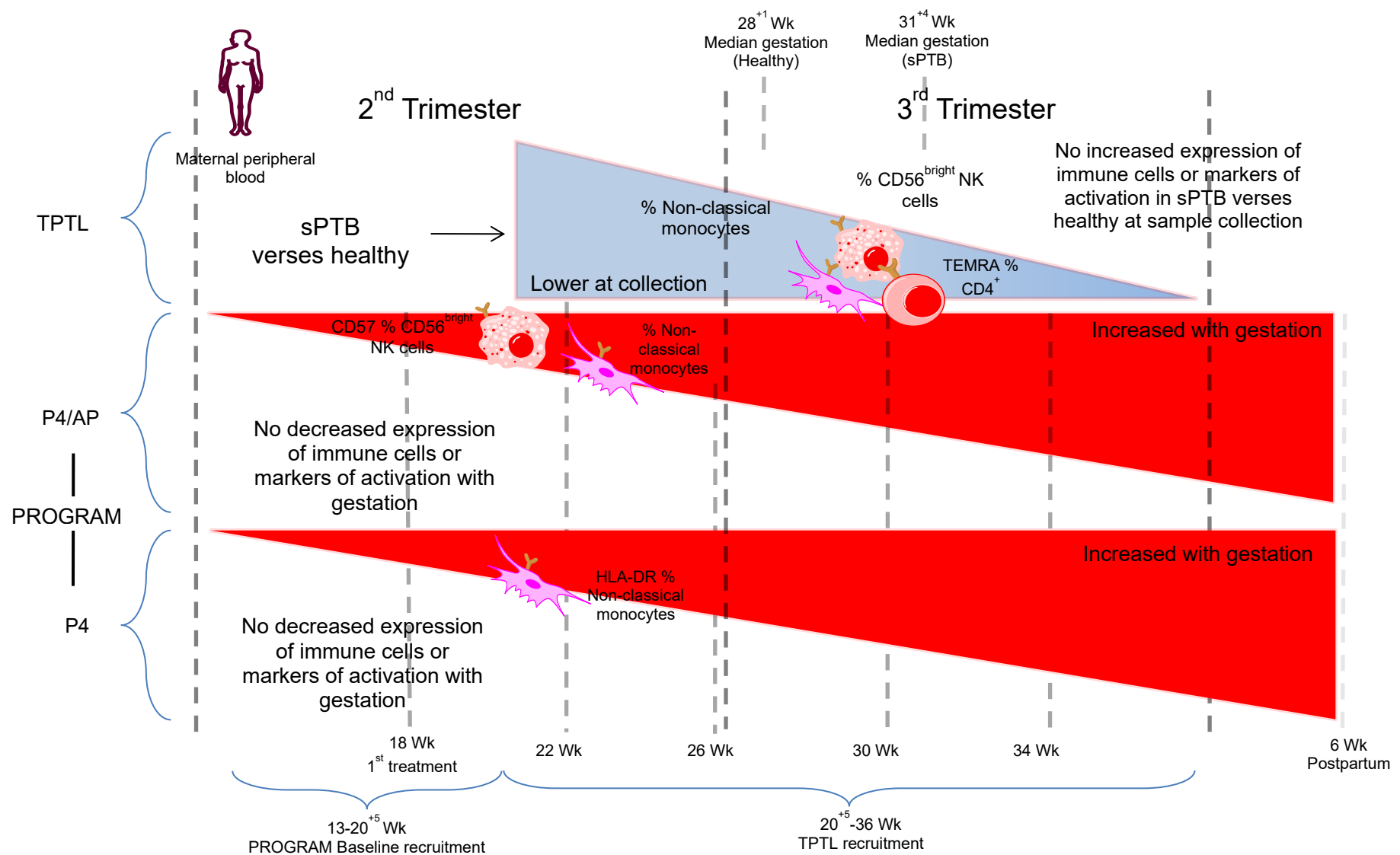


Figure 7.1.1: Summary model of immunological changes associated with high-risk pregnancy.

From top to bottom: TPTL study (top) showing immunological parameters which were lower (blue slope) in sPTB compared to HP women using median gestational age at time of sample collection. Immunological parameters which increased (red slope) as gestation progressed in participants treated with P4/AP (Middle). Immunological parameters which increased (red slope) as gestation progressed in participants treated with P4 only (bottom). Dashed lines (grey) indicate each time point of the PROGRAM study where patient samples were taken. Dashed lines (black) indicate each trimester.

7.2.1 Discussion (Part I) – Insufficient innate cell reinforcement may disrupt the maintenance of pregnancy in high-risk women

The HP participants were found to have a balance in acquired and innate immunity which promoted a regulatory phenotype during pregnancy, however, no changes were observed between the sPTB and HP groups in relation to frequency and markers of activation of T cells and Tregs, which implies that the adaptations observed in the monocyte and NK innate cell immunity, might be contributing towards the adverse pregnancy outcomes found in the sPTB women.

Lower levels of non-classical monocytes was found in the sPTB group, these monocytes can possess an anti-inflammatory phenotype and they have been shown to activate wound-healing macrophages located in inflamed tissue as the first line of defence against pathogenic stimuli [484, 485]. Furthermore, in mice, non-classical monocytes have been shown to induce migration of neutrophils for the removal of damaged and infected cells [486]. This implies that a poorer response to inflammation would be more likely to occur due to the lack of these immune cells. Lower expression of CD56^{bright} NK cells was observed in the sPTB when compared to the HP group. CD56^{bright} NK cells are precursors of CD56^{dim} NK cells [248], which may explain as to why the CD56^{dim} population had a tendency to be lower in the sPTB group. CD56^{bright} NK cells are not cytolytic (like their CD56^{dim} counterpart) due to their high expression of inhibitory CD94/NKG2A receptor [487], however, they are cytokine producing cells and release: INF- γ , GM-CSF and IL-10 when stimulated by monokines [83], added to this, when activated they promote the production of monocyte derived TNF- α [488]. Another way CD56^{bright} NK cells produce INF- γ is upon stimulation with IL-2 derived from T cells. The presence of an IL-2 receptor (IL-2R) on the surface of these NK cells may be used to cross-talk between the innate and adaptive immune system in

response to inflammation [487]. With these data, it suggests that the function of the CD56^{bright} NK cells is to generate a positive feedback loop, producing cytokines to activate other innate cells able to respond and control infection. Therefore, in the sPTB group the failure of innate immunity by the decreased expression of non-classical monocytes and CD56^{bright} NK cells might prevent an adequate adaptive response to deal with antigen exposure. Additionally, the lack of innate immunity provides an insufficient response to endogenous inflammation which may lead to PTB.

7.2.2 Discussion (Part II) – Aminophylline may enhance the anti-inflammatory effect in other immune cell types

There were few immunological parameters which changed in women treated with P4/AP when compared to the P4 only group. Despite the presence of AP *in vivo*, no evidence of an enhanced anti-inflammatory phenotype was observed in high-risk pregnancy for this study. In fact, the converse maybe true, as increased CD57 expression of CD56^{bright} NK cells was observed in the P4/AP group which indicates that these NK cells have a potentially reduced response to infection, as over expression of CD57 on this NK subset has been shown to diminish INF- γ production and their ability to proliferate [489]. A testable model demonstrating the function of these NK cells to better understand their cytotoxic capabilities, cytokine production and ability to proliferate by culturing them under different stimulation conditions can be done. To test any alterations in subset phenotype of NK cells, K562 cells can be used as they are absent of the MHC class 1a ligand and therefore cannot bind to the inhibitory receptor on the NK cell surface [490]. Unpublished work from our group has shown that unstimulated CD56^{bright} NK cell frequency increased whilst CD56^{dim} NK cell frequency decreased upon stimulation with K562 cells in both HIV^{+/-} pregnant women, suggesting that the expansion of the CD56^{bright} population is biased in response to K562 stimulation. However, CD57 expression in these NK cells was not measured prior to experimentation; consequently, these data cannot confirm if over-expression of CD57 might modulate the proliferation of the CD56^{bright} NK subset after stimulation with K562 cells. Therefore, the establishment of a cytotoxic assay such as the one highlighted, could be used in the future to determine the function of this NK subset isolated from AP treated women which have a high expression of CD57.

Increased frequency of non-classical monocytes after the first time point up to 34 weeks of gestation was also found in the P4/AM group. Importantly, non-classical monocyte frequency has been shown to increase in healthy pregnancy after the first trimester [233]. Interestingly, HLA-DR expression on non-classical monocytes was not increased in the P4/AP group unlike in the P4 group, however, innate cell activation has been observed to increase in healthy pregnancy after the first trimester [262]. With these data, it suggests that the longitudinal changes observed in monocyte phenotype are likely gestation related and not as a consequence of treatment with AP or P4.

The AP dose response was aimed to detect any functional changes in response to LPS stimulation, particularly in cytokine production from peripheral monocytes/ $m\phi$. From the results of this chapter, it was unknown as to whether intracellular cAMP levels in monocytes/ $m\phi$ were raised in samples cultured with AP. In fact, it is not well understood as to how cAMP affects the function of these immune cells in general. Drugs like AP which aim to intracellularly raise cAMP by inhibiting phosphodiesterases would require a cAMP analogue which can be used to give a better insight as to which cells are being affected by the change in cAMP [491, 492]. A previous study demonstrated that a cAMP analogue known as 8-pCPT-2'-O-Me-cAMP cultured with human monocytes/ $m\phi$ was shown to upregulate Epac1 expression levels in the mRNA of these immune cells [409]. This activation caused an increase in pro-inflammatory CXCL5, CXCL5 and CCL2 chemokine production in both mRNA and protein [409]. However, another paper using the same cAMP analogue showed that systemic monocyte function such as cytokine and chemokine production was not dependant on exchange protein activated by cAMP (Epac1) activity [493]. Interestingly, Gerlo and colleagues used the same cAMP analogue in addition to using N(6)-benzoyladenosine-cAMP (6-Bnz-cAMP), another cAMP analogue which does not

selectively activate Epac1 but instead protein kinase A (PKA) [492]. What was found when each of these analogues were cultured individually with T cells and monocytes was that, 8-pCPT-2'-O-Me-cAMP upregulated IL-10 production whilst having no effect on TNF- α and IL-1 β production on LPS-induced monocytes. In contrast to this, 6-Bnz-cAMP was found to inhibit T cell cytokine production [492], This might suggest that T cell function maybe mediated by PKA, whereas monocyte function is mediated by Epac. Results of the AP dose response assay were inconclusive as to whether monocyte function was altered in pregnant women when compared to non-pregnant controls. Partly due to the low sample sizes obtained also not measuring the levels of cAMP in the monocytes/m ϕ to confirm the efficacy of AP in culture. Potentially looking at T cell as well as monocyte/m ϕ function using a cAMP analogue and measuring cytokine levels in the protein and mRNA may elucidate the effect of AP towards these immune cells *in vitro*.

7.3 Limitations

A number of limiting factors impeded the progression of the study. No sample collection took place until the end of the first year due to delays regarding ethics and study approval, as well as a lack of staff available to help recruit, consent and collect patient samples. The TPTL study was affected the most because of this interruption, whereby recruitment occurred for only 6 months of the full duration of the study. Added to this, as a result of the COVID-19 pandemic, the recruitment process for the AP dose response experiments was terminated prematurely due to lab closure. Because of this a sample size of 3 HP and NP groups could only be recruited. The recruitment of additional patients would provide more powerful and statistically relevant data to test the hypothesis set out in Chapter 6.

LPS-stimulation of all PBMC had to be performed on frozen cells. All PROGRAM samples were processed and run on fresh blood using flow cytometry on the same day of collection, therefore to run a 6 hour LPS-stimulation alongside was not feasible, PBMC had to be cryopreserved, stored and run at a later date. Additionally, storage in liquid nitrogen was not accessible during this study, and cells had to be stored at -80°C. Silveira and colleagues found that cryopreservation of systemic monocytes affected their ability to differentiate into immature monocyte-derived DC and showed decreased cytokine/chemokine production in response to LPS when compared to fresh cells [494]. Furthermore, all of the cells used for the LPS-stimulation experiments ranged from 6-18 months of storage at -80°C which may have affected cell viability.

7.4 Future Work

A criticism of this study was that no healthy gestational matched pregnant controls were recruited to compare against the immune cell phenotypes of the PROGRAM women. However, the fatty acid profile “FAP” study currently running in our group, obtained PBMC at 12 and 28 weeks of gestation from healthy pregnant women who delivered at term. Analysis and comparison of the phenotype of these immune cells using the same panels would help elucidate whether changes observed in the PROGRAM women was likely due to treatment or gestation related.

The study of cytokines/chemokines and growth factors in maternal serum, cervical vaginal fluid (CVF) and cervical lavage isolated from high-risk pregnant women has had limited research and would be an important addition to the findings of this study. Ekelund and colleagues isolated serum from maternal whole blood taken at delivery before 34 weeks to determine cytokine levels using a Multiplex immunoassay in TPTL subjects. In terms of circulating levels, women who had a lower expression of IL-18 (a

cytokine which induces both a Th1 and Th2 response) with a high expression of IL-12 (a cytokine which induces a Th1 response) had a 2-fold increase of deliver preterm before 34 weeks of gestation when compared to women who delivered after 34 weeks [495]. That being said, no current research has been done on the cytokine profiles in CVF or cervical lavage samples in high-risk pregnancy, and there is evidence to suggest that the cytokine environment in the cervical mucus is influenced by a number of factors. For example, Lieberman and colleagues found that non-pregnant participants who smoked were found to have decreased cytokine expressions of: IL-6, IL-10, INF- γ and IL-1 β . Furthermore, women infected with human papillomavirus (HPV) had lower cytokine levels compared to healthy subjects [496]. This data implies that infection in the female genital tract influences the cytokine milieu with may disrupt fetal-maternal tolerance during pregnancy.

Leukocyte RNA was extracted from every peripheral blood sample taken at each time point from TPTL and PROGRAM participants. After red blood cells were removed, leukocyte RNA was stored in Triazol and kept at -80°C which has been shown to maintain stability over long periods of time [497, 498]. Although, recent literature has explored mRNA gene expression in gestational tissue in both term and PTL, the study of mRNA expression in leukocytes isolated from high-risk pregnant women has yet to be explained. Singh *et al.* explored the gene transcriptome in the myometrium and compared pro and anti-inflammatory gene expression profiles between: term no labour, term early labour, term established labour and preterm no labour samples and found that mRNA expression of: IL-6, CXCL1 and CXCL8 was increased in the term established labour group [499]. Additionally, mRNA gene expression analysis of different sPTB phenotypes using amnion and choriodecidual tissue was performed by the same group, and found that chorioamnionitis-driven PTB has an increased

inflammatory profile when compared to idiopathic PTB [157]. These data explored the transcriptome at delivery in gestational tissue, with the addition of systemic leukocyte mRNA transcriptome taken at multiple time points during high-risk pregnancy may help to give a clearer picture in regards to potential etiologies of PTB.

8. References

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9. Appendix

A)

10/H0801/45

4th October 2018 Version 4

OBSTETRICS AND GYNAECOLOGY,
CHELSEA AND WESTMINSTER HOSPITAL,
369 FULHAM ROAD,
LONDON SW10 9NH

CONSENT FORM

Title of project: Preterm ~~labour~~ study

1. I confirm that I have read and understand the information sheet dated 4th October, 2018 (version 4) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at anytime without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I give consent for the blood, urine, cervical ~~swab~~ and vaginal swabs to be taken. ☐
5. I give consent for the contractions to be monitored ☐
6. I give consent for a myometrial biopsy to be taken if I require a caesarean section ☐
7. I give consent for umbilical cord blood and samples from the placenta after I have given birth ☐
8. I give consent for a stool sample to be taken from the baby after delivery ☐
9. I agree to take part in the above study. ☐
10. I do/do not wish for my GP to be informed. ☐

Name of Patient

Date

Signature

Name of person taking
Consent

Date

Signature

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

B)

Centre Number:

Study Number:

Participant Identification Number for this trial:

CONSENT FORM

Title of Project: PROGRAM: A randomised controlled feasibility study of the tolerability of the combination of PROGesteRone and AMinophylline for the prevention of preterm labour

Name of Principal Investigator: Prof Mark Johnson

Please initial
box

1. I confirm that I have read and understand the information sheet dated (version 1.0 dated 10 Jun 2016) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

☐

3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from SSAT, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

☐

4. I agree my GP being informed of my participation in this study

☐

5. I agree for my vaginal swabs and blood samples to be taken during the study visits

☐

6. I agree for a myometrial samples to be taken if I require a caesarean section for medical reasons

☐

7. I agree to the placenta to be sent for further analysis and for blood to be taken

☐

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Version 1.0 10 Jun 2016

C)

from the umbilical cord after delivery.

Chelsea and Westminster Hospital 
NHS Foundation Trust

8. I agree for the research team to obtain information about my baby's health from my baby's notes

☐

9. I agree to be contacted in the future to see how my baby is getting on and he/she grows up and to access information in health records about you and your baby.

☐☐

10. I agree to take part in the above study.

Name of Participant

Date

Signature

Name of Person taking consent
(principal or co investigator)

Date

Signature

When completed, 1 for participant; 1 for site file; 1 (original) to be kept in medical notes

D)

11/LO/0971

31st July, 2016 Version 3

**OBSTETRICS AND GYNAECOLOGY,
CHELSEA AND WESTMINSTER HOSPITAL,
369 FULHAM ROAD,
LONDON SW10 9NH.**

CONSENT FORM

Patient ID number:

Title of project: Immunosuppression in pregnancy

Name of Researcher:

Please initial
box

1. I confirm that I have read and understand the information sheet dated 31st July, 2016 (version 3) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at anytime without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my record. ☐
4. I agree to take part in the following study and for the blood to be collected at ~~10-13 weeks, 16-20 weeks, 28-30 weeks, 34-36 weeks, at delivery and 2-6 months post-partum:~~ ☐
5. ~~I agree for swabs to be taken from the cervix and vagina during the following times: 10-13 weeks, 16-20 weeks, 28-30 weeks, 34-36 weeks, at delivery and 2-6 months post-partum,~~ ☐
6. I agree to allow any stored plasma/serum samples to be used. ☐
7. ~~I agree for umbilical cord blood and placental samples will be taken immediately after delivery,~~ ☐
8. ~~I agree to have a myometrial biopsy if I undergo a cesarean section.~~ ☐
9. I am happy for my GP to be informed. ☐
10. I do not wish for my GP to be informed. ☐

Name of Patient

Date

Signature

Name of person taking
Consent

Date

Signature

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

Figure 9.1 – Patient consent forms for each of the studies mentioned: TPTL (A), PROGRAM (B, C) and AP dose response (D)