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UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

The Impact of Antiretroviral Therapy and Immunological Factors on Preterm and Small for Gestational Age Deliveries in HIV Infected Pregnant Women

By

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Virology) in the School of Laboratory Medicine and Medical Sciences,

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Declaration

I, Nontlantla Cecilia Mdletshe, declare that this is my original work. Where others have contributed, it has been acknowledged in this thesis. The work presented here has not been submitted in any form for degree purposes to any other university.

The experimental work described in this thesis was conducted at the HIV Pathogenesis Programme Immunology laboratory, Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Professor Thumbi Ndung'u and co-supervision of Doctor Christina Thobakgale Tshabalala.

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As the candidate's supervisor, I agree to the submission of this thesis

Professor Thumbi Ndung'u

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Publications and conference presentations

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Dedication

For my grandmother, Nomathamsanqa Agnes Mdletshe.

“Gogo bek' umthwalo
Kunin' uhlupheka”

My purpose as a woman has grown to portray excellence in all I endeavor to do, to be proud, unapologetic, and live my life without regret. The journey has not always been easy! There have been times, days when things did not go according to plan. People in my life, like my grandmother, taught me that there would be times when you seemingly face insurmountable obstacles, but that this is when you dig deep into your soul for the courage and fortitude needed to complete the task before you. It has taken me time to understand that in spite of life’s detours, there is greatness in me. I dedicate this work to the iconic people in my life who have inspired and strengthened me. I believe that showing gratitude reminds me that I did not arrive at this destination by myself but that an entire village is behind me, rooting for me, praying for me and paving the way for me in ways I myself would not have been able to.

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Abbreviations

AIDS: Acquired Immune Deficiency Syndrome

ANOVA: Analysis of Variance

APC: Antigen Presenting Cell

ART: Antiretroviral therapy

cART: combination Antiretroviral therapy

CCR5: C-C chemokine receptor 5

CD: Cluster of differentiation

CO_2: Carbon dioxide

CTL: Cytotoxic T lymphocytes

CRCR4: CXC chemokine receptor 4

DC: Dendritic cell

DC-SIGN: Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin

DMSO: Dimethyl sulfoxide

FACS: Fluorescent activated cell sorting

FBS: Fetal bovine serum

FCS-A: Forward scatter-area

FSC-H: Forward scatter-height

IFN-γ: Interferon gamma

IL-2: Interleukin 2

IQR: Interquartile range

HIV: Human imunodefiency virus

HAART: Highly active antiretroviral therapy

HLA-DR: Human leukocyte antigen-DR isotype

MHC: Major histocompatibility complex

MIP-1β: Macrophage inflammatory protein-1-beta

NK: Natural killer

NNRTI: Non-nucleoside reverse transcriptase inhibitor

NRTI: Nucleoside reverse transcriptase inhibitor

PBMC: Peripheral mononuclear cells

PD-1: Programmed cell death-1

PI: Protease inhibitor

rpm: Revolutions per minute

TNF-α: Tumor necrosis factor alpha

VL: Viral Load

WHO: World health organization

Mg: Microgram

α: alpha

β: beta

γ: Gamma

µM: Micromal

µl: Microlitre

ºC: Degrees Celsius

mL: Millimetre

mm: Millimetre

mM: Millimetre

ng: Nanogram

x g: Times gravity

Ethics

The research study for this thesis is registered with the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and was approved by this committee (BREC reference number: BE429/15).

The academic leader for Research at the School of Laboratory Medicine and Medical Sciences, College of Health Sciences of the University of KwaZulu-Natal granted approval of the research project towards a PhD degree.

Abstract

Introduction

Antiretroviral therapy (ART) during pregnancy may be associated with an increased risk of adverse pregnancy outcomes, including preterm delivery (PTD) and small-for-gestational-age (SGA) but the underlying biological mechanisms remain unclear. Immune activation as well as the use of ART have been associated with adverse outcomes during pregnancy. We explored the association between adaptive and innate immune cell activation markers ex vivo in HIV-infected women initiating ART during or before pregnancy with PTD or SGA.

Materials and methods

Study participants were women living with HIV drawn from the PIMS cohort, based in Cape Town South Africa and initiated ART during pregnancy or conceived while on ART. Participants were enrolled at median 15 week's gestation; and were analyzed for immune markers, matched on ART initiation timing (15 women initiated pre- and 15 during pregnancy). There were 30 PTD (delivery <37 weeks), 30 SGA (weight for age $\leq 10^{th}$ centile) cases and 30 controls (term, weight for gestational age $>25^{th}$ centile) as outcomes. Immunological parameters were compared T cell activation, antigen presenting cell subsets, activation and function, regulatory T cell phenotypes and functions and plasma cytokine profiles.

Results

We found that $CD8^+$ T cell, monocyte and dendritic cell activation were lower in PTD women initiating ART in pregnancy when compared to SGA cases and AGA controls over time. Classical ($CD14^+CD16^-$) and intermediate ($CD14^+CD16^+$) monocyte frequencies were higher in PTD than in SGA cases and AGA women initiating ART in pregnancy compared to those stable on ART. There was lower inflammatory monocyte ($CD14^{dim}CD16^+$) frequencies over time. Monocytes and mDCs but not pDCs showed higher levels of activation in patients initiating ART compared to those stable on ART. A lower activation of APCs (monocytes,

mDCs and pDCs) was associated with the PTD outcome. When APCs were stimulated with TLR ligands, a lower IFN-α production by monocytes following TLR4 was associated with PTD. A similar trend was also observed for TLR9 and TLR7/8 stimulation at some time points. Some plasma cytokine levels were higher in participants initiating treatment in pregnancy compared to those stable on ART but there was no link of cytokine levels with birth outcomes. Regulatory T cell frequencies did not differ between ART initiators and those stable on ART, did not change over the course of pregnancy and were not associated with pregnancy outcomes.

Conclusion

Overall, we noted that lower levels of monocyte activation and reduced functionality (IFN-α production) of monocytes in response to TLR stimulation were associated with PTD. A similar trend of reduced production of MIP-1β and TNF-α by monocytes was noted for PTD cases. This suggests that reduced responsiveness to antigen stimulation may be an underlying factor for PTD, especially for women initiating ART in pregnancy. The markers of immune activation described here may be potential biomarkers to identify women at risk for PTD. Our results also suggest that PTD and SGA have distinct underlying immunological determinants that warrant further investigation.

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Chapter 1 Introduction to the history, epidemiology, and immune responses to HIV type-1 in pregnant women.

1.1 The global pandemic of HIV

Thirty-eight years after its discovery, HIV remains a public health threat having claimed the lives of 32.7 million people (UNAIDS, 2020b). Since the beginning of the epidemic, an estimated 75.7 million people have been infected with the UNAIDS report estimating that there were 38 million people that are living with HIV in the year 2019, a 0.7% global prevalence (Figure 1.1) (UNAIDS, 2020b). Progress on the prevention of HIV transmission remains slow as there is an estimated 1.7 million people who acquired HIV worldwide in 2019 which marked a 23% decline in new HIV infections since the year 2010, although that was more than three times higher than the milestone of 500 000 that was set for 2020 (UNAIDS, 2020a). Sub-Saharan Africa (SSA) remains the region most affected by the HIV epidemic (UNAIDS, 2020b), where 20.7 million of the people infected worldwide reside (UNAIDS, 2017b). Swaziland has the highest adult prevalence rate of 26.2% followed by South Africa, Mozambique and Namibia at 18.6 %, 11.3% and 12.3% respectively (UNAIDS, 2020b). Women represent half (51%) of all adults living with HIV worldwide. Consequently, HIV has been found to be the leading cause of death among women of reproductive age (UNAIDS, 2015). Three in five new infections in 2019 were among women, and the incidence of HIV infections among adolescent girls and young women (aged 15 to 24 years) remains inordinately high: they are 2.5 times more likely than their male peers to acquire HIV infection. In sub-Saharan Africa, young women between ages of 15-24 account for 24% of all new HIV infections among adults, even though they represent only 10% of the adult population whereas women and girls of all ages accounted for 59% of new HIV infections in sub-Saharan Africa (UNAIDS, 2020a).

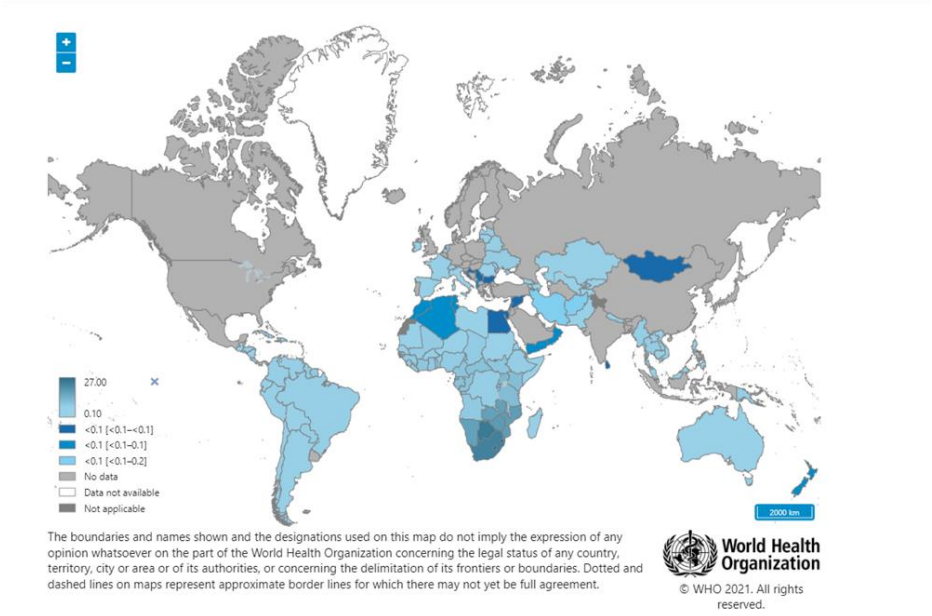

Figure1.1: Prevalence of HIV among adults ages 15 to 49 by country in 2019 ((UNAIDS, 2020b))

1.1.1 HIV prevalence in South Africa

South Africa has the biggest HIV epidemic in the world, with an estimated 7.5 million people living with HIV in 2019 (UNAIDS, 2019). It accounts for a third of all new HIV infections in southern Africa. In 2019, there were 200,000 new HIV infections and 72,000 South Africans died from AIDS-related illnesses(UNAIDS, 2019). The prevalence of HIV remains high among the general population at 20.4%, but prevalence varies markedly between regions. For example, HIV prevalence is 27% in KwaZulu-Natal compared with 12.6% in the Western Cape(Avert, 2020). There are several target groups that are most affected by HIV infection in South Africa. Women and adolescent girls are the most affected, with HIV prevalence among young women in South Africa being nearly four times greater than that of men their age (Avert, 2019). Approximately one-fifth of South African women in their reproductive ages (15 and 24 years) are HIV positive and made up the majority of new infections in 2017 (HSRC, 2018). Factors such as poverty, the low status of women and gender-based violence (GBV) have been cited as reasons for the disparity in HIV prevalence between genders. Indeed an estimated 20–25%

of new HIV infections in young women have been attributed to GBV (Van Damme et al., 2008, Feucht et al., 2007). In an effort to reduce these rates, young women and adolescent who are at high risk during their high risk season are now being offered pre-exposure prophylaxis (PrEP) (Avert, 2019).

1.2 HIV structure and life cycle

1.2.1 HIV classification

The human immunodeficiency virus is a lentivirus which is a subgroup of retrovirus family that leads to acquired immunodeficiency syndrome if left untreated. The classification is based on the morphology, genetic and biological properties of the virus. Particularly, HIV is enveloped, contains reverse transcriptase and comprises of two identical copies of positive sense, linear ribonucleic acid (RNA) genome (Garry, 1989, Levy, 1993). Two types of HIV have been described thus far; HIV-1 (Barre-Sinoussi et al., 1983, Gallo et al., 1983) and HIV-2 (Clavel et al., 1986). HIV-1 is responsible for the global epidemic compares to HIV-2 which is mostly geographically restricted to West Africa and is known to be less pathogenic and less transmissible. HIV-2 infected patients have survived longer when compared to HIV-1 infected patients, with lower transmission rates and viral loads, and a slower $CD4^+$ T cell decline is seen in HIV-2 infected patients (Jaffar et al., 1997, Marlink et al., 1994, Whittle et al., 1994, Esbjörnsson et al., 2019, Vidya Vijayan et al., 2017).

1.2.2 HIV structure

HIV is a retrovirus, as such it is made up of RNA as its genetic material. Each virion contains two positive strand copies of the viral genome containing a 5’ cap and a 3’ poly(A) tail (Steckbeck et al., 2013). HIV is an irregular, roughly spherical virus with a characteristic electron dense conical core surrounded by a lipid envelope that is derived from the host cell

during the budding process (Figure 1.2) (Steckbeck et al., 2013, Luciw et al., 2002). The outer membrane is comprised of the lipid bilayer where the surface glycoprotein (gp120) is anchored through its interaction with the transmembrane glycoprotein (gp41) (Turner and Summers, 1999, Wang et al., 2000). Host cellular proteins such as the major histocompatibility antigens, actin and ubiquitin also contribute to the formation of the outer membrane (Arthur et al., 1992). The inner membrane is made up of the matrix proteins (p17 or MA). While the nucleocapsid protein (p24 or CA) encompasses the two RNA molecules which are anchored in a ribonucleoprotein complex formed by several nuclear capsid proteins (p7 or NC), integrase (p13 or IN) reverse transcriptase (p66/p51 or RT) and protease (p11 or PR) (Freed, 1998).

Upon entry into the host cell, the RNA undergoes reverse transcription forming complementary DNA by the viral reverse transcriptase, which is contained in the infecting virion. The DNA is then transported into the nucleus with host proteins forming part of a pre-integration complex and integrated into the host genome with the help of another viral enzyme integrase and other cellular factors. The viral DNA is now able to function as a template to produce many copies of the virus in the host cell (Freed, 1998, Smith and Daniel, 2006).

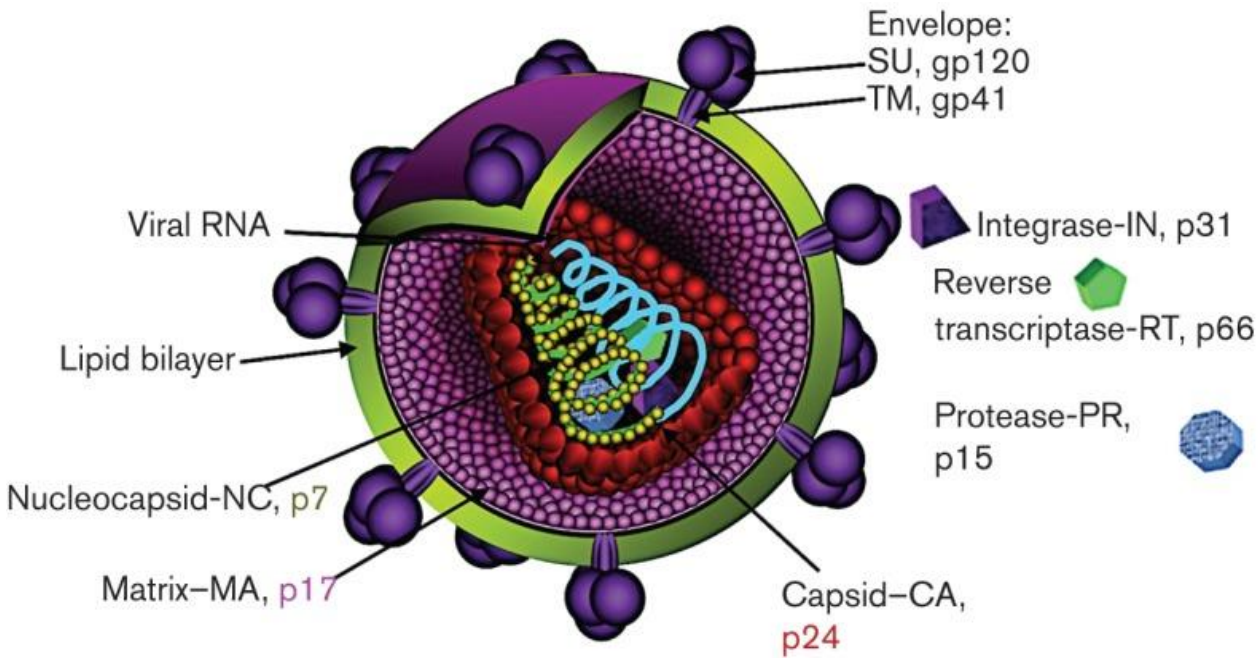

Figure 1.2: HIV virion structure. Schematic representation of a mature HIV virion detailing the localization of viral proteins and the approximate virion structure (Steckbeck et al., 2013).

1.3 HIV infection

When HIV encounters the target host cell, viral replication will occur where infection and further production of the new virions takes place. For viral replication to successfully happen, a number of steps occur: virus attachment, fusion and entry, uncoating and reverse transcription, importing of the pre-integration complex into the nucleus, integration, transcription and translation, assembly and budding and finally maturation (Sakuma et al., 2012). As shown in figure 1.3, upon cell entry capsid proteins are uncoated, resulting in the release of RNA genome and viral enzymes (RT, IN and PR). The positive sense RNA (green) is converted by RT into double-stranded DNA (dark pink) in the cytoplasm and imported into the nucleus followed by integration into the host genome. After transcription, viral mRNAs are processed by cellular machinery. During the early viral life cycle, only fully spliced viral mRNAs (i.e. tat, nef and rev) can be exported from the nucleus to the cytoplasm. After Rev is synthesized, the Rev protein is imported into the nucleus, and singly spliced (i.e. vif, vpr, vpu and env) and unspliced (i.e. gag and gag-pol) mRNA, which contains RRE

as a cis-element, are exported from the nucleus through interaction with the Rev protein. Once the viral mRNAs are synthesized (most viral proteins are synthesized in the cytosol and Env proteins are synthesized through the endoplasmic reticulum), viral genome and proteins are assembled at the plasma membrane. New HIV particles are then released from the host cell. Immediately after virus budding, the multimerization of Gag and Gag-Pol activate the viral PR, which leads to the structural rearrangements and gives rise to the mature infectious virions (Sakuma et al., 2012).

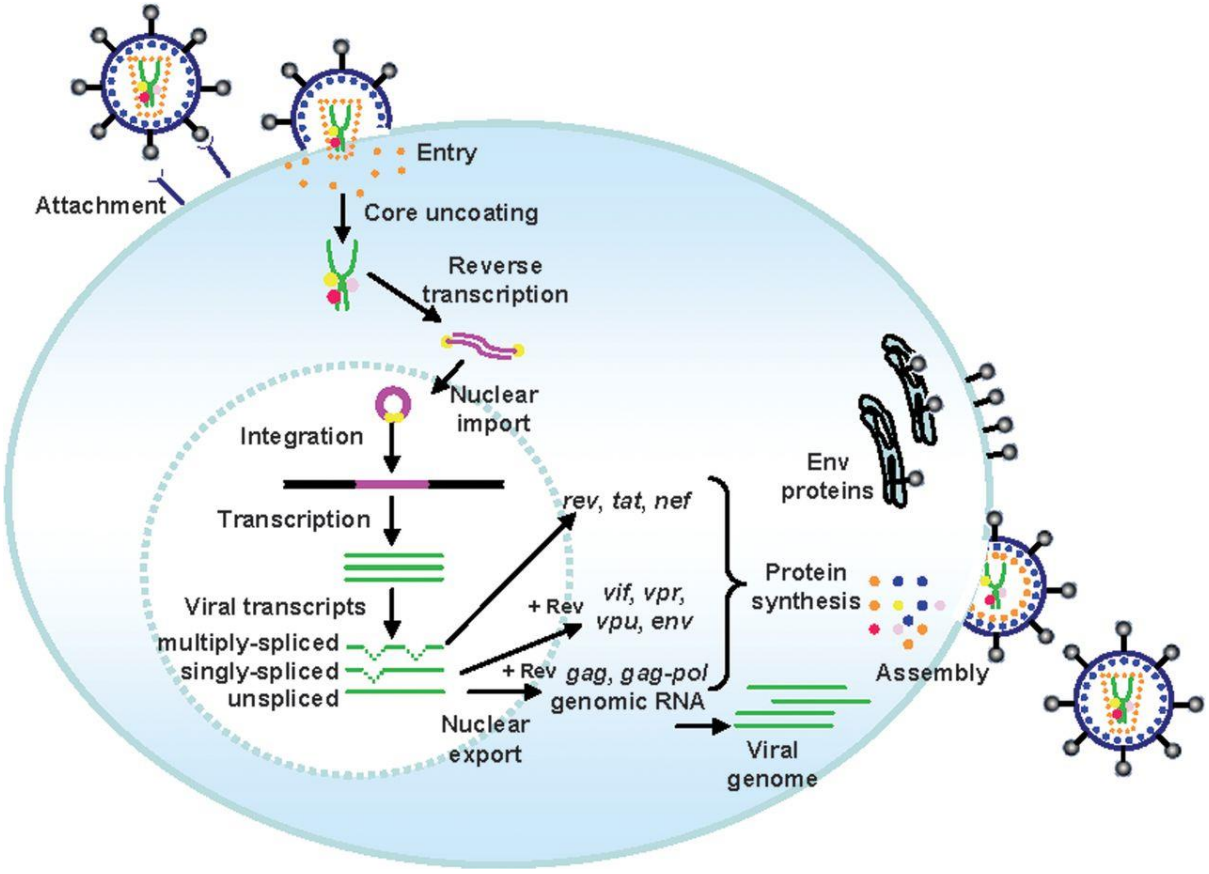

Figure 1.3: Schematic diagram of the HIV-1 life cycle.

1.4 HIV transmission

HIV is known to be transmitted sexually, perinatally, and parenterally (Berkley, 1991, Cohen, 2016). The transmission of HIV-1 results from virus exposure at mucosal surfaces (the genital

and rectal mucosa) or from percutaneous inoculation (Shaw and Hunter, 2012, Mayer and de Vries, 2018). Transmission occurs via direct transfer or exchange of bodily fluids. The relative percentage of HIV infection caused by each of these routes depends upon the prevalence of infection among groups of the population and on their shared behaviors. Heterosexual transmission is responsible for nearly 70% of HIV-1 infections worldwide with the remainder largely attributable to men who have sex with men (MSM), maternal-infant infection, and injection drug use (Shaw and Hunter, 2012). Due to the fact that it is difficult to directly analyse such exposures in humans; studies of HIV-1 epidemiology, viral and host genetics, risk factor and behavior analyses, animal models, human explant tissues, and in vitro studies of virus-target cell interactions have aided in better understanding of transmission events (Shaw and Hunter, 2012).

1.4.1 Sexual transmission of HIV

Sexual transmission is by far the most common mode of HIV transmission globally. The likelihood of one being infected with HIV is primarily dependent on sexual behavior. Chances of HIV infection vary greatly and depend on a number of factors. Male to female penile-vaginal transmission appears to be 2–3 times more efficient than female to male transmission and there is some evidence that first sexual intercourse for females may be associated with particularly high transmission probabilities (Carias and Hope, 2019, Boily et al., 2009). Receptive anal intercourse appears to be more risky than receptive vaginal intercourse with implications for spread amongst MSM (Morison, 2001).

Transmission can only occur when HIV contained in one of the bodily fluids enters the body of another person. This generally occurs when the virus encounters the other person's mucosal membranes, for example the membranes that line the vagina or rectum, though it can also occur through breaks in the skin. The sexual transmission of HIV from one person to another requires

four conditions: a fluid known to transmit HIV—in the case of sex the fluids are blood, semen (including pre-ejaculate), vaginal and anal fluids; the fluid makes contact with an area of the body—a mucosal membrane lining the vagina, rectum or parts of the penis, a lesion or a break in the skin through which transmission can occur; entry into the body of sufficient virus to establish infection; and an initial infection within immune cells of the mucosal membranes is established and a subsequent spread of the infection to other immune cells in the body (Powers et al., 2008, Boily et al., 2009).

1.4.2 Parenteral transmission of HIV

Parenteral transmission occurs when an infectious agent is acquired into the body through other methods besides the gastrointestinal (or enteral) route, examples include subcutaneous, intravenous, intramuscular, and via intrasternal injections (Berkley, 1991, Carias and Hope, 2019). Parenteral transmission of HIV occurs most commonly amongst injecting drug users (IDUs) when needles are shared. The transmission is due in part to the fact that before injecting, IDUs draw back on the syringe plunger, pulling blood into the syringe to verify that the needle is in a vein (McCoy et al., 1998).

1.4.3 Perinatal transmission of HIV

Since the beginning of the epidemic, an estimated 5.1 million children world-wide have been infected with HIV (Clark, 2006). Mother-to-child transmission (MTCT) is believed to be responsible for more than 90% of these infections. Mother-to-child transmission of HIV infection is defined as transmission of HIV from an infected mother to her child during gestation, labour, or postpartum through breastfeeding all of which is more likely to take place if the mother has a high viral load (Teasdale et al., 2011, John and Kreiss, 1996). How and when transmission occurs is different depending on mode; for example in non-breastfed

infants, the late in utero and intrapartum period appears to be the time during which most vertical transmissions occur whereas transmission that occurs via breastfeeding has been found to be related to the duration of breastfeeding (John and Kreiss, 1996).

During the past two decades, remarkable progress has been made in the risk reduction of perinatal HIV-1 transmission. Knowledge about the timing of HIV-1 transmission to infants has allowed the development of appropriate interventions. The risk of HIV-1 transmission to the infant is about 25% at delivery in the absence of interventions, with most of the risk arising after 36 weeks and especially intrapartum (Kourtis et al., 2006). HIV-1 transmission happens at a rate of 8.9 per 100 child-years of breastfeeding after the fourth week, with higher rates during the first 4 weeks (Lewin and Rouzioux, 2011). Mixed feeding roughly doubles the risk of HIV-1 transmission compared with exclusive breastfeeding (Kourtis et al., 2006). Prolonged breastfeeding is the norm in most resource-poor settings, where the risk of transmitting HIV-1 to children reached about 40% without interventions. Recommended mother-to-child HIV-1 transmission interventions have resulted in a tenfold reduction in this risk, and complete elimination of mother-to-child HIV-1 transmission is now considered feasible (Vrazo et al., 2018).

1.5 Prevention of mother-to-child transmission (PMTCT)

Antiretroviral therapy is the mainstay of prevention of mother-to-child HIV-1 transmission. Antepartum zidovudine monotherapy, augmented with one dose of nevirapine during labour, was an effective and affordable intervention, but was then superceded by standard combination antiretroviral therapy for women who did not qualify for continuing antiretroviral therapy at the time when universal treatment guidelines had not been established. It is now evident that

combination antiretroviral therapy is more effective at prevention (de Vincenzi, 2011), and has the additional advantages of reducing sexual HIV transmission and reducing HIV-associated morbidity and mortality and is now recommended for all HIV infected persons. In resource-limited settings the reduction in infant HIV transmission by formula feeding is offset by a higher infant mortality (Thior et al., 2006), which shows the crucial role that breastfeeding has in child health. As of September 2015 WHO released guidelines recommending that all pregnant women living with HIV be immediately provided with lifelong treatment, regardless of CD4 count (WHO, 2013, WHO, 2015, UNAIDS, 2016, UNAIDS, 2017c).

It is important to note that ART alone would not reach the goal of elimination of prevention of mother-to-child HIV-1 transmission. Access to antenatal care, HIV testing, and mother-to-child HIV-1 transmission interventions needed to be substantially increased in regions with a high HIV prevalence (Wettstein et al., 2012). In Africa, the uptake of mother-to-child HIV-1 transmission interventions was improved if the male partner was involved, but this is often difficult to achieve (Wettstein et al., 2012). It is common that in low and middle-income countries, many women present late for antenatal care or deliver without having had antenatal care. This then poses a challenge with regards to the timely prevention and therefore efficiency of transmission. A further challenge to the implementation of antiretroviral therapy for mothers to prevent transmission from breastfeeding is that antiretroviral therapy adherence post-partum has been found to be significantly lower than antepartum as is reported in a recent meta-analysis (Nachega et al., 2012). The uptake of ART is working because between 2009 and 2013, the MTCT rate reduced from 28 to 18% in sub-Saharan Africa (Endalamaw et al., 2018). In 2016, the rate of vertical transmission of HIV is estimated at 1.4-5.9%, with an average of 3.5% (Psaros et al., 2015).

1.5.1 PMTCT in South Africa

In the past few years, South Africa has made great progress in reducing mother-to-child transmission of HIV due to the improvements in the choice of ART and the widespread accessibility of the PMTCT program. In the year 2016, more than 95% of HIV-positive pregnant women received ART to reduce the risk of MTCT (UNAIDS, 2017a). As a result MTCT rates have fallen from 3.6% to 1.5% between 2011 and 2016, achieving the national target for 2015 of a transmission rate below 2% (SANAC, 2016). A study that was conducted with the aim of reviewing the national-, provincial- and district-level birth HIV testing data from routine National Health Laboratory Services (NHLS) in South Africa records between April 2016 and March 2017, found that the *in utero* mother-to-child transmission (MTCT) rate was 0.9% (Goga et al., 2018). WHO guidelines, adopted by South Africa recommend combination antiretroviral therapy (cART) for life for HIV-1-infected pregnant women with a CD4 count <350 cells/mm^3, and cART during pregnancy and breastfeeding for women with a CD4 count ≥350 cells/mm^3 (Mutevedzi et al., 2010, DOH, 2014). In 2013, the fixed-dose combination pill (FDC) made up of the regular three drugs used in the first-line regimen (TDF, FTC/3TC and EFV) was introduced to improve adherence and retention (NDOH, 2015). South Africa is on track to eliminate MTCT as the number of children born with HIV has now fallen to below 6,000 in 2015 (SANAC, 2016). The ultimate goal is that of zero transmissions and in order to succeed at this, the focus has to be on getting mothers to adhere to treatment throughout breastfeeding as well as during pregnancy and birth (SANAC, 2017). Together, the PMTCT and ART programs have ensured that nearly all children born to HIV-1-infected women are themselves uninfected, although they are all exposed to antiretroviral drugs during fetal and early infant life, an important time in their development, with little data as to the potential consequences in the short- and medium-term for them and their families.

1.6 Antiretroviral therapy

The early 2000s saw an advancement in ART roll out in most countries due to changes enforced by the World Health Organization (WHO, 2013). Most international guidelines, including those for low-income and middle-income countries then increased the CD4 criterion for initiation of antiretroviral therapy to 500 cells per μL or lower (Sabin et al., 2013). There was increased access to ART and it was demonstrated that ART could reduce HIV transmission. In sub-Saharan Africa, 57% of people were expected to complete eligibility assessment for antiretroviral therapy, and only 65% of people who start treatment remain in care after 3 years (Kranzer et al., 2012, Dwyer-Lindgren et al., 2019).

1.6.1 ART program in South Africa

South Africa currently has the largest ART program in the world. In April 2018, more than 4.4 million people were receiving ART, which equates to 61% of people living with HIV in the country (UNAIDS, 2018). Early on it was the Western Cape that was at the forefront of ART distribution with the help of several projects that had started in the province. The first such project began providing ART in Khayelitsha in May 2001 followed by a project in Gugulethu in September 2002 (Boulle et al., 2008). The turnaround for the South African ART program was seen in 2008, and the dire projections that had been envisioned for the HIV positive in the country was turned around as the number of people on ART increased from 366,000 in 2007 to 1.72 million in 2011. In 2016, in keeping with the changing WHO guidelines, South Africa implemented ‘test and treat’, which meant that every individual with a positive diagnosis was eligible to start antiretroviral treatment increasing the number of people eligible for treatment from 3.39 million in 2015 to 7.7 million in 2018 (UNAIDS, 2018). In 2018, 62% of all HIV positive patients were receiving ART (Figure 1.4), this has risen to 71% in 2019 (Avert, 2020, UNAIDS, 2020b).

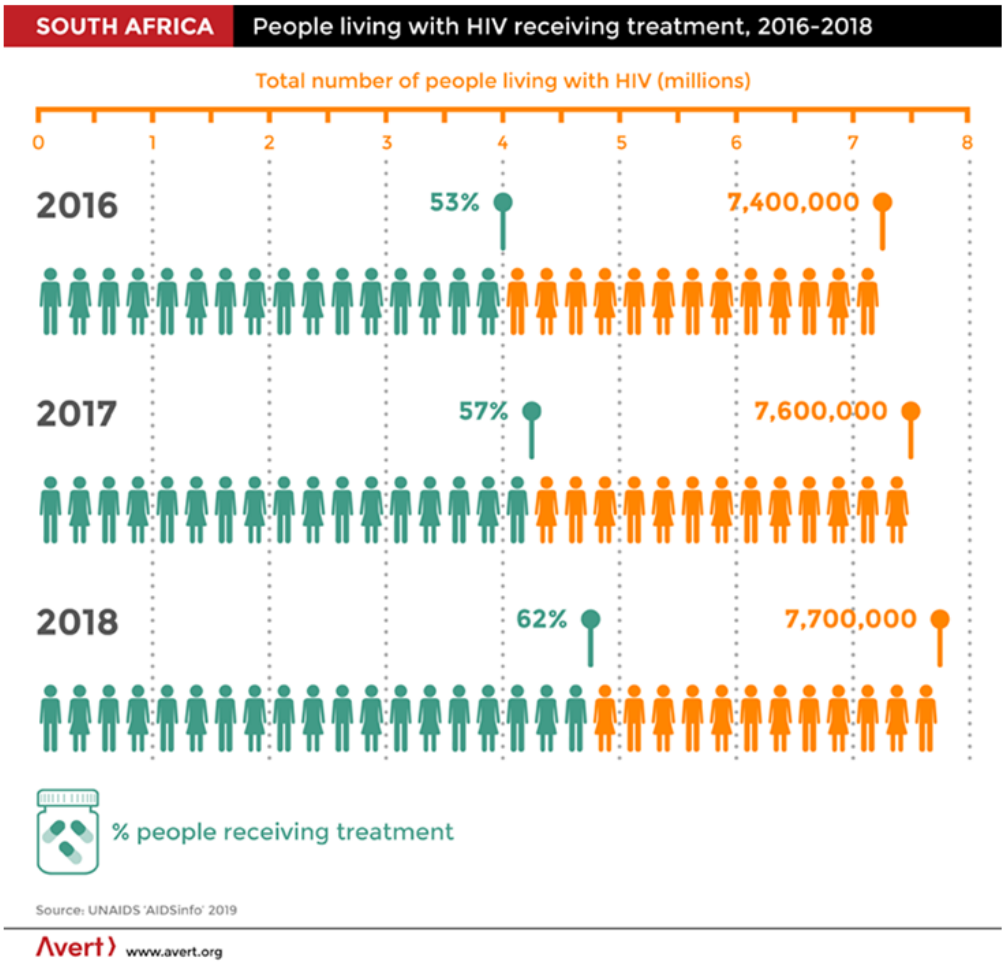

Figure 1.4: Antiretroviral treatment in South Africa (Avert, 2020)

1.7 Adverse effects associated with ART use

To date, there is no cure or vaccine for HIV. The aim remains to treat HIV such that viral replication is maximally suppressed and a plasma viral load below the level of detection is maintained with the use of ART (Ghosn et al., 2018). ART does not eliminate non-replicating provirus; therefore, lifelong ART is mandatory to ensure complete viral suppression. An ART regimen can be deemed as successful if it is able to suppress viral replication without resistance, minus associated costs, which may include toxicity, long-term adverse effects, drug interaction risk, and impact on quality of life (Ghosn et al., 2018).

There exist large knowledge gaps about the safe use of ART in pregnancy. Pregnancy-specific safety data is not common in the literature, given the dependence on observational data. It is

known that untreated HIV infection in pregnancy is associated with increased risk of various adverse birth outcomes, including preterm birth, low birthweight, babies born small for gestational age, and stillbirth, with the highest risk seen among women with advanced HIV disease or immunosuppression (Powis et al., 2011). It is important then to highlight that although ART reduces the risk of adverse birth outcomes associated with poor maternal health or infant HIV infection, cART can simultaneously increase the risk, particularly of preterm birth, via mechanisms that are not completely understood (Bailey et al., 2018). Some proposed mechanisms are known to account for these outcomes. These include an ART-induced disruption or reversal of the shift from T-helper type 1 to T-helper type 2 cells which is a normal feature of pregnancy, resulting in predisposition to early labour, and an increase in the inflammatory response following ART initiation, possibly as a result of immune reconstitution (Short and Taylor, 2014).

1.8 Gestational age

The accurate dating of pregnancy is critically important for pregnancy management from the first trimester to delivery, and is particularly necessary for determining viability in premature labor and in post-dates deliveries (Henry, 2012). In the past, the common means of determining gestational age relied on estimates using clinical dating, particularly based on menstrual history. It is worth mentioning that even if the menstrual history is correct; the exact time of ovulation, fertilization, and implantation cannot be known (Butt et al., 2014). Another method is that of dating based on clinical examination where the size of the uterus, estimated through pelvic or abdominal examination, can be roughly correlated with gestational age; however, factors that affect uterine size (such as fibroids) and maternal body characteristics (such as obesity) will affect such an estimate (Butt et al., 2014). The third method is that of gestational age determination based on ultrasound. Ultrasound biometric measurements determine

gestational age based on the assumption that the size of the embryo or fetus is consistent with its age. Biological variation in size is less during the first trimester than in the third trimester. Ultrasound estimation of gestational age in the first trimester is therefore more accurate than later in pregnancy (Butt et al., 2014). As can be understood, the accurate determination of gestational age is required for many aspects of antenatal care. In the past, it was probably felt that a few days of inaccuracy was acceptable; however, emerging data suggests that a few days of inaccuracy can affect factors such as the performance of maternal serum screening, the assessment of post-dates pregnancy, and the subsequent induction of labour. Research in the years has shown that the use of ultrasound derived dates is the best method to determine gestational age for clinical use.

1.9 Fetal growth monitoring

Fetal growth is the primary method of assessment of fetal well-being and is an important determinant of health later in life. The monitoring of fetal growth is particularly important as the early detection of growth abnormalities may help to prevent fetal demise and manage perinatal complications. Abnormalities in fetal growth are diagnosed using criteria which includes low birth weight, macrosomia, small-for-gestational age (SGA) and large-for-gestational age (LGA) (Mayer and Joseph, 2013). To measure growth restriction, a few methods have been proposed and will be discussed below.

1.9.1 Symphysis fundal height

Used more in the surveillance of uncomplicated pregnancies, the symphysis fundal height (SFH) measurement is performed using a non-elastic tape to measure the top of the uterine fundus to the upper boarder of the symphysis pubis (Henry, 2012). This method is commonly

used and is inexpensive (Neilson, 1998). The measurement is then plotted on a customised growth chart to aid in estimation of the growth of the fetus. There are opposing views as to whether the method is useful and revelant when compared to for example abdominal palpitation (Neilson, 1998).

1.9.2 Ultrasound estimate of gestational age

To evaluate fetal growth, weight tables are used along with sonographically estimated fetal weight prenataly. It is important that the gestational age used is as accurate as possible, there has to be precise weight measurements and a weight curve that is a true representation of the population (Curran, 2019). The use of ultrasound imaging has therefore become essential for assuring correct gestational age and for fetal size assessment. Evidence is emerging at population level that use of ultrasound biometry increases the rate of detection of fetal growth restriction and the identification of those at increased risk of neonatal morbidity (Kiserud et al., 2017).

1.9.3 Estimated Fetal Weight Percentile (EFW)

After obtaining a reliable gestational age and best estimate of the fetal weight, a growth chart or weight table may be used to assign an EFW growth percentile as shown in Figure 1.5.

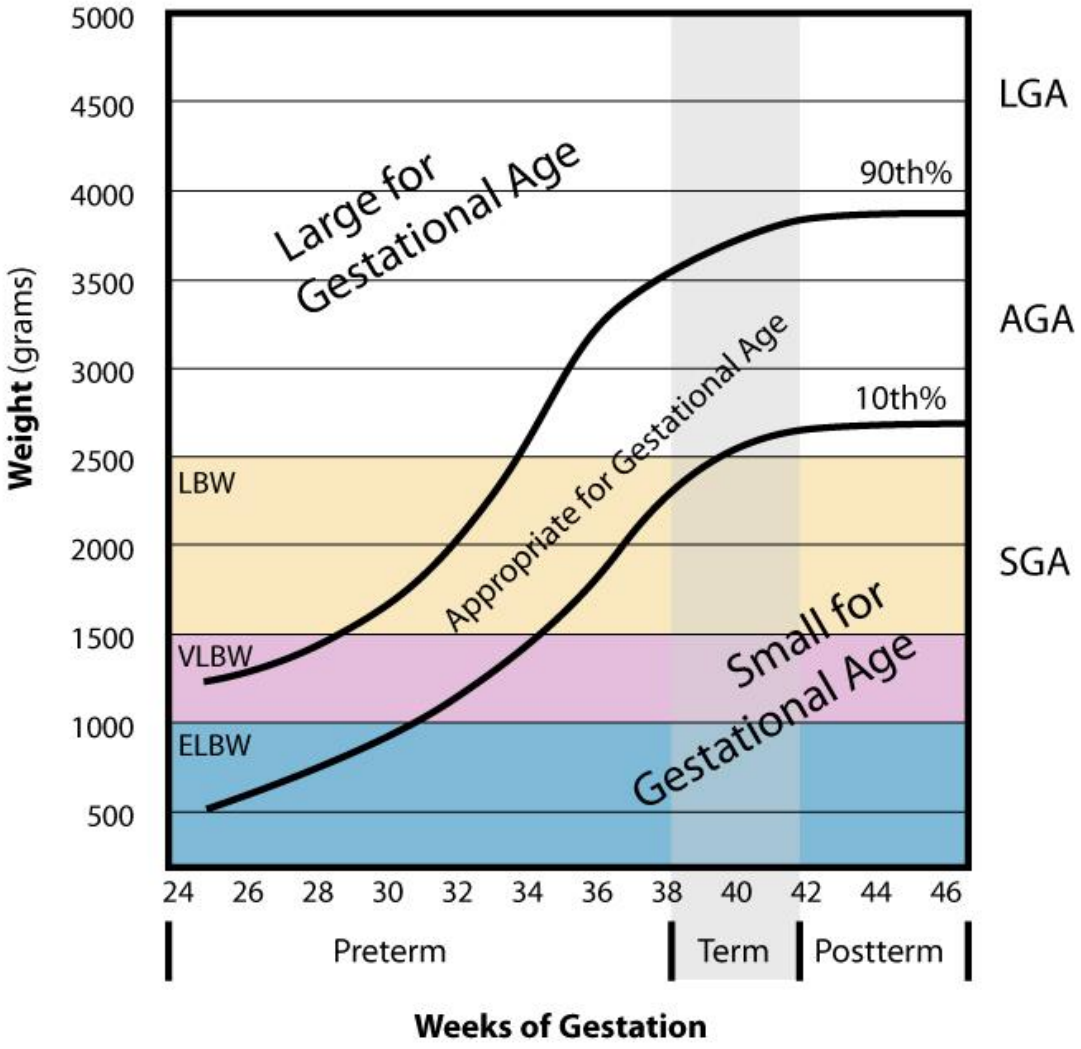

Figure 1.5: Gestational age and fetal growth abnormalities. Classification of size, SGA-Small for Gestational Age with a weight below 10th percentile. AGA-Appropriate for gestational age characterized by weight between 10 and 90th percentiles (between 2.5 kg and 4kg). LGA-Large for gestational age with a weight above the 90th percentile. Classification by gestational age: where Preterm birth is <37 weeks, full term is 37-40 weeks and postterm is >42 weeks

1.10 Association of cART with an increased risk of premature delivery

Combination ART was first associated with preterm birth in Europe in 1998. In subsequent studies, results have been different depending on ART regimen, timing of initiation, and setting. Protease inhibitors (PI) (particularly when boosted with ritonavir) have been widely implicated in observational studies. This was also seen in the MmaBana trial in Botswana, which showed a preterm birth rate of 21·4% among women randomised to receive zidovudine, lamivudine, ritonavir-boosted lopinavir at 26–34 weeks' gestation, versus 11·8% among those

randomised to receive zidovudine, lamivudine, and abacavir. In a study conducted in a facility-based public health program in Botswana; HIV-1-infected women, on either HAART or PMTCT, were at increased risk of adverse pregnancy outcomes, including stillbirth, premature delivery, small-for-gestational age infants and early neonatal death (Powis et al., 2011). This effect was driven by maternal HAART, which was found to be associated with an independent 1.2 to 1.8-fold increase in adverse pregnancy outcomes (Chen et al., 2012).

Although maternal HIV, or short-term ZDV PMTCT prophylaxis, has not been associated with significantly increased risk of preterm delivery (Ndirangu et al., 2012), there have however been reports from Europe and the USA of an increased risk of preterm delivery with HAART (Thorne, 2004b, Thorne and Newell, 2005, Townsend et al., 2010a). Reports from studies such as the MmaBana trial in Botswana and earlier work from the Ivory Coast also suggested an association between HAART and risk of preterm delivery/low birth weight (Ekouevi et al., 2011, Powis et al., 2011).

1.11 Immune activation and other possible PTD causes

The process of labor is identified by several clinical events. These include increased uterine contractility, cervical dilation, and rapture of the chorioamniotic membranes (Romero et al., 2014c, Romero et al., 2006). Preterm birth is thought to occur when a switch in the myometrium goes from an inactive to a contractile state. This process is thought to be accompanied by a shift in signaling, from an anti-inflammatory to pro-inflammatory pathway. A process that is inclusive of chemokines (IL-8), cytokines (IL-1 and IL-6) and contraction-associated proteins (oxytocin receptor, connexion 43, prostaglandin receptors). Progesterone maintains uterine quiescence by repressing the expression of these genes. In preparation for dilation, the cervix

ripens; this process is mediated by changes in extracellular matrix proteins. Including a loss in in collagen cross-linking, an increase in glycosaminoglycans, as well as changes in the epithelial barrier and immune surveillance properties (Mahendroo, 2012). All these activities decrease in the tensile strength of the cervix which is key for cervical dilation. An increased expression of inflammatory cytokines (TNF-α, IL-1) and chemokines, increased activity of proteases (matrix metalprotease MMP 8 and 9), dissolution of extracellular matrix components such as fibronectin, and apoptosis have been implicated in this process (Figure 1.6) (Romero et al., 2014b). The common assumption when treating preterm labor is that it is a single condition. There is however accumulating evidence that it is rather a syndromic occurrence, which has multiple aetiologies instead of the latter. Examples of the possible mechanisms as suggested by work by Romero et al are illustrated in Figure 1.6, worth highlighting is intra-uterine infection which has been linked to spontaneous preterm delivery. The idea is that preterm labor is a syndrome that is associated with multiple mechanisms of disease. There are a few other possible aetiologies of inflammation which is then thought to induce the labor process. One of the most defined is microbially induced inflammation. Studies have proven that the genital tract is able to be a conduit for infection. Inflammation is a key marker during the process where microbes and their products are sensed by pathogen recognition receptors such as toll-like receptors (TLRs). These then induce the production of chemokines, prostaglandins, and proteases, leading to the activation of the common pathway of parturition. Other causes are decidual hemorhage and vascular disease, decidual senescence, and the decline in progesterone action. In summary, the fetus is a semi-allograft, and tolerance is needed to maintain the pregnancy and the breakdown of this structure leads to a pathological state as shown in the figure below.

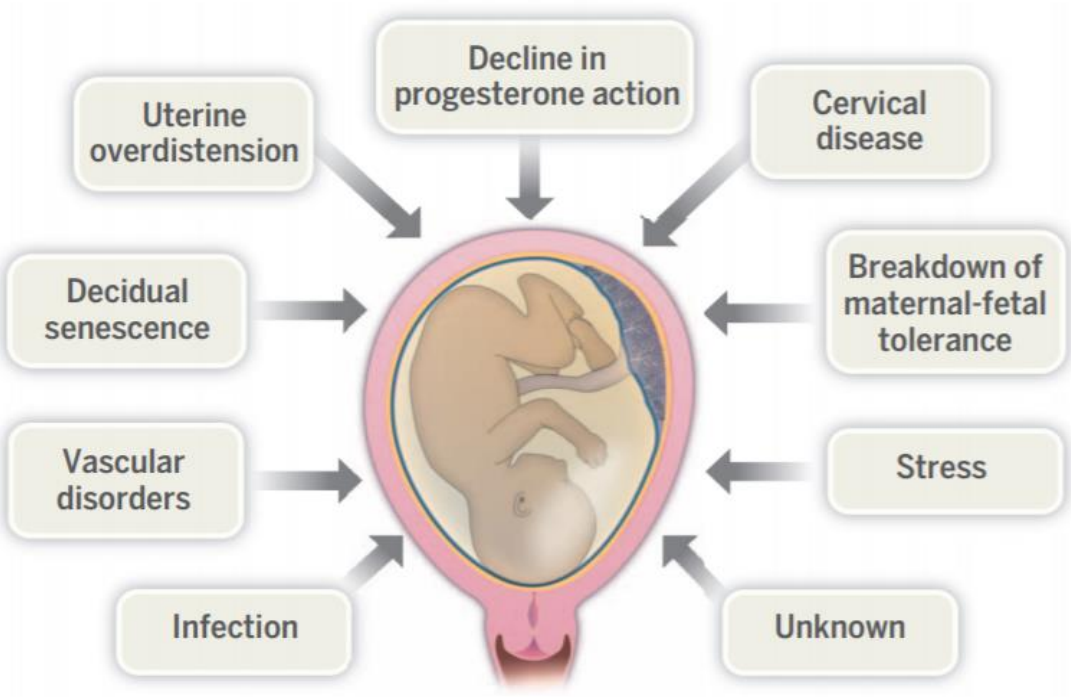

Figure 1.6: **Proposed mechanisms of disease implicated in spontaneous preterm labour**. Genetic and environmental factors are likely contributors to each mechanism.

1.12 Association of ART with an increased risk of SGA delivery

There exists some, although not much, data that addresses or describes a potential link between SGA and HIV infection, ART and PI-based regimens (Briand et al., 2009b, Chen et al., 2012, Ndirangu et al., 2012, Phiri et al., 2015, Parekh et al., 2011). Of the studies which exist, the data demonstrate mixed associations (Aaron et al., 2012, Briand et al., 2009b, Chen et al., 2012, Phiri et al., 2015). One study found an increased risk of SGA in HIV-positive women who were not receiving cART (Ndirangu et al., 2012). In a study conducted by Arron et al, (2013), the authors found that among a cohort of 183 pregnant HIV-positive women, comprising 117 women receiving PIs and 39 receiving NNRTIs, there were high rates of SGA. In this cohort, SGA was found to be associated with HIV disease severity, as well as with high rates of cigarette smoking (38%) and illicit drug use (35%). Women taking NNRTIs were less likely to

have SGA infants below both the 10th and the 3rd percentile (Aaron et al., 2012). Most of the women receiving NNRTIs (74.9%) initiated cART during pregnancy.

Chen *et al*. found that SGA occurred at an overall rate of 18% (AOR, 1.8; 95% CI, 1.6, 2.1) among their population of 9,504 pregnant HIV-positive women from Botswana. From this same cohort, SGA occurred at a rate of 26.1% among those women who had begun cART prior to pregnancy and continued with it during pregnancy (n = 2,851 on NNRTIs and n = 312 on PIs) (Chen et al., 2012). They also found a higher rate of SGA in HIV-positive women. A French study comprising 2,630 women on PIs and 508 on NNRTIs found no significant association between the type and duration of cART and the proportion of SGA infants (Briand et al., 2009a). This study however did not compare cART that was initiated either before or during pregnancy whilst Phiri *et al*. concluded that exposure to a PI during the first trimester of pregnancy lowered the risk of SGA in comparison to non-PI-exposure throughout pregnancy (Phiri et al., 2015).

The studies outlined above have involved NNRTI-based and PI-based regimens, and most found an association between initiation of NNRTI prior to conception and risk of SGA (Chen et al., 2012). In another study, most women, whether they were receiving NNRTI- or PI-based regimens, had viral suppression, and only 4.3% of all women had a CD4+ cell count below 200 cells/µl (Snijdewind et al., 2018).

One hypothesis for reduced fetal growth, resulting in SGA, may be placental insufficiency due to vascular damage caused by HIV infection or cART-triggered endothelial injury (Ronsholt et al., 2013). Whether a specific cART regimen reduces endothelial injury associated with HIV infection or actually contributes to further endothelial cell activation is still unknown (de Gaetano Donati et al., 2004).

Overall, the possible cause of adverse pregnancy outcomes on growth may be changes in the HIV-positive mother's cytokine profile due to the influence of cART (Chen et al., 2012). Chen *et al*. further comments that successful pregnancy maintenance and HIV infection are both associated with Th2 cytokine predominance and as cART acts to reverse Th2 to Th1 cytokine predominance, this conversion could result in the modification of cytokine levels and immune milieu that may influence pregnancy outcome.

1.13 Immunological changes during pregnancy associated with differential birth outcomes

Significant progress has been made in the understanding of the mechanisms underlying immunological changes during pregnancy, and their consequences for pregnancy outcome. It has been shown that mammals have evolved the ability to specifically expand the extrathymic differentiation of regulatory T lymphocytes (Tregs) to enforce maternal-fetal tolerance (Samstein et al., 2012), and adoptive transfer of regulatory T cells can reduce spontaneous abortion in mice (Yin et al., 2012). It has also been demonstrated that sex-hormones can modulate the responses of dendritic cells to stimulation, in particular in response to stimulation with Toll-like receptor ligands (TLRs), and furthermore changes in TLR signaling during pregnancy have a significant impact on pre-eclampsia (Panda et al., 2012, Berghöfer et al., 2006, Meier et al., 2009). However, little is known about the immune-related changes necessary for a successful pregnancy in women whose immune status is altered by HIV infection, and how HAART initiated during pregnancy affects these immune responses. Some reports suggest increased adverse pregnancy outcomes in HIV-1-infected women compared to uninfected controls, but whether that is an effect of HIV per se or of associated disease progression is unclear; a separate study found this effect to be largely explained by HAART use in the infected women (Chen et al., 2012, Rollins et al., 2007).

It could thus be hypothesized that reconstitution of immune responses under HAART in pregnant women will also result in enhanced immunity against the fetus and increases in preterm birth (most likely of a child appropriate for gestational age). If HAART in African populations was shown to be associated with increased preterm delivery risk, implications would be considerable for the mothers, their children and the health care system given the large numbers of infected women receiving HIV treatment. Overall, the complex relationship between pregnancy, HIV, host immunity, antiretroviral treatment, immune reconstitution, and preterm birth is poorly understood and needs urgent clarification.

1.14 Immune tolerance in term and pre-term labor

As mentioned earlier, pregnancy demonstrates the capabilities of the human immune system. The fetus, a semi-allogeneic graft, grows and develops within the mother without succumbing to immunological rejection, a process that depends on the proper establishment of fetomaternal tolerance. This tolerance is said to be initiated by the presentation of the paternal–fetal antigen from semen and is facilitated by seminal plasma factors. Antigen is processed by dendritic cells (DCs) and then presented to T cells in the uterine draining lymph nodes. As a result, antigen-specific regulatory T cells (Tregs) proliferate in order to create peripheral tolerance towards fetal antigens and allow conceptus implantation.

During late pregnancy, it is proposed that circulating maternal leukocytes (innate and adaptive) are recruited into reproductive tissues (cervix and myometrium) and to the maternal/fetal interface (decidual tissues) by chemotactic processes, (Gomez-Lopez et al., 2009, Gomez-Lopez et al., 2010, Gomez-Lopez et al., 2012) where a pro-inflammatory state develops and leads to labor and delivery of the baby (Romero et al., 1989, Romero et al., 2006, Gotsch et al., 2009). It is thought that the premature activation of this pro-inflammatory pathway can lead to

a breakdown of fetomaternal tolerance and play a role in the induction of labor, which subsequently can result in preterm birth (Törnblom et al., 2005).

1.15 Monocytes during pregnancy

During normal pregnancy, the circulation of peripheral blood through the placenta results in contact of maternal immune cells with the placenta. This may activate circulating immune cells, especially monocytes (Mellembakken Jan et al., 2002). In cases such as pre-eclampsia monocytes are highly activated together with other inflammatory cells such as granulocytes and endothelial cells (Faas et al., 2014). At the beginning of a healthy pregnancy, there is an increase of innate immune cells such as macrophages and NK cells at the maternal–fetal interface (Wallace et al., 2012). These macrophages and NK cells may have a local immune function; however, they also appear to be important for placental development by promoting trophoblast recruitment, spiral artery remodeling and angiogenesis. Monocytes and macrophages may thus play an important role in healthy pregnancy as well as in the pathophysiology of adverse outcomes. Further insight into the role of these cells in these conditions, may lead to a better understating of the inflammatory response in normal pregnancy as well as in adverse pregnancy outcomes.

Monocytes arise from precursors in the bone marrow and comprise about 5–10% of the circulating blood leukocytes. They circulate in the blood for a few days before migrating into tissues to become macrophages or dendritic cells (Gordon and Taylor, 2005). They have important roles in homeostasis, tissue repair, and inflammation and it has become clear that circulating monocytes are a heterogeneous population (Gordon and Taylor, 2005, Saha and Geissmann, 2010). In humans, monocyte subsets can be distinguished based on the expression of CD14, the lipopolysaccharide (LPS) receptor. The main subset (comprising about 90–95% of the monocytes) is a subset expressing high levels of CD14, but lacking CD16 (FcγR-III)

expression. Since this is the main subset and until recently thought to be the only subset, this subset is usually called “classical subset”. The second subset of monocytes is characterized by low expression of CD14 together with CD16. This subset is often called the non-classical subset. More recently, a third, intermediate subset of monocytes has been defined, called the intermediate subset (Ziegler-Heitbrock, 2015)

Classical monocytes are professional phagocytes that can generate reactive oxygen species (ROS) and produce cytokines in response to toll-like receptor (TLR) dependent activation by LPS. Non-classical monocytes are weak phagocytes and do not generate ROS, but are more efficient producers of pro-inflammatory cytokines after TLR dependent activation (Gordon and Taylor, 2005, Mukherjee et al., 2015). This subset has been shown to have a longer half-life and localize to both resting and inflamed tissue (Gordon and Taylor, 2005). These cells crawl on the luminal side of the endothelium and survey endothelial cells and tissues for damage and infection (Ziegler-Heitbrock, 2015). Upon damage or infection, they may rapidly invade the tissue and initiate the inflammatory response (Auffray et al., 2007). Non-classical monocytes have been shown to be increased in various inflammatory diseases (Ziegler-Heitbrock, 2015, Zimmermann et al., 2010)

In a normal pregnancy the female immune system must adapt to the presence of the semi-allogeneic fetus. Many changes in the peripheral circulation have been observed, both in the specific and innate immune response. In the specific immune response, a decreased Th1/Th2 ratio has been observed in both T cells as well as in NK cells (Borzychowski et al., 2005, Veenstra van Nieuwenhoven et al., 2002). These changes may be associated with changes in regulatory T cells and Th17 cells (Ernerudh et al., 2011, Saito et al., 2010). It has been suggested that the innate immune response also has to adapt to pregnancy in order to

compensate for such changes in the specific immune response. This adaptation has most often been shown by an increased numbers of circulating monocytes and granulocytes, resulting in increased number of total leukocytes during pregnancy (Veenstra van Nieuwenhoven et al., 2003). Some of these changes include an increase in leukocyte numbers as a sign of generalised inflammation and a phenotypical activation in these monocytes (Naccasha et al., 2001).

In relation to monocytes subsets, data has been contradictory. Some studies have found decreased numbers of classical monocytes and an increased number of intermediate monocytes in healthy pregnancy (Faas et al., 2014). These findings were in line with the suggestion that pregnancy is an inflammatory condition, since in other inflammatory diseases, this intermediate subset has also been shown to be increased (Moniuszko et al., 2009, Rossol et al., 2012). Another study however found increased numbers of classical monocytes and decreased numbers of non-classical monocytes in pregnant vs. non-pregnant women (Al-ofi et al., 2012).

1.16 Dendritic Cells (DCs) in term and preterm labor

DCs are specialized in antigen recognition and presentation. DCs exhibit properties that include induction of antigen-specific T-cell activation, T-cell suppression, Treg generation and peripheral tolerance (Banchereau and Steinman, 1998, Steinman et al., 2000). DCs contribute to fetomaternal tolerance during early pregnancy (Plaks et al., 2008). In mice, uterine DCs have a DC2 phenotype at 15 days post conception, which suggests that these cells contribute to the tolerogenic state by inducing a local anti-inflammatory (Th2) response during late gestation. The fact that immature DCs express the anti-inflammatory cytokine IL-10, a potential early biomarker of preterm birth, suggests that these cells may participate in the etiology of preterm labor (Blois et al., 2004, Ruiz et al., 2012). Further research is needed to establish a role for DCs during late gestation, labor and preterm labor.

The adaptive immune system creates memory and responds to specific antigens. During pregnancy, the adaptive immune limbs of both the mother and the fetus must tolerate each other to maintain pregnancy until term. A breakdown of this fetomaternal tolerance may lead to labor. In term pregnancy, lack of the tolerogenic state results in physiologic labor, however, a premature retreat of this tolerogenic state might lead to preterm labor (Gomez-Lopez et al., 2014).

1.17 T cells

During pregnancy, maternal T cells recognize fetal antigens through interactions with antigen-presenting cells (Bytautiene et al., 2004). Fetal antigen-specific T cells maintain fetomaternal immune tolerance across pregnancy (Rowe et al., 2012). It is proposed that maternal circulating T cells infiltrate into the maternal/fetal interface prior to delivery and during labor at term (Gomez-Lopez et al., 2009, Gomez-Lopez et al., 2011). It has also been noted that decidual T cells are activated and have both a regulatory and an effector phenotype (Sindram-Trujillo et al., 2003, Tilburgs et al., 2009a, Tilburgs et al., 2009b, Tilburgs et al., 2010). A breakdown of this fetomaternal tolerance may lead to labor. In term pregnancy, lack of the tolerogenic state results in physiologic labor but a premature retreat of this tolerogenic state might lead to preterm labor.

1.17.1 Effector T cells

There is evidence that decidual $CD4^+$ T cells are involved in term parturition (Gomez-Lopez et al., 2013). Specifically, decidual $CD4^+$ T cells are more abundant in term than in preterm

gestations without labor. These T cells express CD45RO, but not CD45RA, which suggests that they are memory cells that were generated early in pregnancy when fetal–antigen presentation occurs (Gomez-Lopez et al., 2013, Rowe et al., 2012). These decidual $CD4^+$ T cells express IL-1β, TNF-α and MMP-9 during spontaneous labor at term (Gomez-Lopez et al., 2013). Decidual T cells have been shown to express activation markers such as CD25 and labor mediators implicated in both term and preterm labor. This suggests that the adaptive arm of the immune system is active during labor (Gomez-Lopez et al., 2014, Sindram-Trujillo et al., 2004). During term labor T cells are preferentially recruited into the rupture zone of the fetal membranes by chemotactic processes facilitated by CXCL10 and CCL5 (Gomez-Lopez et al., 2011, Gomez-Lopez et al., 2012). However, T-cell attraction to the rupture zone is significantly diminished in premature ROM cases. These data suggest that T-cell recruitment into the maternal/fetal interface is required for term pregnancy, and the dysregulation of this recruitment may lead to pathological rupture of membranes.

Fetal T cells might also play a role during preterm labor. Memory fetal T cells ($CD45RO^+RA^-$) are present in higher proportions in cord blood from cases of preterm labor compared to term labor. These fetal T cells are also activated ($CD25^+CD69^+$) during preterm labor (Luciano et al., 2011). These results suggest that fetal T cells; in some cases, can contribute to the pathophysiology of preterm labor.

Cytotoxic T cells (CTLs) are present at the maternal/fetal interface in term gestations in the absence of labor, where they express perforin and granzyme B (Tilburgs et al., 2006, Tilburgs et al., 2009a). In placenta, CTLs are abundant in cases with villitis of unknown etiology and

express T-cell chemokine receptors (CXCR3 and CCR5) (Kim et al., 2009). In peripheral circulation, CD300a^{+} CTLs have an effector memory phenotype, and their proportion is higher in women with chronic chorioamnionitis than in women without this lesion (Xu et al., 2012).Taken together, these data suggest that CTLs may participate in pathological inflammation associated with preterm birth, but their role during spontaneous labor at term and preterm requires further exploration.

1.17.2 Tregs

There are two main Treg subsets: thymic Tregs (tTregs) and extrathymic or peripheral Tregs (pTregs). During pregnancy, CD4^{+}pTregs have been categorized into four subsets: DR^{high+}CD45RA^{-}, DR^{low+}CD45RA^{-}, DR^{-}CD45RA^{-} and naïve DR^{-}CD45RA^{+} (Steinborn et al., 2012). The proportion of each subset seems to be relevant in the pathophysiology of pregnancy complications such as preterm labor. Women with preterm labor have a reduced proportion of naïve DR^{-}CD45RA^{+} Tregs, accompanied by higher proportions of DR^{-}CD45RA^{-} and DR^{low+}CD45RA^{-} Tregs within their total pTreg pool (Steinborn et al., 2012, Schober et al., 2012b). Indeed, the suppressive activity of pTregs is strongly reduced in term and preterm labor (Schober et al., 2012b), which is correlated with a reduction in the expression of HLA-DR in preterm cases (Kisielewicz et al., 2010a). This suggests that the lack of suppressive function during late pregnancy could trigger the onset of parturition at term and preterm gestations (Gomez-Lopez and Laresgoiti-Servitje, 2012). At term pregnancy, Tregs are found at the maternal/fetal interface, have a unique phenotype (CD4^{+}CD25^{bright}FoxP3^{+}CD69^{+}HLA-DR^{+}CTLA-4^{+}), and exhibit suppressive function *in vitro* (Tilburgs et al., 2006, Tilburgs et al., 2008). However, the role of decidual Tregs remains

undetermined. Currently, we are investigating the function and phenotypic characteristics of these cells during term and preterm labor.

Several mechanisms of Treg suppression have been suggested (Jenabian et al., 2013). They do this by the secretion of inhibitory cytokines (IL-10, TGF-ß or IL-35), induction of apoptosis by IL-2 deprivation, perforin/Granzyme B or by CTLA-4 and GITR interactions pathways (Sakaguchi et al., 2010). Treg cells also use CD39 (nucleoside triphosphate diphosphorylase-1) and CD73 (ecto-5′-nucleotidase) for their suppressive activity. These ecto-enzymes hydrolyse extra-cellular pools of inflammatory ATP into adenosine diphosphate (ADP) and/or adenosine monophosphate (AMP) to adenosine (Figure 1.7) (Fausther et al., 2012, Pulte et al., 2007). Another known important physiological regulator of the immune response is extracellular adenosine, known to be an by inhibiting T cell proliferation and IFN-γ/IL-2 production and these effects are mediated through the adenosine-receptor A2A (A2AR) by stimulating the generation of intracellular cyclic AMP (cAMP) (Ohta et al., 2009). It has been recently shown that Treg inhibit HIV replication in conventional T cells through cAMP-dependent mechanisms (Moreno-Fernandez et al., 2011). A study Nikolova (2011) evaluated the impact of CD39/adenosine pathway in HIV pathogenesis and reported that expanded Treg/$CD39^+$ in infected patients correlate with immune activation and $CD4^+$ cell depletion. They showed that these Treg exerted a strong suppressive effect on effector CD8 T cell functions and these inhibitory effects were relieved by using an anti-CD39 monoclonal antibody (Nikolova et al., 2011).

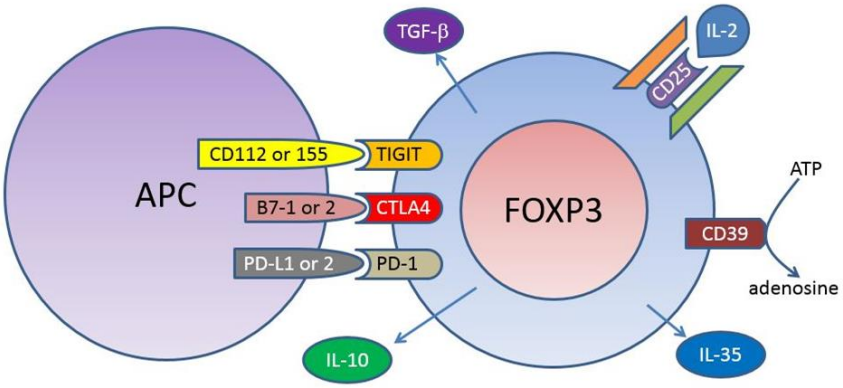

Figure 1.7: Proposed mechanism for Treg function. Tregs may mediate their inhibitory function through multiple soluble cell-surface factors. CTLA-4, TIGIT and PD-1 interact with costimulatory molecules on antigen presenting cells (APC). CD25 binds to T cell growth factor IL-2. CD39 converts local ATP to adenosine. The cytokines IL-10, IL-35 and TGF-βhave suppressive functions on nearby immune cells (Lord, 2015).

1.18 Immune activation

HIV infection is also characterized by a marked increase in immune activation, which includes both the adaptive and innate immune systems, and abnormalities in coagulation (Lichtfuss et al., 2011). The drivers for immune activation include the direct effect of HIV as a ligand for the Toll-like receptor (TLR-7 and TLR-8) expressed on plasmacytoid dendritic cells, leading to production of interferon-α (Meier et al., 2009); microbial translocation, with lipopolysaccharide as a potent activator or TLR-4 leading to the production of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor α (TNF-α); co-infection with viruses such as cytomegalovirus that induce profound expansion of activated cytomegalovirus-specific T cells; and a reduced ratio of T-helper-17 and regulatory T cells, especially in the gastrointestinal tract (Prendergast et al., 2010, Brenchley et al., 2006, Hsue et al., 2006). Evidence of residual inflammation or increased immune activation exists, even in

HIV infected patients with adequate CD4 T-cell restoration on antiretroviral therapy (Figure 1.8). Although many studies have identified associations between different biomarkers of inflammation and adverse clinical events, causation in studies in people has been difficult to establish.

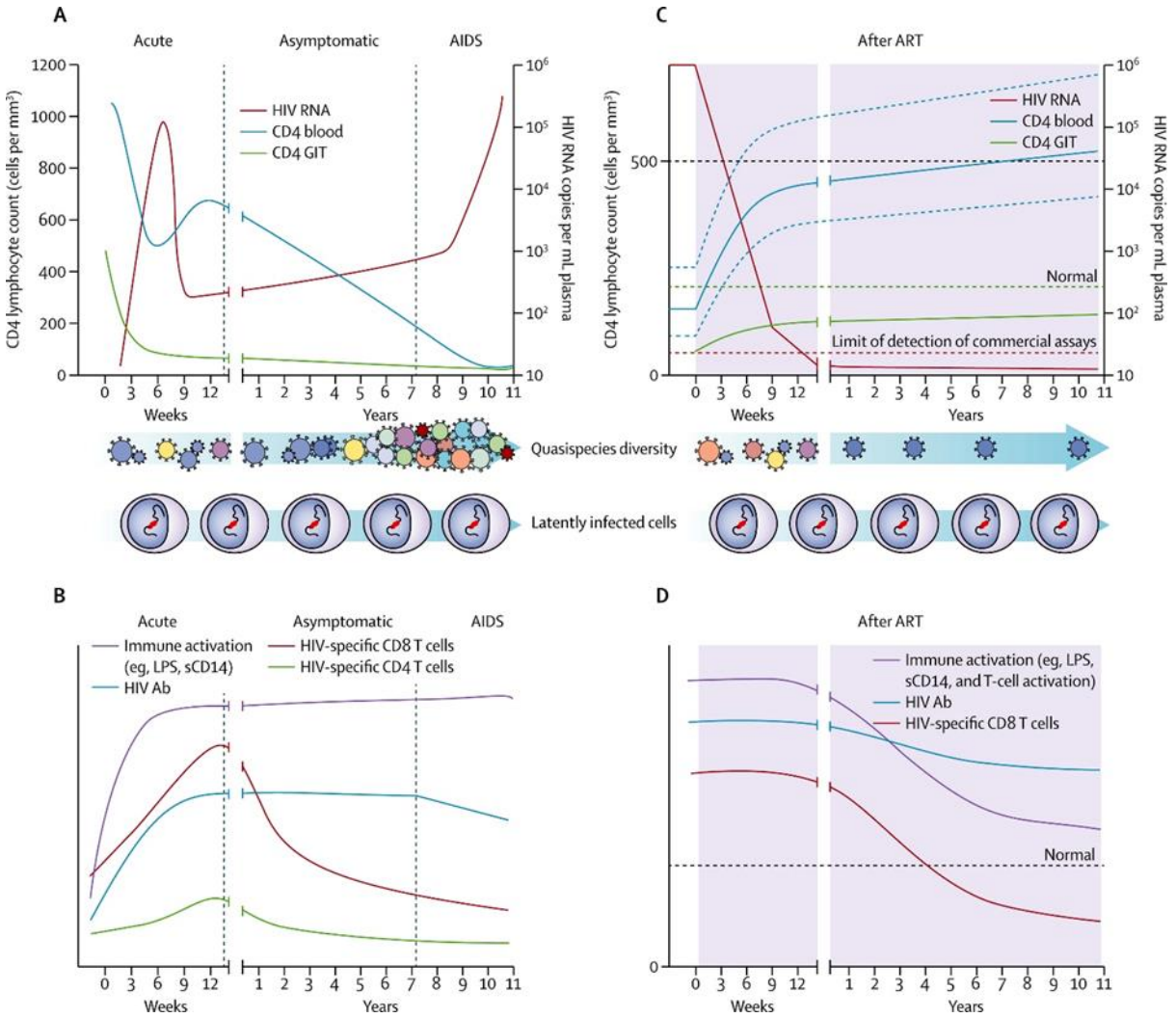

Figure 1.8: Natural history of untreated HIV infection and changes after antiretroviral therapy. (A) In untreated HIV infection, CD4 T cells are progressively lost in blood but CD4 T cells in the gastrointestinal tract are rapidly depleted early on. (B) The acute response to HIV infection includes a dramatic increase in markers of immune activation and production of non-neutralizing antibodies and HIV-specific CD4 and CD8 T cells that are associated temporally with a decrease in HIV RNA in blood. (C) After antiretroviral therapy, HIV RNA significantly decreases followed by recovery of CD4 T cells, which varies between individuals (panel). By contrast, recovery of CD4 T cells in the gastrointestinal tract is reduced. (D) With reduction of HIV RNA and viral antigen, HIV-specific T cells decrease after antiretroviral therapy, whereas antibody persists in all patients. Immune activation decreases after antiretroviral therapy but in most patients remains significantly increased compared with healthy controls. GIT=gastrointestinal tract. LPS=lipopolysaccharide (Maartens, 2014).

1.19 Immune reconstitution disease

Immune reconstitution disease, also called immune reconstitution inflammatory syndrome, is an immunopathological response resulting from the rapid restoration of pathogen-specific immune responses to pre-existing antigens combined with immune dysregulation, which happens shortly after initiation of antiretroviral therapy (Lawn and Meintjes, 2011). Most commonly, the antigens triggering immune reconstitution disease are from opportunistic infections, notably tuberculosis, cryptococcal meningitis and cytomegalovirus retinitis (Muller et al., 2010). Immune reconstitution disease is common, with an overall incidence of 16·1% reported in a systematic review (Muller et al., 2010). The disease causes high morbidity, but mortality is low (4·5%), except with cryptococcal meningitis immune reconstitution disease where upto 20·8% mortality has been reported (Muller et al., 2010). Immune reconstitution disease happens more commonly when antiretroviral therapy is started in patients with low CD4 counts or soon after starting treatment for the opportunistic infection. Strategies to reduce immune reconstitution disease include initiation of antiretroviral therapy at high CD4 counts, delayed initiation of antiretroviral therapy in patients with an infection (especially if infection includes the central nervous system (CNS), and screening for and prevention of opportunistic infections before initiation of antiretroviral therapy (Muller et al., 2010).

Overall, collaboration between the innate and adaptive limbs of the immune system is required to sustain pregnancy until term. A disruption of either limb at term may lead to physiological labor, and an untimely disruption could result in pathological preterm labor. Research targeting the immune cells involved in the process of labor might reveal new strategies to prevent preterm labor and consequently preterm birth.

1.20 Study rationale and gaps in knowledge

South Africa is a country in a region highly burdened with HIV infections. Of those infected, women are in the lead and the majority of those are of childbearing age. Vertical transmissions of HIV to infants was a major concern in the past. The massive roll out of ARTs has greatly reduced HIV mortality. Consequently, the use of lifelong combination ART during pregnancy has seen the rates of mother-to-child-transmission dwindle to impressively low numbers such that infants born to HIV positive mothers are free of infection. This has changed the face of the pandemic and has been a positive sign in the fight against HIV.

Previous research has shown that the use of ART during pregnancy resulted in consequences resulting in adverse pregnancy outcomes, in particular preterm and SGA delivery, although the data is conflicting with some studies not finding these outcomes whilst others attributed the differences to the regimen used. However, there is no clear association and there is a lack of understanding of the underlying mechanisms. This is of significant public health concern in sub-Saharan Africa, considering the large number of HIV infected women, the majority of whom are now on ART. So far, most of the available explorative research on the impact of HIV or ART on detrimental pregnancy outcomes has been conducted in European countries and yet the bulk of the pandemic is in Africa, specifically southern Africa where the HIV-1 subtype C strain predominates. Little is known about the underlying mechanisms leading to detrimental pregnancy outcomes and no predictive biomarkers exist, thus complicating clinical interventions to curb this problem.

1.21 Innovation

This work not only addresses a research question of high scientific and public health significance, but is also very innovative. With an increase in cART use during pregnancy in HIV-1-infected women, there is an increase in the possibility of consequences for adverse pregnancy outcomes. In particular that of preterm and SGA delivery whose underlying mechanisms must be better understood to optimize their clinical management and to maximize the PMTCT impact. The proposed study will provide critical new insights by quantifying the extent to which cART used to reduce MTCT impacts differential birth outcomes and will also test the hypothesis that immune dysregulation occurring during immune reconstitution under ART leads to preterm delivery.

(i) Testing the hypothesis that immune dysregulation occurring during immune reconstitution under ART leads to preterm delivery.

The findings from this will contribute importantly to the understanding of the biological mechanisms leading to preterm birth, will inform policy, and eventually save lives.

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1.22 Study aims and objectives

To quantify the association between cART use in pregnancy and pregnancy outcomes, specifically preterm and small for gestational age (SGA), the following aims are addressed:

Aim 1: Assessment of antigen-presenting cells (APCs) function upon TLR stimulation following initiation of ART during pregnancy and pre-term delivery.

Hypothesis: We hypothesize that antigen presenting cell function will be altered in HIV infected women who initiate ART during pregnancy, contributing to differential birth outcomes.

Aim 2: Assessment of regulatory T cells (Tregs) functional changes associated with initiation of ART during pregnancy and pre-term delivery.

Hypothesis: Reconstitution of immune responses under ART in pregnant women will result in enhanced immunity against the fetus and increased preterm birth driven by Treg responses.

Aim 3: Quantification of plasma cytokine profiles associated with the initiation of ART during pregnancy and their impact on preterm birth.

Hypothesis: We hypothesize that an ART mediated cytokine shift in HIV infected women who initiate ART could be responsible for an increase in preterm deliveries.

Chapter 2 Cohort characteristics, materials and methods

2.1 Study participants and cohort characteristics

The Prematurity Immunology and HIV-infected Mothers and their infants Study (PIMS) is a prospective cohort study that was conducted among HIV-infected women seeking antenatal care (ANC) at a community-based public sector primary care facility in Cape Town, South Africa. Participants were enrolled between April 2015 and October 2016. Between this time, pregnant women who were ≥18 years old at their first ANC visit were enrolled at the Gugulethu Midwife Obstetric Unit (MOU) which is in a low-income high HIV-prevalence sub-district of Cape Town, South Africa. The facility serves a catchment population of approximately 350,000, with an estimated antenatal HIV seroprevalence of 30% and ANC uptake of 95% in 2014 (specimens for the study were collected during this period). All women in this setting routinely have gestational age estimated based on last menstrual period and symphysis-fundal height (SFH) at the first ANC visit.

Ultrasound assessments were available for PIMS participants enrolling at less than 20 weeks of pregnancy (Malaba et al., 2017). Women were enrolled into PIMS and those already stable on ART had 3 study visits at 4-week intervals whereas those initiating made four study visits (the extra study visit, two weeks after enrolment was for ART initiation). At each visit, a blood sample was drawn into sodium heparin tubes (BD Biosciences, New Jersey, USA) and transported to the lab where peripheral blood mononuclear cells (PBMCs) were isolated within 4 hours of collection, frozen and stored into liquid nitrogen.

2.1.1 Routine care services

As part of routine ANC services, gestational age (GA) was estimated based on date of last menstrual period (LMP) and symphysis-fundal height (SFH). All women without a previous HIV diagnosis underwent HIV testing, with universal ART eligibility. HIV-infected women conceiving while on ART continued their current regimen throughout pregnancy; regimens included NNRTIs such as efavirenz (EFV) or a protease inhibitor (PI, predominantly used after failure of first-line therapy). For women initiating ART in pregnancy, the fixed-dose combination of tenofovir (TDF) + emtricitabine (FTC) + efavirenz (EFV) was used throughout.

2.1.2 Recruitment

Following screening of all women attending their first ANC visit, those ≥18 years were eligible and approached to participate in the study. Women who agreed to participate had their routinely collected LMP-based GA and SFH-based GA reviewed by the counsellor; women estimated to be ≤24 weeks were referred for a research ultrasound scan (US) for formal pregnancy dating by a research sonographer blinded to the midwife assessment. HIV-infected women who were ≤24 weeks' gestation on US were then recruited into a nested cohort (Group 2); half of these had initiated lifelong ART prior to conception and half initiated ART during pregnancy.

Women in Group 2 participated in up to eight scheduled study visits, from the start of ANC through to 12 months postpartum. Women on ART from before pregnancy had three antenatal visits at <24 weeks, 28 and 34 weeks; women who initiated ART during pregnancy had an additional study visit two weeks after the ART initiation (which in most women took place on the same day, or close to, the first study visit). At all study visits, data was collected on maternal health (HIV care and ART use, clinical care and inter-current clinical history).

2.1.3 Definition of groups and sub-groups

To investigate the proposed hypothesis that immunological changes resulting from maternal ART exposure are associated with adverse birth outcomes, women enrolled into the cohort were intensively sampled, with repeated phlebotomy throughout pregnancy for immunological investigations. These investigations compare 90 women who delivered preterm (30 PTD cases) or had small-for-gestational age infants (30 SGA cases) and those from appropriate controls (30 term AGA). PTD was defined as delivery < 37 weeks, SGA as weight for gestational age $\leq 10^{th}$ centile, AGA controls were term, with weight for gestational age $\geq 25^{th}$ centile. Controls and cases were matched on timing of ART initiation and analysed blinded. Investigations included longitudinal quantification of plasma cytokine profiles, phenotypic and functional characterisation of T cells including regulatory T cells (Tregs) as well as antigen-presenting cells (APCs).

2.1.4 Specimen collection

A total of 90 patients were chosen for the investigations described in this thesis. Baseline characteristics at first ANC visit are shown in Table 2.1 Median age was 32 years (IQR: 26-36). It was the first pregnancy for 15% of women (n=14), and 26% (n=23) were nulliparous, with no significant difference by timing of ART initiation. Of the 90 women, 47 initiated ART before pregnancy (stable on ART), and 43 initiated ART at their first ANC (initiators). Most women (n=79, 88%) were on TDF-3TC-EFV, one (1%) was on TDF-3TC-NVP and three (3%) on other NNRTI-based regimens. Six (6%) women were on a PI regimen, one of whom started during pregnancy and the other five were stable on ART. Median $CD4^+$ T cell count overall was 436 cells/µl (IQR: 392-573); 371 (IQR: 251-537) for women stable on ART and 468 (344-612) for initiators. Viral load (VL) information was measured for all women at baseline.

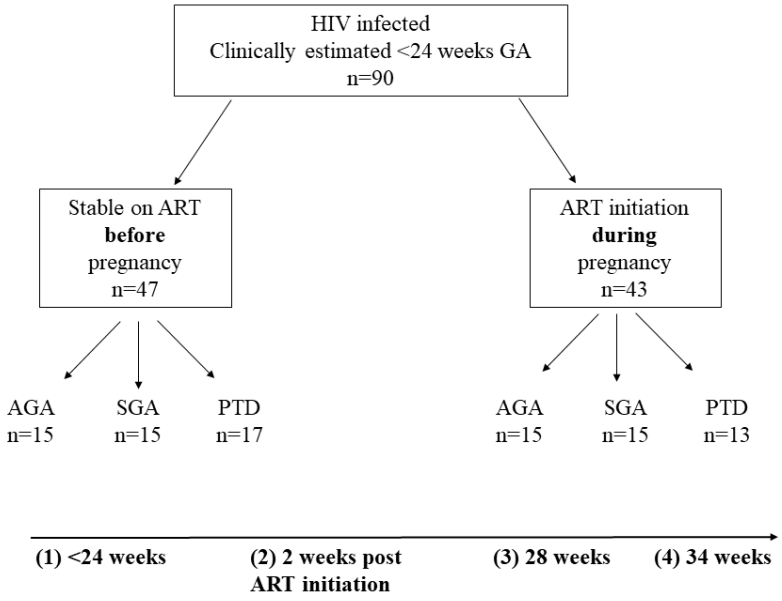

Figure 2.1: Definition of subgroups and time points for sample collection for PIMS patients throughout the study.

Table 2.1 Demographic and clinical characteristics of women who initiated ART during or before pregnancy

	Total	Initiation before pregnancy				Initiation during pregnancy			
	N=90	N=47				N=43			
		AGA	PTD	SGA	P-value	AGA n=15	PTD n=13	SGA n=15	P-value
		N=15	N=17	N=15					
Maternal characteristics									
Age, years: median (IQR)	32 (26-36)	33 (28-35)	36 (32-38)	36 (28-39)	0.205	28 (24-34)	26 (25-31)	31 (25-37)	0.768
Education (finished high school)	32 (35)	5 (33)	5 (29)	5 (33)	0.962	7 (47)	6 (46)	4 (27)	0.450
Employments status: Employed (%)	33 (36)	5 (33)	7 (41)	6 (40)	0.889	6 (40)	3 (23)	6 (40)	0.565
Socioeconomic status (SES)*					0.741				0.860
Lowest	28(31)	3 (20)	6 (35)	5 (33)		4(28)	4(30)	6(40)	
Medium	30(33)	6 (40)	5 (30)	5 (33)		5(35)	5(38)	4(26)	
Highest	29(29)	6 (40)	6 (35)	4 (27)		6(46)	3(23)	4(26)	
Missing	3 (3)	0 (0)	0 (0)	1 (7)		0 (0)	1(7)	2(13)	
Obstetric characteristics									
Gravidity: median (IQR)	3 (2-3)	3 (2-4)	3 (2-3.5)	4 (3-4)	0.420	2 (2-3)	2 (2-3)	2 (1-2)	0.347
Parity: median (IQR)	1(0-2)	1 (1-2)	1 (1-2)	2 (1-2)	0.244	1 (0-1)	1 (0-2)	1 (0-2)	0.315
Previous Preterm*: Yes	9 (10)	0 (0)	2 (12)	5 (33)	0.034	1 (7)	0 (0)	1 (7)	0.635
Gestational age at booking/enrolment: median weeks (IQR)	15 (11-18)	13 (9-15)	16 (9-17)	15 (9-18)		14 (12-17)	19 (14-21)	16 (12-18)	
Height, (cm): median (IQR)	158 (155.5-162.5)	162 (156-166)	158 (155.5-161)	157 (150.5-163)	0.343	160 (156.5-169.5)	158 (155.5-160)	159 (152.5-161.1)	0.161

Haemoglobin (g/dl): median (IQR)	11,4 (10-3-12.4)	11 (11.1-12)	11.8 (11-12.7)	11.6 (10.6-12.9)	0.283	10.5 (10-12)	11.3 (10.2-11.7)	10.9 (10.1-11.6)	0.697
Weight (kg): median (IQR)	70.15 (59.4-83.9)	74.9 (64,30- 85,95	76 (67,30-85,45)	61.7 (52,50-74,70)	0.126	80,10 (61,70-98,40)	61,35 (58,28-67,48)	64,00 (56,70-92,00)	0.413
HIV-associated parameters									
Current ART regimen, self-report					0.199				0.353
TDF-3TC-EFV	79 (88)	13 (86)	13	13 (86)		14 (93)	11 (84)	15 (100)	
TDF-3TC-NVP	1 (1)	0 (0)	0	0 (0)		1 (7)	0 (0)	0 (0)	
Other NNRTI-based regimen	3 (3)	0 (0)	3	0 (0)		0 (0)	0 (0)	0 (0)	
PI-based regimen	6 (6)	2 (14)	1	2 (14)		0 (0)	1 (8)	0 (0)	
Missing	1 (1)	0 (0)	0 (0)	0 (0)		0 (0)	1 (8)	0 (0)	
CD4 cell count, (cells/µL)* median (IQR)	436 (392-573)	416 (353-566)	529 (309-638)	485 (333-584)	0.509	368.5 (247-535)	314 (219-457)	493 (283.5-926.5)	0.724
Missing	14 (16)	2 (13)	2 (11)	0 (0)		3 (20)	4 (30)	3 (20)	
Viral load, copies/ml (baseline A1), median (IQR)	281 (20-80000)	20 (20-27)	25 (20-528)	20 (20-65)	0.342	21900 (6120-59900)	4730 (1300-7950)	2120 (741-18800)	0.681
Viral load, copies/ml (A1.5), median (IQR)	198 (37-431)					431 (275-512)	99 (20-137)	89 (25-307)	0.172

*CD4 results abstracted from routine records and are nearest in time to the first ANC visit

*14 patients who did not have CD4 were excluded from the analysi

2.2 Preparation of peripheral blood mononuclear cells for the analysis of antigen presenting cells.

Leukocytes are routinely isolated from whole blood and comprise monocytes (macrophage precursors), lymphocytes (natural killer cells, B and T cells), and a small percentage of other immune cells, such as dendritic cells. These cells are isolated from freshly drawn blood, using Ficoll density gradient centrifugation (Panda and Ravindran, 2013). These cells can then be cryopreserved at ultra-low temperatures in liquid nitrogen allowing them to remain viable for long periods of time with no significant changes to their viability or functionality. After a careful thawing procedure, cryopreserved peripheral blood mononuclear cells (PBMCs) can be stimulated or treated with various immunomodulatory agents or drugs in vitro to aid in immunological research.

2.3 Isolation of PBMCs from whole blood

In brief, PBMCs were isolated as follows: Whole blood samples were collected from donors using sodium heparin (BD Vacutainer, USA) and were processed within 6 hours of collection. Blood samples were poured into 50ml conical tubes for PBMC processing. A 50ml tube of whole blood was then centrifuged at 500xg for 10 minutes at room temperature. After spinning for 10 minutes, the supernatant (plasma), was carefully removed and stored at -80 ºC ultra-freezer. The remaining whole blood was diluted with equal amount of Dulbeco's Phosphate Buffered Saline (DPBS) (Life Technologies, UK) that contained 1% of penicillin-streptomycin solution and gently mixed by inverting. Diluted blood was overlaid into a 50ml conical tube separation medium (15ml of Histopaque-1077, Sigma Aldrich) at room temperature.The tube was centrifuged for 30 minutes at 500xg at room temperature. At this point, the whole blood had divided into different cell layers (Figure 2.2).

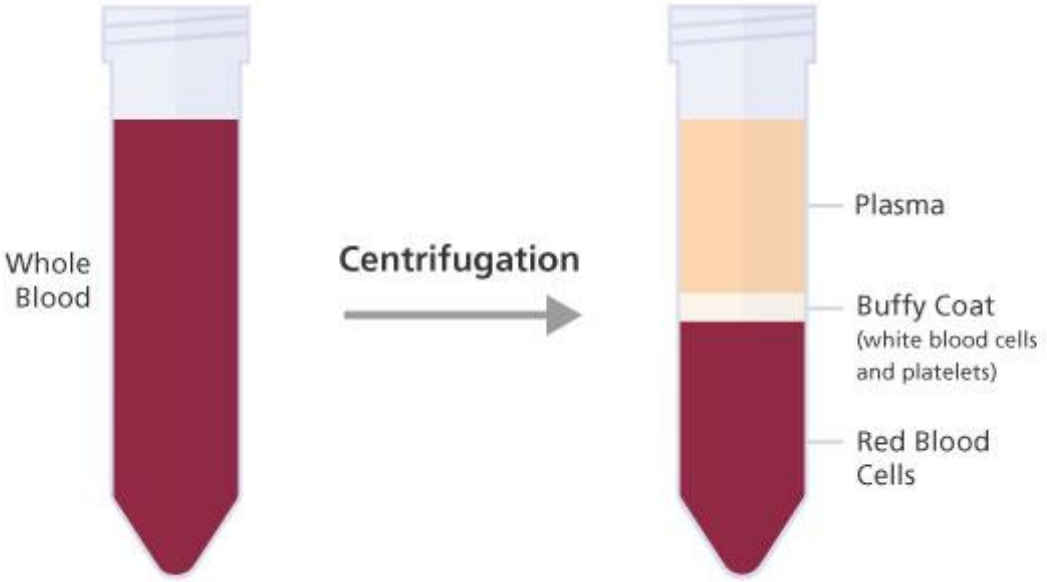

Figure 2.2 PBMC Ficoll density gradient separation

The lymphocytes which are in the buffy coat layer, were then removed from the interface and transferred to another sterile 50ml conical tube, where DPBS was added to wash the lymphocytes. The cells were centrifuged at 500xg for 10 minutes at room temperature, this wash step was repeated twice. After the second wash, the supernatant was decanted and the lymphocyte pellet was suspended in 20 mL of R10 (RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin, 1.7Mm sodium glutamate and 1% of Hepes buffer). Isolated PBMCs were counted using a trypan blue solution (Life Technologies, USA). The dye was prepared at a 1:10 dilution by mixing 20 µl PBMCs and 180 µl trypan blue. The mixture was vortexed and incubated for 5 minutes before counting. Then 10 µl of the mixture was loaded onto a haemocytometer slide and counting was done manually using a light microscope before resuspension of the cells at final concentration of $10x^6$/per millilitre of R10 medium. PBMCs were kept at -80^{o}C overnight before being trnsfered to liquid nitrogen for long term storage.

2.4 Thawing of cryopreserved PBMCs

PBMCs that had been previously been cryopreserved in freezing medium (10% dimethyl sulfoxide (DMSO) in filtered heat inactivated fetal calf serum (FCS) (Gibco, NY, USA)) were thawed quickly using a water bath set at 37 ^{o}C (LabFix, Durban, SA) and immediately transferred into a 15 ml falcon tube containing 9ml of R10 medium (RPMI 1640 medium (Sigma, St. Louis, MO, USA)) supplemented with 10% FCS (Gibco), 1% L-Glutamine (Sigma), and 1% penicillin/streptomycin (Gibco)). This was followed by centrifugation (Eppendorf centrifuge 5810R, Merck, Germany) at 1800 rpm for 10 minutes after which the supernatant was discarded into a waste bucket containing 2% Virkon (DuPont de Nemours, South Africa). The cells were then washed twice with 2% FCS in phosphate buffered saline (PBS) and an aliquot was taken for immediate surface staining and the remainder left to rest for 2 hours in R10 medium at 37 ^{o}C with 5% CO_2.

2.5 Cell counting

A haemocytometer was used to manually carry out cell counts in a ratio of 1:9 with trypan blue (Sigma) staining solution. Trypan blue works by penetrating the cell membranes of dead cells which can then be seen under the microscope with live cells being translucent. Briefly, 10 µl of the cells suspended in 10 ml of R10 were added to an Eppendorf tube containing 90 µl of trypan blue and resuspended thoroughly. The cells and trypan blue (10 µl) mixture was then loaded onto a haemocytometer and cells were counted under an electron microscope (Olympus CH20 upright microscope). A ten times (x10) magnification was suitable for the identification of PBMCs. To determine cell viability, all the cells within five squares of the haemocytometer were counted to give an estimate of the total number of cells in the suspension. Viability was

then determined using the formula: $\left|\frac{Number\ of\ live\ cells}{Number\ of\ live\ cells+number\ of\ dead\ cells} \times 100\right|$. Only cells with a viability of 80% were assessed.

2.6 Characterization of the APCs and T cells using flow cytometry

Flow cytometry in principle is the passage of cells in a single file in front of a laser so they can be detected, counted and sorted with the aid of a ligh source. Cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths as shown in figure 2.3 (Adan et al., 2017). Size, granularity, and fluorescent features of the cells which are derived from either antibodies or dyes, are the parameters used to analyze and differentiate cells. The underlying principle of flow cytometry is related to light scattering and fluorescence emission, which occurs as light from the excitation source (commonly a laser beam) that strikes the moving particles. This analysis was performed following thawing and counting of PBMCs as mentioned above.

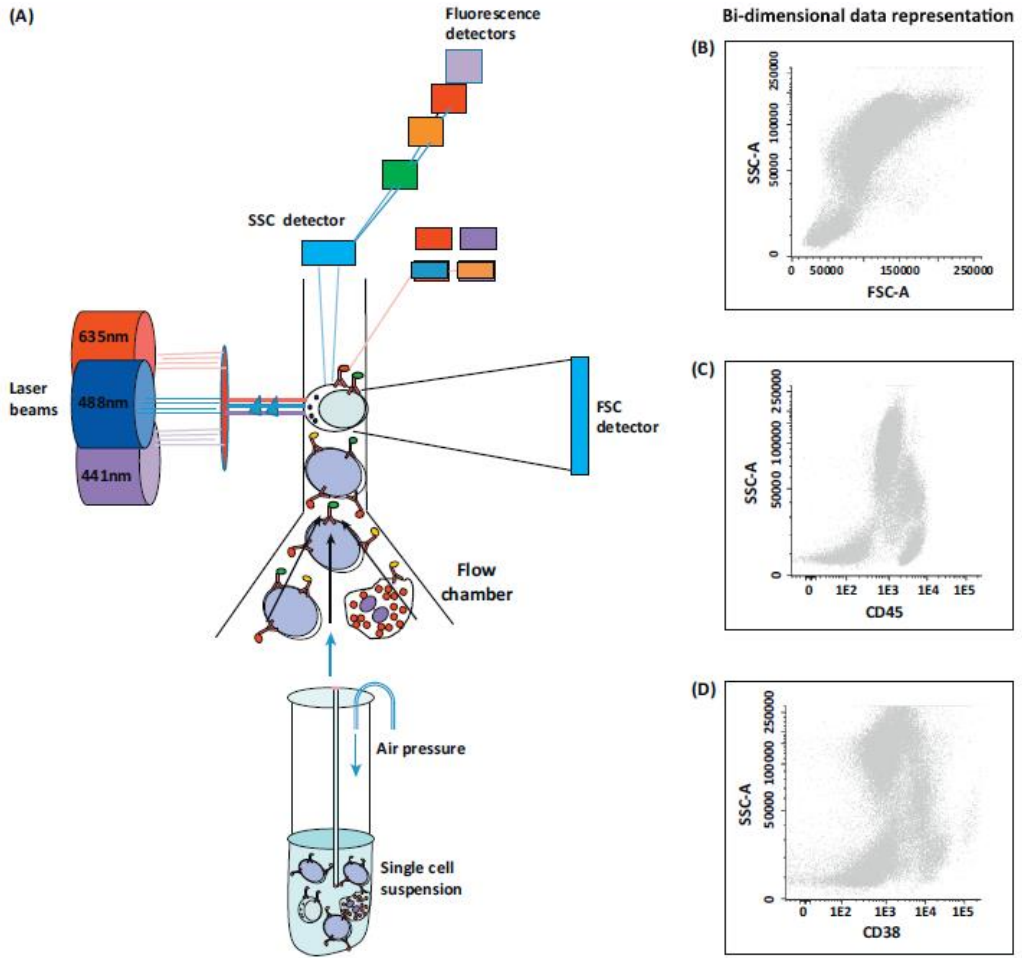

Figure 2.3: Schematic representation of flow cytometry (FC) data generation. Individual cells from a single cell suspension are pushed to pass aligned and one-by-one through a flow chamber in front of three laser beams; a forward light scatter (FSC) detector is placed in front of the laser beams and collects light at low angles ($<10^0$), whereas the sideward light scatter (SSC) detector and the fluorescence detectors collect light scattered and the fluorescence emissions at an angle of 90^0 with respect to the intersection between the cell flow and the laser beams (A). The digital signal generated by the amount of light reaching the different detectors is processed by a computer equipped with FC data analysis devoted software. Representative 2D dot plot representations of the data generated are shown in Panels B–D: FSC versus SSC (B), SSC versus CD45 (C) and SSC versus CD38 (D) (Pedreira et al., 2013).

2.7 Surface staining of cells for flow cytometry analysis

After counting, 1 x10^6 cells/well were added into a 96-well round-bottomed plate. A surface stain was performed for each of the samples with antibodies directed against the following antigens: CD3 (Clone: OKT3, BioLegend (BL)), CD4 (Clone: RPA-T4, BL), CD11c (Clone: 3.9, BL), CD123 (Clone: 6H6, BL), CD8 (Clone: RPA-T8, BD), CD56 (Clone:HCD56, BL), CD19 (Clone: HIB19, BL) (used to exclude natural killer (NK) and B cells respectively), CD14

(Clone: HCD14, BL), CD16 (Clone: 3G8, BD) (for the identification of monocytes), HLA-DR (Clone: G46-6, BD), CD38 (Clone: HB-7, BL), CD69 (Clone: FN50, BL), CD86 (Clone: 2331, BL) (markers of activation). For each experiment, a fluorescence minus one (FMO), which is the control that contains all fluorochromes of interest except the one being measured, was included for each marker to allow subsequent gating. Aqua (Life Technologies, USA) viability dye was included for all samples. The cells were incubated for 20 minutes in the dark at room temperature. After this incubation, cells were then washed with 2% FCS/PBS. The plate was then centrifuged at 2000rpm for 6 minutes at room temperature and the supernatant was decanted thereafter. A fixative, 40 µl (Perm A, Merck), was added to the cells and incubated for 20 minutes in the dark at room temperature. The cells were washed again, the supernatant decanted and cells resuspended in 200µl of 2% FCS/PBS before acquisition on a multicolour BD LSRFortessa flow cytometer (BD). Between 500 000 to 1 000 000 events were acquired and the data was analysed using Flowjo software version 9.8.5 (FlowJo LLC, Ashland, Oregone). A flow diagram depicting the experimental design can be found in Figure 2.4. For analysis, initial gating was on lymphocytes followed by gating for single cells, excluding of B cells, NK cells (which were all on one marker), along with dead cells. Thereafter, $CD3^+$ T cells were gated on followed by gating on $CD4^+$ and $CD8^+$ T cells. The subsequent gates done were to show that activation was measured by the expression of HLA-DR and CD38 on $CD4^+$ and $CD8^+$ T cells respectively.

For the identification of blood monocyte subsets by flow cytometry, gating was done for single cells and successive exclusion of NK cells and B cells as well as gating on live cells. This was followed by gating for CD3 negative and positive cells. HLA-DR expression was gated on from the CD3 negative cells followed by CD14 vs CD16 to differentiate three (classical, intermediate and inflammatory) monocyte subsets.

For the identification of bulk $CD14^+$, mDC and pDC subset and measurement of activation by flow cytometry, forward versus side scatter and all cells was gated on. This was followed by the exclusion of NK and B cells, along with dead cells. HLA-DR expression was gated from CD3 negative cells followed by CD14 expression. The subsequent plots were based on the expression of CD86 and CD69 on $CD14^+$ cells for monocyte activation and on CD14 negative for CD11c and CD123. Fluorescence minus one (FMO) controls were used to determine the respective gates. Activation in each of the cell populations was based on the expression of CD86 and CD69 markers.

2.8 Intracellular cytokine staining (ICS) measurement of APCs

Cytokine production following toll-like receptor (TLR) ligand stimulation on a single cell level was assessed using flow cytometry as described in section 2.5 above. To do this, thawed PBMCs were left to rest for 2hrs at 37 ^{o}C in 5% CO_2 prior to use. After 2 hours incubation, cells were counted and one and a half million were stimulated under each condition: 1 µg/ml LPS (TLR 4) (Merck), 1 µg/ml CL097 (TLR 7/8) (Invivogen) or 500 µM CpG ODN2216 (TLR 9) (Invivogen). Unstimulated cells served as negative controls. Five µg/ml brefeldin A (Sigma) (inhibitor of protein transport) was immediately added to each well following the addition of TLR ligands to inhibit cellular cytokine release. The second experimental condition stimulated cells without the addition of the protein transport inhibitor for all the conditions. Intracellular cytokine content of APCs was determined after 18 hours of incubation with the respective TLR ligands. Following overnight stimulation, the plate was centrifuged at 2000 rpm for 8 minutes and the supernatant was discarded for the BFA condition. For the wells without the inhibitor, supernatant was collected, and the pellet resuspended in 1 ml Trizol (Sigma) and immediately stored at -80 ^{o}C for further analysis (Figure 2.4). Cells were then washed with 100 µl of wash buffer (2% FCS in DPBS (Life Technologies, UK)) and centrifuged at 2000 rpm for 8 minutes.

A cocktail of aqua dye to ascertain viability (100 µl) (Life Technologies, UK) and surface stain markers: CD3 (Clone: OKT3, BL), CD4 (Clone: RPA-T4, BL), CD11c (Clone: 3.9, (BL), CD123 (Clone: 6H6, BL), CD8 (Clone RPA-T8, BD), CD56 (Clone: HCD56, BL), CD19 (Clone: HIB19, BL) (used to exclude natural killer (NK) and B cells respectively), CD14 (Clone: HCD14, BL), CD16 (Clone: 3G8, BD) (for the identification of monocyte populations depending on the expression of these markers) and HLA-DR (Clone: G46-6, BD), a marker of activation (Table 2.2). This was incubated for 20 min in the dark at room temperature and this

was followed by a wash step, then fixed with Perm A (Sigma) for 20 minutes in the dark and washed.

Following a wash step, the cells were permeabilized with 100 µl of Perm B (Sigma) and stained with anti-tumor necrosis factor (TNF)-α PerCPy5.5 (Clone: Mab11, BL), anti-interleukin (IL)-12 (Clone: C11.5, BL), anti-interferon (IFN)-α (Clone: 7N4-1, BL) and macrophage inflammatory protein (MIP)-1β (Clone: D21-1351, BD) for 20 minutes in the dark followed by a wash step. After incubation, the cells were washed with 2% FCS/PBS buffer and the cell pellet resuspended in 200µl of wash buffer for acquisition on the multicolour BD LSRFortessa flow cytometer (BD). At least 500 000 events were acquired, and the data was analysed using Flowjo software version 9.8.5 (FlowJo LLC, Ashland, Oregon). A flow diagram depicting the experimental design can be found in Figure 2.4. Table 2.2 below summarizes all antibodies used in phenotypic and functional experiments.

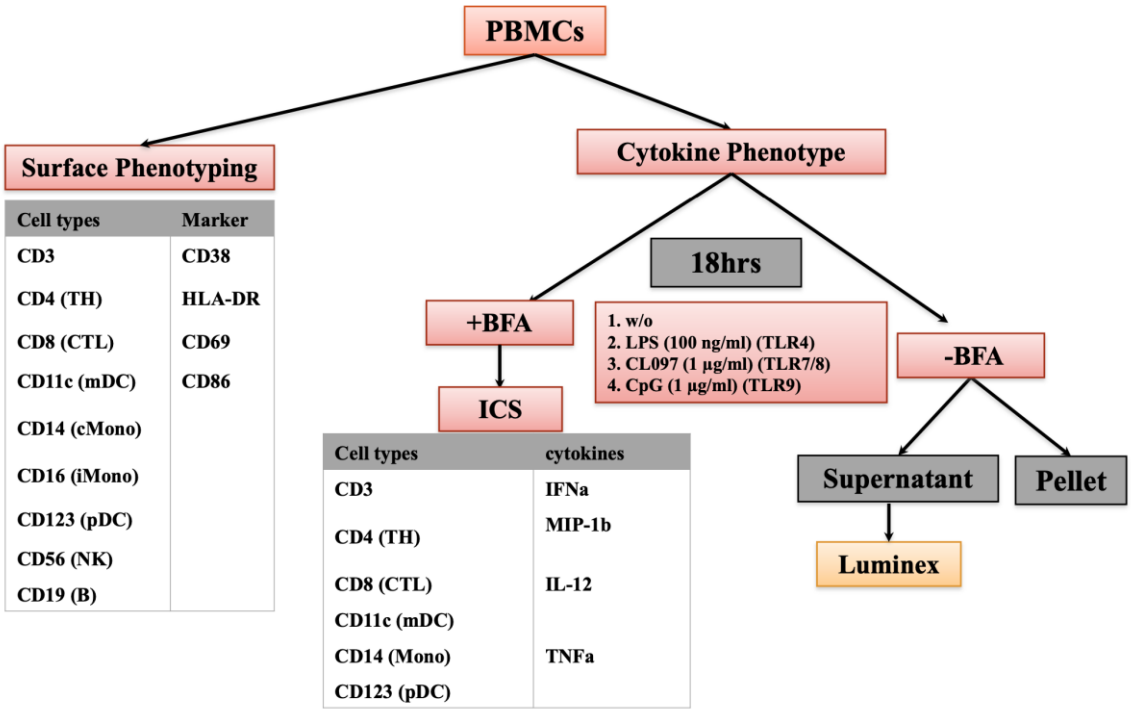

Figure 2.4: Experimental design used for the antigen presenting cell panel.

Table 2.2 Antibody panel for APC analysis

Antigen	Flourochrome	Clone	Isotype	Company	Volume used (µl)
CD3	BV650	OKT3	Mouse IgG1	BioLegend	2.5
CD4	APC	RPA-T4	Mouse IgG1	BioLegend	1
CD8	FITC	RPA-T8	Mouse IgG1	BD	1
CD11c	PE-Cy5	3.9	Mouse IgG1	BioLegend	1
CD14	APC-Cy7	HCD14	Mouse IgG1	BioLegend	1
CD16	BV786	3G8	Mouse IgG1	BD	1
CD19/56	BV421	HIB19	Mouse IgG1	BioLegend	2.5
CD38	PE	HB-7	Mouse IgG1	BioLegend	2
CD69	Per-CP-Cy5.5	FN50	Mouse IgG1	BioLegend	2.5
CD86	PE-Cy7	2331	Mouse IgG1	BioLegend	1
CD123	BUV395	6H6	Mouse IgG1	BioLegend	1
HLA-DR	PE-Texas Red	G46-6	Mouse IgG1	BD	1
MIP-1β	FITC	D21-1351	Mouse IgG1	BD	1
IFNα2	PE	7N4-1	Mouse IgG1	BD	2
IL-12	APC	C11.5	Mouse IgG1	BioLegend	1
TNF-α	BV605	Mab11	Mouse IgG1	BioLegend	1

2.9 Treg cell isolation and staining

2.9.1 Thawing of cells for use in regulatory T cell experiments

To prepare for thawing the cells, R1 (1% FBS in RPMI (Sigma, Germany)) media was warmed in a 37 ^{o}C water bath. The cells were then thawed in the 37 ^{o}C water bath until only a speck of ice crystal remains. Upon removal, the vial was wiped with ethanol. The cells were transferred to a clean 50 ml Falcon tube where 10 ml of warm R1 was added drop wise while swirling. This was then topped up to 25 ml with R1 and the cells centrifuged at 1200 rpm for 10 minutes. The supernatant was then decanted, and the pellet resuspend in 500 µl of DNAse (Roche, Basel) solution and incubated for 3 minutes at room temperature. The cells were then washed with 25 ml of R1 and centrifuged at 1200 rpm for 10 minutes. The supernatant was then discarded, and the pellet resuspended in 5 ml R10 (10% FBS in RPMI). The cells were then rested for 2 hours at 37 ^{o}C in a CO_2 incubator with the lid loosely fit.

2.9.2 Surface staining for regulatory T cells using flow cytometry

After resting, the cells were centrifuged at 1200 rpm for 10 minutes, the supernatant was then discarded, and the cell pellet gently resuspend. After counting, approximately 1 million cells per well were transferred into a U-bottom plate. The cells were centrifuged at 2100 rpm for 3 minutes, the supernatant was then discarded, and the cells washed with 200 µl PBS. The cells were then centrifuged at 2100 rpm for 3 minutes and the wash step was repeated. The next step was to make up the VIVID (ThermoFisher) working stock (1 µl stock + 39 µl PBS); this was always kept on ice and covered in foil from light. An aliquot of 4 µl of VIVID (LifeTech) was added into each well and resuspend thoroughly, followed by incubation in the dark for 20 minutes at RT. PBS (150 µl) was added into each well and the cells were centrifuged at 2100 rpm for 3 minutes. The supernatant was discarded, and the pellet washed with 200 µl PBS, and centrifuged at 2100 rpm for 3 minutes. An antibody cocktail was then prepared as follows:

CD8 (Clone: SK1, BioLegend (BL)), CD39 (Clone: A1, BL), CD25 (Clone: 2A3, BD), CD127 (Clone: A019D5, BL), CD14 (Clone: MHCD1417, Life Technologies), CD4 (Clone: SK3, BL), CD3 (Clone: UCHT1, BL), TIGIT (Clone: MBSA43, eBiosciences), CD45 (Clone: HI30, BD), CD28 (Clone: CD28.2, BL), CD45RA (Clone: H100, BL), PD-1 (Clone: EH12.1, BD) (Table 2.3). Fifty (50) µl of the antibody mix was then added to the cells, resuspended thoroughly, and incubated for 30 minutes at RT in the dark. Thereafter, 150 µl FACS wash was added and centrifuged at 2100 rpm for 3 minutes. The cells were washed again with 200 µl FACS wash and centrifuged at 2100 rpm for 3 minutes. The cell mixture was then ready for intracellular staining.

2.9.3 Intracellular staining of regulatory T cells with FoxP3 and CTLA4

After the final wash, the supernatant was discarded. The pellet was then resuspended thoroughly in 200 µl of Fixation/Perm (BD buffer and incubated for 40 minutes at RT in the dark. The cells were then centrifuged at 1800 rpm for 3 minutes at RT, and the supernatant discarded. Then 200 µl of 1x Perm buffer was added, the cells were then centrifuged at 1800 rpm for 3 minutes at RT. The pellet was resuspended thoroughly in 50 µl of FoxP3 + CTLA4 prepared in perm buffer and incubated for 45 minutes at RT in the dark. Thereafter 150 µl of 1x perm buffer was added, and the cells centrifuged at 1800 rpm for 3 minutes at RT, and the supernatant was then discarded. The pellet was then resuspended in 200 µl of 1X perm buffer, centrifuged at 1800 rpm for 3 minutes and the supernatant discarded. The cells were then resuspended in 100 µl of cell fix and transferred to FACS tubes and were kept at 4 ^{o}C until it was ready for acquisition on the LSRFortessa (BD) within 24 hours. Compensation tubes were prepared for each experiment. Table 2.3 below summarizes all antibodies used in phenotypic and functional experiments for regulatory T cell assays

For analysis, frequencies of T cells were enumerated by staining PBMCs that were thawed, stained and analyzed on a LSRII. When looking solely at antigen expression, Tregs are often defined as CD3+, CD4+, CD25hi, FOXP3+, and CD127lo.

Table 2.3: Treg antibody panel.

Antigen	Flourochrome	Clone	Company	Volume used (µl)
CD8	PerCP-Cy5.5	SK1	BioLegend	1 µl
CD39	FITC	A1	BioLegend	5 µl
CD25	PE-Cy7	2A3	BD	1.25 µl
CD127	PE-Cy5	A019D5	BioLegend	1.25 µl
CD14	PE-Texas Red	MHCD1417	Life Tech.	1 µl
CD4	APC-H7	SK3	BioLegend	0.3 µl
CD3	AF-700	UCHT1	BioLegend	1 µl
TIGIT	APC	MBSA43	eBio	3 µl
CD45	BV786	HI30	BD	1.25 µl
CD28	BV711	CD28.2	BioLegend	1.25 µl
CD45RA	BV650	H100	BioLegend	0.3 µl
PD-1	BV510	EH12.1	BD	1.25 µl
VIVID	PacBlue		Life Tech.	

2.10 Multiplex serum cytokine and chemokine analysis using Luminex technology.

Cytokine functions are complex, and the understanding of cytokine profiles is important for understanding immune responses. It therefore becomes critical to possess the ability to analyze the function of a complete set of cytokines expressed within microenvironments (e.g., a site of inflammation). Although there have been technologies that have aided us in this regard, one of the major limitations such as the need for a large sample volume or detection of precursor proteins rather than native secreted proteins (de Jager et al., 2003). Luminex has so far broken these barriers as it allows the analysis of a multiple number of cytokines within a single well. Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals (Figure 2.5) (Harris and Chen, 2019). EMD Millipore combines the streamlined data acquisition power of Luminex xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex instrument (Merck, Burlington, Massachusetts, United States).

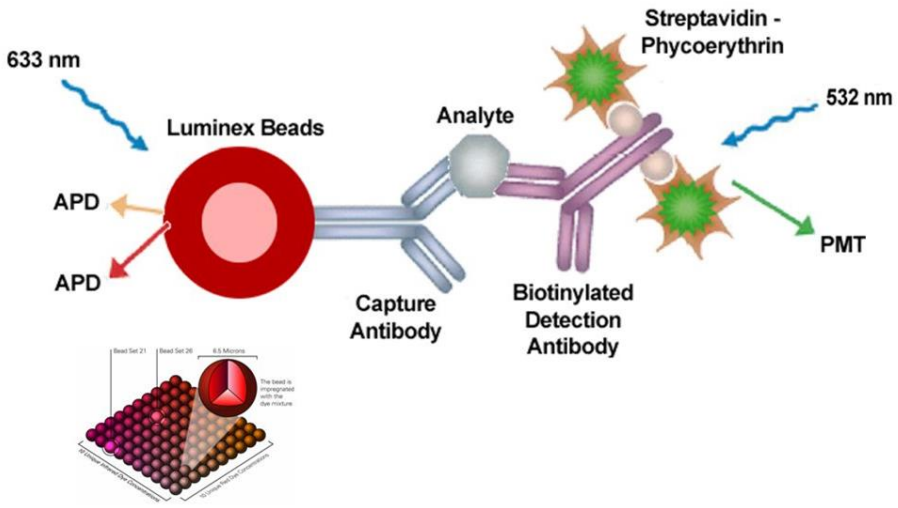

Figure 2.5: Luminex assay principle.

The Milliplex® Map human cytokine/chemokine system (Merck Millipore), based on a Luminex bead array platform as described above, was used to measure the concentrations of 15 analytes. Levels of interleukin (IL)-1α, IL-1β, IL-2, -IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p40), IL-12 (p70), inducible protein (IP)-10, macrophage inflammatory protein (MIP)-1β, tumour necrosis factor (TNF)-α, interferon (IFN)-α and IFN-γ. Plates were read using the BioRad Luminex®100™ system and analysed with integrated Bioplex Manager Software v6.1 (BioRad Corp., USA). Values above or below the concentration range for each analyte (3.2 to 10,000 pg/ml) were derived from analyte-specific standard curves by extrapolation. Below the lowest extrapolated value, a concentration of zero was recorded. All samples and standards were measured in duplicate, and measurements were normalized to quality controls for each plate run.

2.11 Data analysis and statistics

For the analysis of flow cytometry data, Flowjo software version 9.8.5 (FlowJo LLC, Ashland, Oregone) was used. GraphPad Prism software version 5.01 (GraphPad software Inc) was used for the graphical representation and non-parametric univariate analyses of the data. To determine the associations of different immune markers and PTD, allowing for baseline CD4 count, a regression model was developed in Stata version 15 (GraphPad software Inc); frequencies of classical and intermediate monocytes were summed because they were all significant univariately. Mann-Whitney U test, Wilcoxon paired test and student t test were used as appropriate and are detailed in the results for each analysis. The p value of below or equal to 0.05 was considered significant

Chapter 3 Low immune activation in early pregnancy is associated with preterm delivery but not small-for-gestational age outcome in HIV infected women initiating antiretroviral therapy in pregnancy: a PIMS case-control study in Cape Town, South Africa

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3.1 Introduction

Antiretroviral therapy (ART) improves survival, and prevents mother-to-child HIV transmission (Abrams and Myer, 2013). Untreated, advanced HIV disease is associated with adverse birth outcomes (Rollins et al., 2007); ART in pregnancy has been associated with preterm delivery (PTD), low birth weight (LBW) and/or small-for-gestational age (SGA) infants in some (Chen et al., 2012, Bytautiene et al., 2004, Thorne et al., 2004, Thorne, 2004b, Townsend et al., 2010a, Ekouevi et al., 2008a, Fowler et al., 2016), but not all (de Vincenzi, 2011) studies, possibly driven by specific ART regimen (Powis et al., 2011, Sebikari et al., 2019). HIV infected people, including pregnant women, are offered ART immediately at HIV diagnosis (WHO, 2015, SANAC, 2017). Research is needed to inform understanding of potential biological mechanisms underlying any association between HIV or ART and pregnancy outcome (Watts and Mofenson, 2012).

Increased immune activation is required for the maintenance of pregnancy to term, with a physiological shift towards increased peripheral immune cells activation over pregnancy (Mikyas et al., 1997, Yuan et al., 2009, Shah et al., 2017, Loewendorf et al., 2014) . ART reduces systemic immune activation (Giorgi et al., 1998, Giorgi et al., 1993, Hunt et al., 2014), which although demonstrated to be overall clinically beneficial may also alter immune regulatory pathways linked to immune activation that are essential for normal pregnancy. However, it has also been suggested that excessive systemic immune activation, inflammation at the maternal-fetal interface and other immune dysfunction may be linked to PTD and other adverse pregnancy outcomes (Arenas-Hernandez et al., 2019, Gomez-Lopez et al., 2017, St. Louis et al., 2016). Overall, associations between PTD, SGA and immunological- and infection-related events are complex, with the exact mechanisms not fully understood (Romero et al., 2014a, Bonney and Johnson, 2019, Manning et al., 2019, Amabebe and Anumba, 2018)

. We established the Prematurity Immunology in HIV-infected Mothers and their Infants Study (PIMS) in Cape Town, South Africa, to investigate the association between timing of ART initiation (pre-conception or during pregnancy), immunological parameters and PTD or SGA (Malaba et al., 2020). This work was aimed at investigating the consequences of increased pro-inflammatory immune response following initiation of cART in pregnant women at baseline. We hypothesized that HIV or ART modulation of immune cell activation status or alteration of immune cells subsets during pregnancy would be associated with PTD or SGA. We focused on T cells, monocytes and dendritic cells because these cells play a central immune effector or immunoregulatory role and alterations in their activation status or other perturbations have been reported in HIV infection and pregnancy.

3.2 Methods

PIMS is a prospective cohort study of HIV-infected women in antenatal care (ANC) at a public sector facility in Cape Town, South Africa (Malaba et al., 2020). HIV infected women ≤24 weeks gestation, as assessed by ultrasound, were enrolled and followed with three study visits for those on ART pre-conception (stable on ART) at <20 weeks (baseline), 28 and 34 weeks of pregnancy, plus an additional study visit two weeks after ART initiation for women newly identified as HIV infected and initiated on ART at their first ANC visit. At each visit, blood was drawn into sodium heparin tubes (BD Vacutainer, New Jersey, USA) and peripheral blood mononuclear cells (PBMCs) isolated within 4 hours of blood collection by density gradient centrifugation, counted by the trypan blue method and stored in liquid nitrogen. For the study presented here, 30 cases of PTD, 30 SGA cases and 30 appropriate-for-gestation age (AGA)/term controls as outcomes were selected. Controls and cases were matched on timing of ART initiation and analysed blinded. The median gestational age at enrolment was 15 weeks both for women initiating and stable on ART. PTD was defined as delivery <37 weeks, SGA

as weight for gestational age ≤10th centile, AGA controls were term, with weight for gestational age ≥25th centile (Malaba et al., 2020). Baseline information was collected by trained study nurses. CD4 cell counts were closest to the visit on which the sample was taken. Viral loads were determined for the first two study visits.

Ethical clearance was obtained from the Human Research Ethics Committee of the University of Cape Town (reference number 739/2014), the University of Southampton Faculty of Medicine Ethics Committee (reference 12542 PIMS) and the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (reference BE429/15). All participants provided written informed consent.

3.2.1 Cellular immunophenotyping and intracellular cytokine staining

Flow cytometry was performed following thawing and counting of PBMCs. A surface stain was performed with antibodies directed against the following antigens: CD3 (Clone:OKT3, BioLegend (BL)), CD4 (Clone:RPA-T4, BL), CD11c (Clone:3.9, (BL), CD123 (Clone:6H6, BL), CD8 (Clone RPA-T8 BD Biosciences (BD)), CD56 (Clone:HCD56)/CD19 (Clone: HIB19, BL) (used to exclude natural killer (NK) and B cells respectively), CD14 (Clone: HCD14-BL), CD16 (Clone:3G8) (BD) (for the identification of monocyte populations depending on the expression of these markers), HLA-DR (Clone:G46-6) (BD), CD38 (Clone:HB-7) (BL), CD69 (Clone:FN50) (BL), CD86 (Clone 2331, BL) (markers of activation). Aqua (Life Technologies) viability dye was included for all samples. This was followed by fixation using Perm A (Merck) for 20 minutes in the dark at room temperature. Cell populations were enumerated, and markers of activation were measured. Samples were acquired on the LSR-II (BD Biosciences). Cell populations were defined as: $CD4^+$ T cells: $CD3^+CD4^+$, activated $CD4^+$ T cells: $CD3^+CD4^+$ $CD38^+HLA\text{-}DR^+$, $CD8^+$ T cells: $CD3^+CD8^+$,

activated $CD8^+$ T cells: $CD3^+CD8^+$ $CD38^+HLA\text{-}DR^+$ (Figure 3.1), monocytes (lineage-$HLA\text{-}DR^+CD123^-CD14^+$) (Figure 3.2), monocytic dendritic cells (mDCs) (lineage-$HLA\text{-}DR^+CD11c^+CD123^-CD14^-$), plasmacytoid dendritic cells (pDCs) (lineage-$HLA\text{-}DR^+CD11c^-CD123^+CD14^-$) (Figure 3.3).

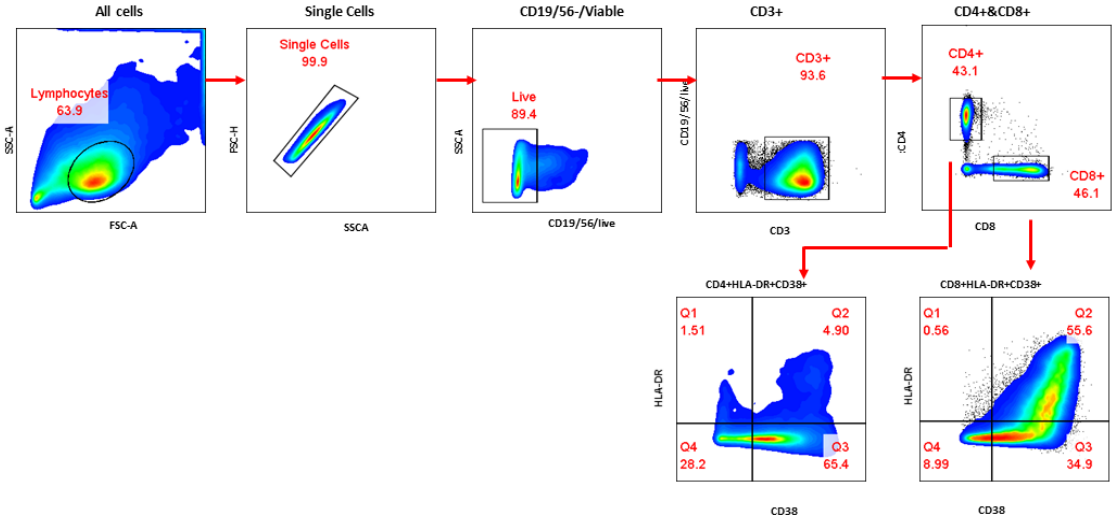


Figure 3.1: Representative gating strategy for the identification of $CD4^+$ and $CD8^+$ T cell subset activation by flow cytometry. Initial gating was on lymphocytes followed by singlets, exclusion of B cells, NK cells, along with dead cells. Thereafter, $CD3^+$ T cells were gated on followed by gating on $CD4^+$ and $CD8^+$ T cells. The subsequent plots show activation was measured the by expression of HLA-DR and CD38 on $CD4^+$ and $CD8^+$ T cells respectively.

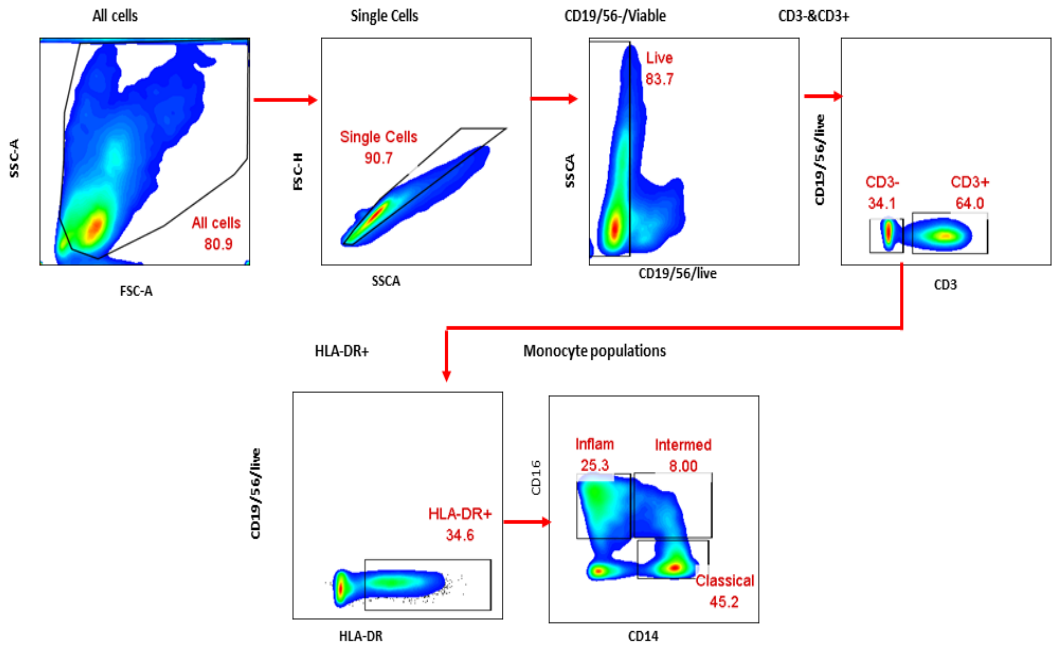


Figure 3.2: Identification of blood monocyte subsets by flow cytometry. Gating strategy for identification of monocyte subsets showing firstly gating for single cells and successive exclusion of NK cells and B cells as well as gating on live cells. This was followed by gating for CD3 negative and positive cells. HLA-DR expression was gated on from the CD3 negative cells followed by CD14 vs CD16 to differentiate three (classical, intermediate and inflammatory) monocyte subsets.

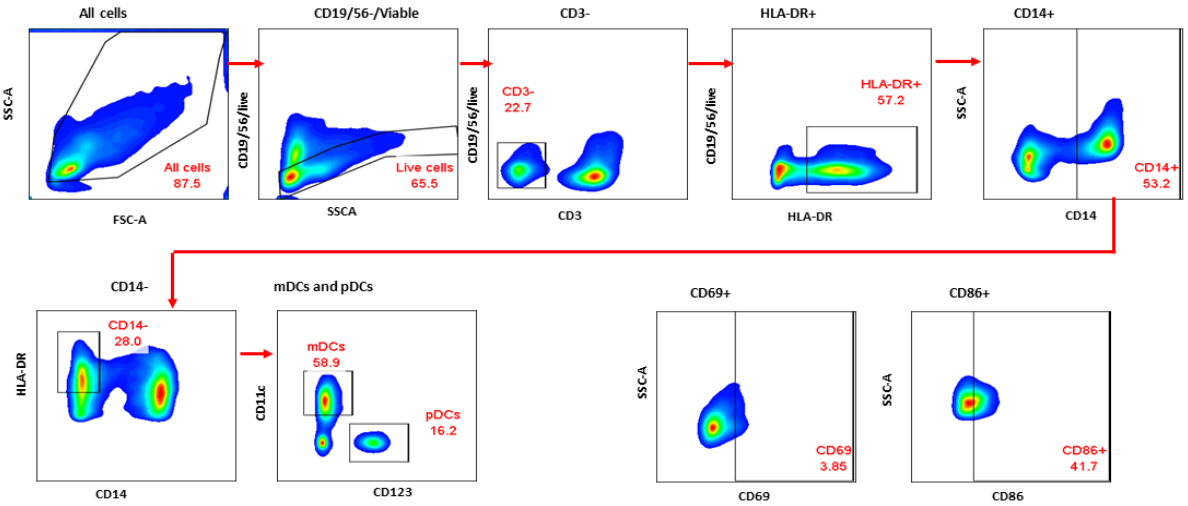


Figure 3.3: Representative gating strategy for the identification of bulk CD14, mDC and pDC subset and measurement of activation by flow cytometry. The first dot plot shows forward versus side scatter and all cells were gated on followed by exclusion of NK and B cells, along with dead cells. HLA-DR expression was gated from CD3 negative cells followed by CD14 expression. The subsequent plots were based on the expression of CD86 and CD69 on $CD14^{+}$ cells for monocyte activation and on CD14 negative for CD11c and CD123. Fluorescence minus one (FMO) controls were used to determine the respective gates. Activation in each of the cell populations was based on the expression of CD86 and CD69 markers.

Cytokine production following toll-like receptor ligand stimulation was determined by flow cytometry. One and a half million PBMCs were stimulated with 1 μg/ml LPS (Merck), 1 μg/ml CL097 (Invivogen) or 500 μM ODN2216 (Invivogen). Unstimulated cells served as negative controls. 5 μg/ml brefeldin A (Sigma) was immediately added to each tube following the addition of TLR ligands to inhibit cellular cytokine release. Intracellular cytokine content of cells was determined after 18 hours of incubation with the respective TLR ligands. All samples were acquired on the LSR II. The percentage of cytokine-producing monocytes, mDCs and pDCs was determined by FlowJo (Treestar Inc). The gating strategy is shown (Figure 3.4).

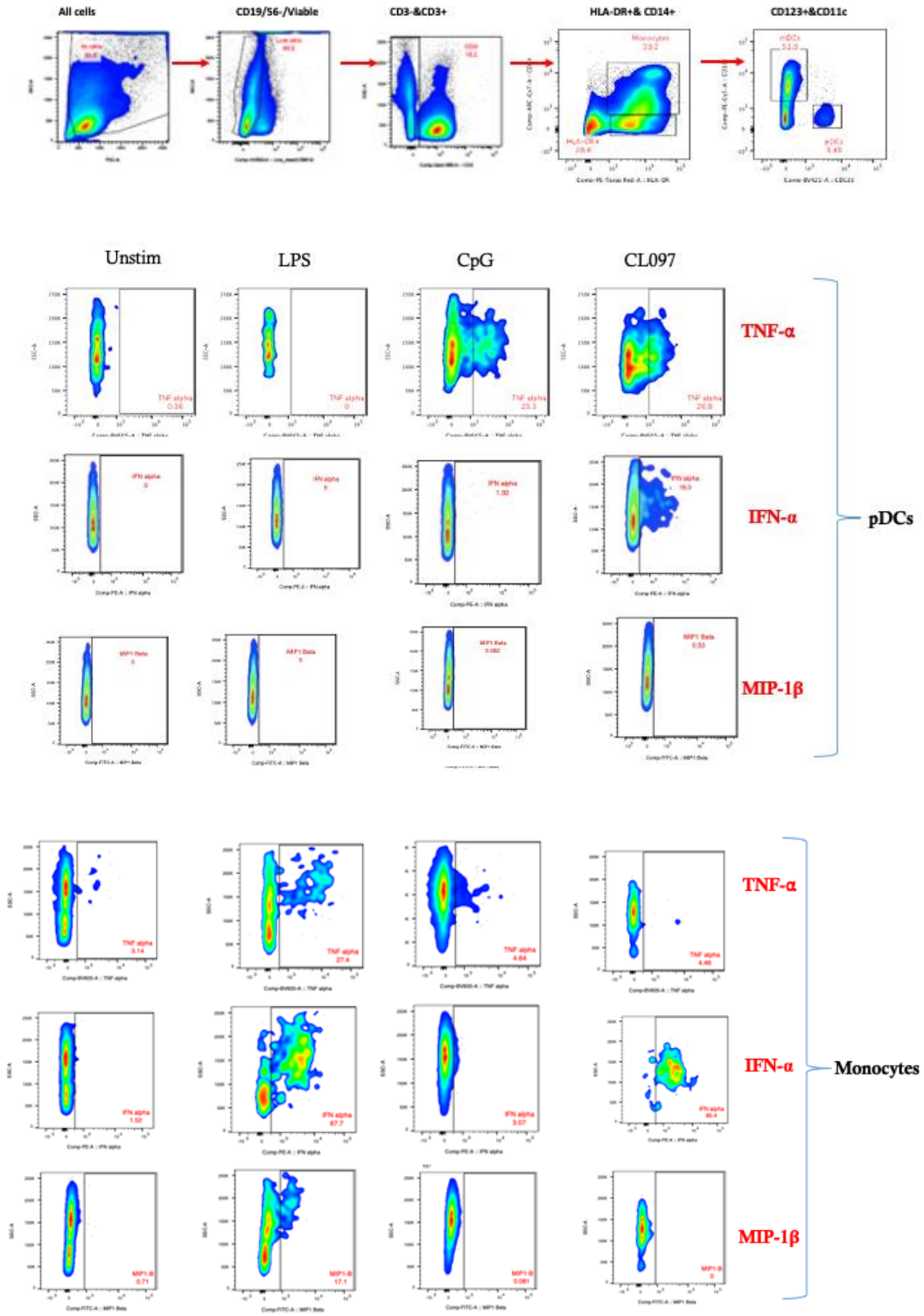


Figure 3.4: Representative gating strategy for determining the percentage of cytokine-producing monocytes, mDCs and pDCs. The first dot plot shows forward versus side scatter and all cells were gated on followed by exclusion of NK and B cells, along with dead cells. HLA-DR and CD14 expression was gated from CD3. Monocytes were CD14 positive and from these we determined cytokine expression. From the HLA-DR positive population, we further gated on expression ofCD11c (mDCs) and CD123 (pDCs) and also determined cytokine expression. The representative plots below are for TNF-αexpression when unstimulated, stimulated with LPS, CpG and CL097.

3.2.2 Statistical analysis

For the analysis of flow cytometry data, FlowJo version 10.5.2, and GraphPad Prism version 5.01 (GraphPad Inc) were used for the graphical representation and non-parametric univariate analyses. Comparisons of paired samples between time points within the same group of individuals were assessed using Wilcoxon matched pairs signed rank test. Comparisons between different groups of individuals were assessed using Wilcoxon rank-sum test (Mann-Whitney U test). To determine the associations of different immune markers and PTD, allowing for baseline viral load, regression models were developed in Stata version 15 (Stata Corp); frequencies of classical and intermediate monocytes were summed because their significant associations with PTD in univariate analyses had similar directionality. To explore the potential use of monocyte activation as a biomarker for PTD, we estimated the sensitivity and specificity of bulk $CD14^{+}CD86^{+}$ cells thresholds and used the receiver operating characteristic (ROC) curves to assess its ability to discriminate between PTD and AGA in the logistic regression models. The optimal cut-off for the bulk monocytes expressing CD86 was descriptively determined as the intersection between sensitivity and specificity estimated at various predefined cut-off values.

3.3 Results

Median age was 32 years (IQR: 26-36) (Table 1). Of the 90 women, 47 initiated ART pre-pregnancy (stable on ART), and 43 at first ANC (initiators); most (n=79, 88%) were on TDF-3TC-EFV.

Table 3.1. Demographic and clinical characteristics of women who initiated ART during or before pregnancy.

	Total	Initiation before pregnancy				Initiation during pregnancy			
	N=90	N=47				N=43			
		AGA	PTD	SGA	P-value	AGA n=15	PTD n=13	SGA n=15	P-value
		N=15	N=17	N=15					
Maternal characteristics									
Age, years: median (IQR)	32 (26-36)	33 (28-35)	36 (32-38)	36 (28-39)	0.205	28 (24-34)	26 (25-31)	31 (25-37)	0.768
Education (finished high school)	32 (35)	5 (33)	5 (29)	5 (33)	0.962	7 (47)	6 (46)	4 (27)	0.450
Employments status: Employed (%)	33 (36)	5 (33)	7 (41)	6 (40)	0.889	6 (40)	3 (23)	6 (40)	0.565
Socioeconomic status (SES)*					0.741				0.860
Lowest	28(31)	3 (20)	6 (35)	5 (33)		4(28)	4(30)	6(40)	
Medium	30(33)	6 (40)	5 (30)	5 (33)		5(35)	5(38)	4(26)	
Highest	29(29)	6 (40)	6 (35)	4 (27)		6(46)	3(23)	4(26)	
Missing	3 (3)	0 (0)	0 (0)	1 (7)		0 (0)	1(7)	2(13)	
Obstetric characteristics									
Gravidity: median (IQR)	3 (2-3)	3 (2-4)	3 (2-3.5)	4 (3-4)	0.420	2 (2-3)	2 (2-3)	2 (1-2)	0.347
Parity: median (IQR)	1(0-2)	1 (1-2)	1 (1-2)	2 (1-2)	0.244	1 (0-1)	1 (0-2)	1 (0-2)	0.315
Previous Preterm*: Yes	9 (10)	0 (0)	2 (12)	5 (33)	0.034	1 (7)	0 (0)	1 (7)	0.635

Gestational age at booking/enrolment: median weeks (IQR)	15 (11-18)	13 (9-15)	16 (9-17)	15 (9-18)		14 (12-17)	19 (14-21)	16 (12-18)	
Height, (cm): median (IQR)	158 (155.5-162.5)	162 (156-166)	158 (155.5-161)	157 (150.5-163)	0.343	160 (156.5-169.5)	158 (155.5-160)	159 (152.5-161.1)	0.161
Haemoglobin (g/dl): median (IQR)	11,4 (10-3-12.4)	11 (11.1-12)	11.8 (11-12.7)	11.6 (10.6-12.9)	0.283	10.5 (10-12)	11.3 (10.2-11.7)	10.9 (10.1-11.6)	0.697
Weight (kg): median (IQR)	70.15 (59.4-83.9)	74.9 (64,30-85,95	76 (67,30-85,45)	61.7 (52,50-74,70)	0.126	80,10 (61,70-98,40)	61,35 (58,28-67,48)	64,00 (56,70-92,00)	0.413
HIV-associated parameters									
Current ART regimen, self-report					0.199				0.353
TDF-3TC-EFV	79 (88)	13 (86)	13	13 (86)		14 (93)	11 (84)	15 (100)	
TDF-3TC-NVP	1 (1)	0 (0)	0	0 (0)		1 (7)	0 (0)	0 (0)	
Other NNRTI-based regimen	3 (3)	0 (0)	3	0 (0)		0 (0)	0 (0)	0 (0)	
PI-based regimen	6 (6)	2 (14)	1	2 (14)		0 (0)	1 (8)	0 (0)	
Missing	1 (1)	0 (0)	0 (0)	0 (0)		0 (0)	1 (8)	0 (0)	
CD4 cell count, (cells/µL)* median (IQR)	436 (392-573)	416 (353-566)	529 (309-638)	485 (333-584)	0.509	368.5 (247-535)	314 (219-457)	493 (283.5-926.5)	0.724
Missing	14 (16)	2 (13)	2 (11)	0 (0)		3 (20)	4 (30)	3 (20)	
Viral load, copies/ml (baseline A1), median (IQR)	281 (20-80000)	20 (20-27)	25 (20-528)	20 (20-65)	0.342	21900 (6120-59900)	4730 (1300-7950)	2120 (741-18800)	0.681

Viral load, copies/ml (A1.5), median (IQR)	198 (37-431)		431 (275-512)	99 (20-137)	89 (25-307)	0.172

* Study participants are denoted by number with percentages in brackets

*SES was measured using a composite socioeconomic status score, based on current employment, housing type and access to household assets, that was used to categorize women as ‘high’, ‘mid’ or ‘low’ SES. A median of these is shown above.

*CD4 results abstracted from routine records and are the nearest in time to the first ANC visit.

*All study participants were normotensive. Data was missing for 10 patients initiating therapy during pregnancy and 4 patients stable on ART.

*P values denote to the comparison across the three groups (AGA, SGA and PTD).

3.3.1 CD8+ but not CD4+ T cell activation at baseline is associated with PTD

The co-expression of CD38 and HLA-DR on CD4+ T cells declined significantly between baseline and the last time point for patients initiating ART but not in the ART-stable group (Figure 3.5A). In initiators at both baseline (ART-naïve) and two weeks post-ART initiation, CD4+ T cell activation levels were similar for women with AGA, SGA or PTD (Figure 3.5B); likewise, there was no significant difference by pregnancy outcome in women stable on ART (Figure 3.5C). There was a decrease in CD8+ T cell activation for patients initiating ART, with a lower magnitude but significant decline for participants stable on ART (Figure 3.5D). Activation levels were lowest for the PTD cases in the initiating group both at baseline (ART-naïve) and 2-weeks thereafter (ART-exposed) compared to the AGA controls and SGA cases (Figure 3.5E). There was no significant difference in $CD8^+$ T cell activation by outcome for women stable on ART; this was true also for later time points (Figure 3.5F and data not shown). Interestingly, in the initiators, PTD and SGA cases had significantly lower median viral load compared to controls; lower viral loads were noted for those stable on ART but there were no significant differences by pregnancy outcome (5). Overall, these data show reduced activation in $CD8^+$ but not $CD4^+$ T cells for women initiating ART during pregnancy; further, PTD cases who initiate ART in pregnancy have low activation of $CD8^+$ T cells and this is partly explained by lower viremia in the PTD group.

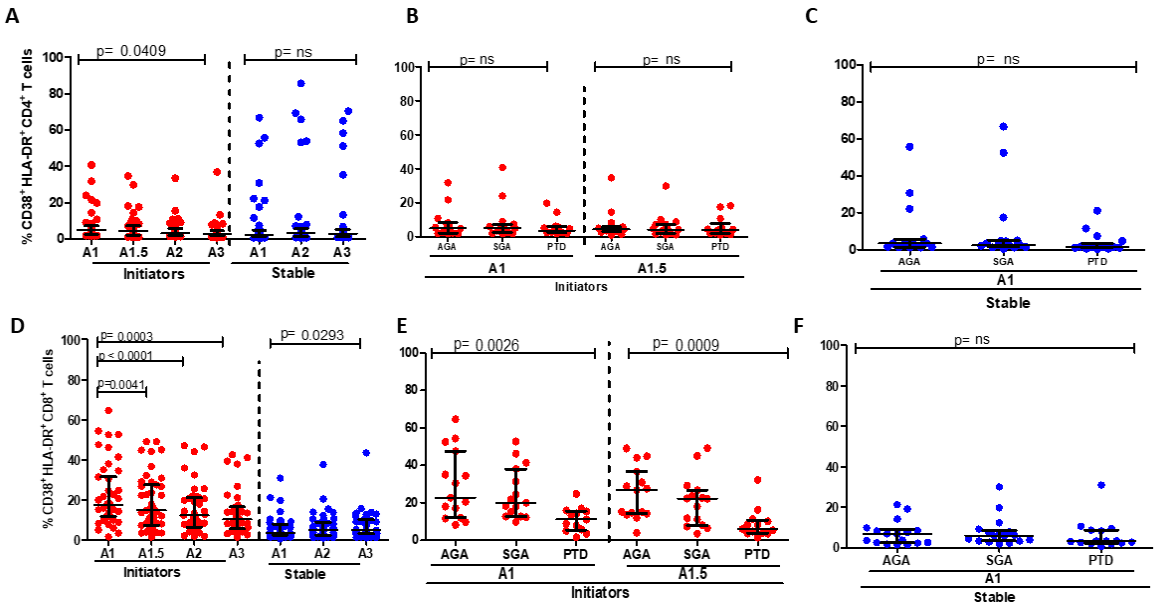


Figure 3.5: CD4 and CD8 activation. A) $CD4^+$ T cell activation levels for women initiating (red circles) and stable on ART (blue circles) over time and not stratified by birth outcome. B) $CD4^+$ T cell activation levels by birth outcomes for women initiating ART (in red) at baseline (A1) and two weeks post ART initiation (A1.5) (C) $CD4^+$ T cell activation levels by birth outcomes for women stable on ART (blue) at baseline (A1). D) $CD8^+$ T cell activation levels for women initiating (red circles) and stable on cART (blue circles) over time. E) $CD8^+$ T cell activation levels by birth outcomes for women initiating ART (in red) at baseline (A1) and two weeks post ART initiation (A1.5). (F) $CD8^+$ T cell activation levels by birth outcomes for women stable on ART (blue) at baseline (A1).

3.3.2 Monocyte subsets are associated with PTD

Classical monocyte ($CD14^{+}CD16^{-}$) frequencies increased significantly over time for initiators, but not for women stable on ART (Figure 3.6A). Stratified by ART timing and pregnancy outcome, classical monocytes frequencies were consistently higher for PTD cases than SGA cases and AGA controls, initiating ART with no significant difference observed for those stable on ART (Figure 3.6B & C). We observed significant changes in intermediate monocyte frequencies in women initiating and stable on ART when looking at patients over time (Figure 3.6D). Notably, intermediate monocyte frequencies were higher in PTD than in SGA cases and AGA controls for initiators, both at baseline and 2 weeks post-ART initiation (Figure 3.6E), but no differences were observed in those stable on ART (Figure 3.6F). Inflammatory monocyte frequencies did not significantly change over time in either initiators or stable on ART (Figure 3.6G). Among initiators, inflammatory monocytes frequencies were lower in PTD than SGA cases and AGA controls (Figure 3.6H), with no significant differences for those stable on ART (Figure 3.6I). In summary, these data show an increase in the classical and intermediate monocyte populations in women initiating ART, with a reduction in the inflammatory subset.

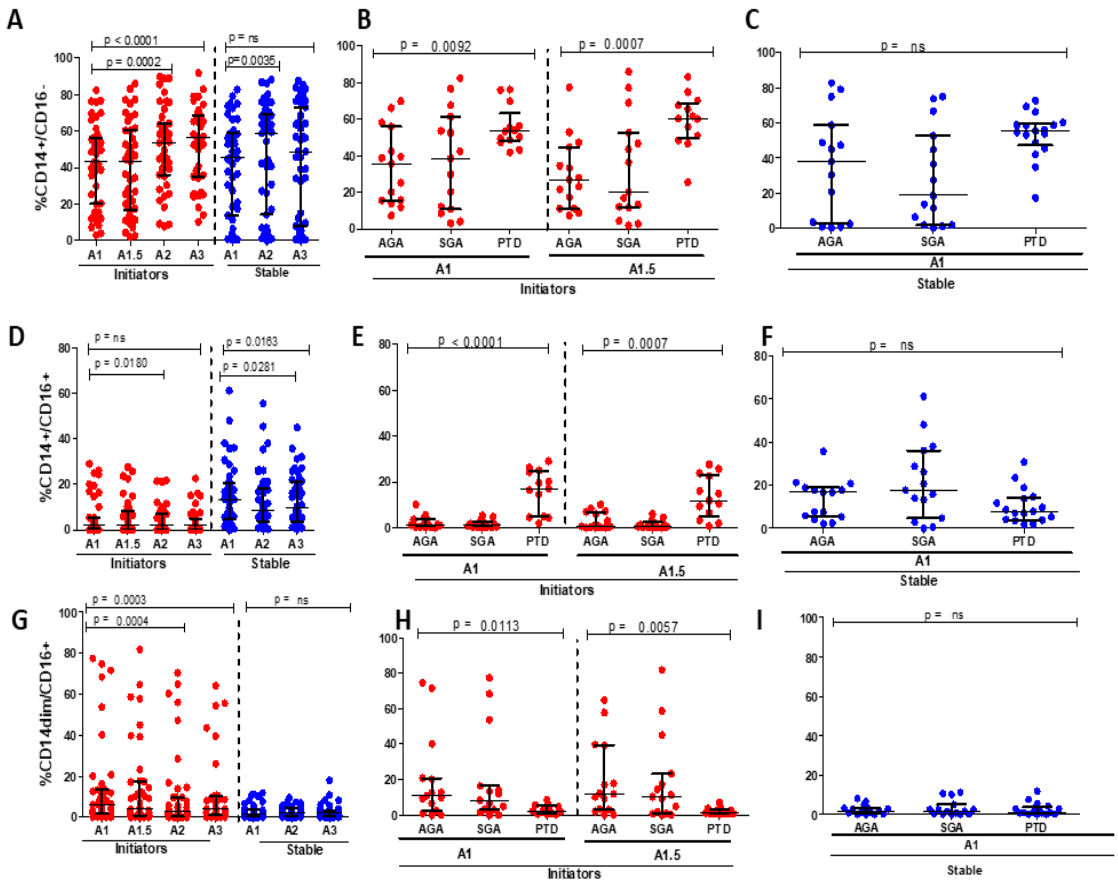


Figure 3.6: Monocyte populations. A) Classical monocyte (CD14+CD16-) levels for women initiating (red circles) and stable on ART (blue circles) over time and not stratified by birth outcome. B) Classical monocyte levels by birth outcome for women initiating ART (in red) at baseline (A1) and two weeks post ART initiation (A1.5). (C) Classical monocytes levels by birth outcomes for women stable on ART (blue) at baseline (A1). D) Intermediate monocyte (CD14+CD16+) levels for women initiating (red circles) and stable on ART (blue circles) over time and not stratified by birth outcome. E) Intermediate monocyte levels by birth outcomes for women initiating ART (in red) baseline (A1) and two weeks post ART initiation (A1.5) (F) Intermediate monocyte levels by birth outcomes for women stable on ART (blue) at baseline (A1). G) Inflammatory monocyte (CD14dimCD16+) levels for women initiating (red circles) and stable on ART (blue circles) over time and not stratified by birth outcomes. H) Inflammatory monocyte levels by birth outcomes for women initiating ART (in red) baseline (A1) and two weeks post ART initiation (A1.5) (I) Inflammatory monocyte levels by birth outcomes for women stable on ART (blue) at baseline (A1).

3.3.3 Lower frequencies of bulk monocyte, mDC and pDC activation (CD86+ and CD69+) are associated with PTD

There was no significant difference in levels of CD86 and CD69 expression in bulk monocytes over time for both initiators and those stable on ART (Figure 3.7A & D). Stratified by outcome, expression of both CD86 (Figure 3.7B & C) or CD69 (Figure 3.7E & F) on monocytes was significantly lower in PTD cases in both ART groups.

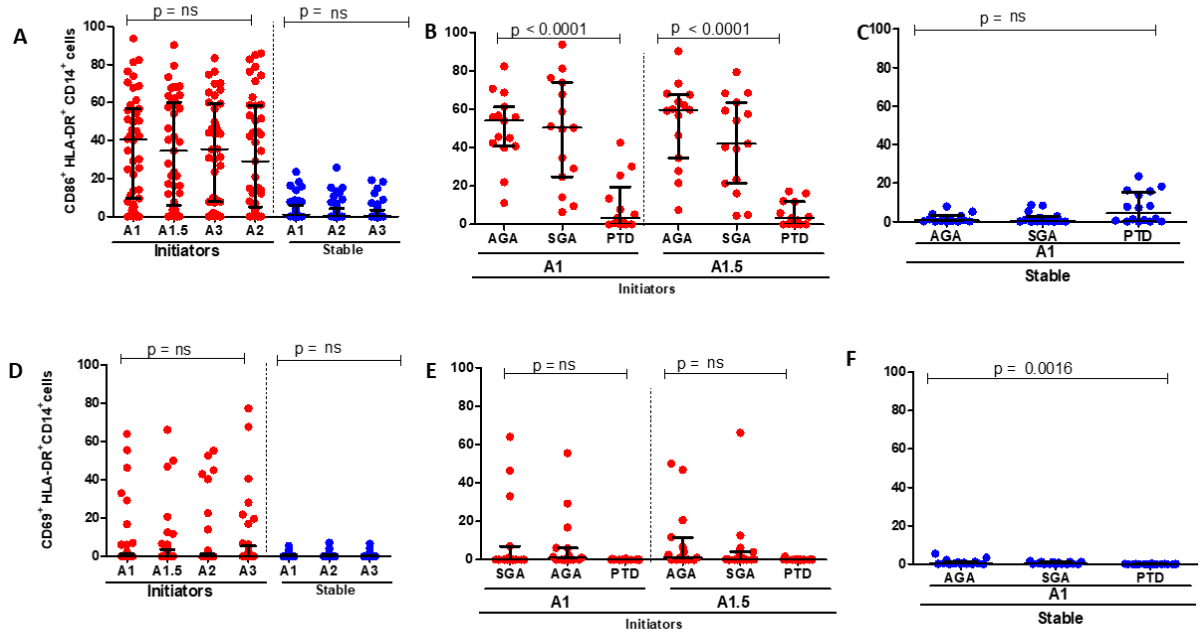


Figure 3.7: **Monocyte activation**. A) $CD86^{+}HLA\text{-}DR^{+}$ expression in bulk monocytes in women initiating (red circles) and stable (blue circles) on ART over time, not stratified by outcome. B) $CD86^{+}HLA\text{-}DR^{+}$ expression outcomes in bulk monocytes for women initiating ART (in red) at baseline (A1) and two weeks post ART initiation (A1.5). C) $CD86^{+}HLA\text{-}DR^{+}$ expression by birth outcomes in bulk monocytes for women stable on ART (blue) at baseline (A1). (D) $CD69^{+}HLA\text{-}DR^{+}$ expression in bulk monocytes in women initiating (red circles) and stable (blue circles) on ART over time. E) $CD69^{+}HLA\text{-}DR^{+}$ expression outcomes in bulk monocytes for women initiating ART (in red) baseline (A1) and two weeks post ART initiation (A1.5). (F) $CD69^{+}HLA\text{-}DR^{+}$ expression outcomes in bulk monocytes for women stable on ART (blue) at baseline (A1).

Levels of CD86 and CD69 expression on mDCs was not significantly different in initiators and those stable on ART; although expression was higher in initiators when ART-naïve than those stable on ART (Figure 3.8A & D). CD86 levels were significantly lower in PTD cases than in AGA or SGA for both ART timing groups (Figure 3.8B and C); results were similar for CD69 expression but for women initiating ART only (Figure 3.8E & F).

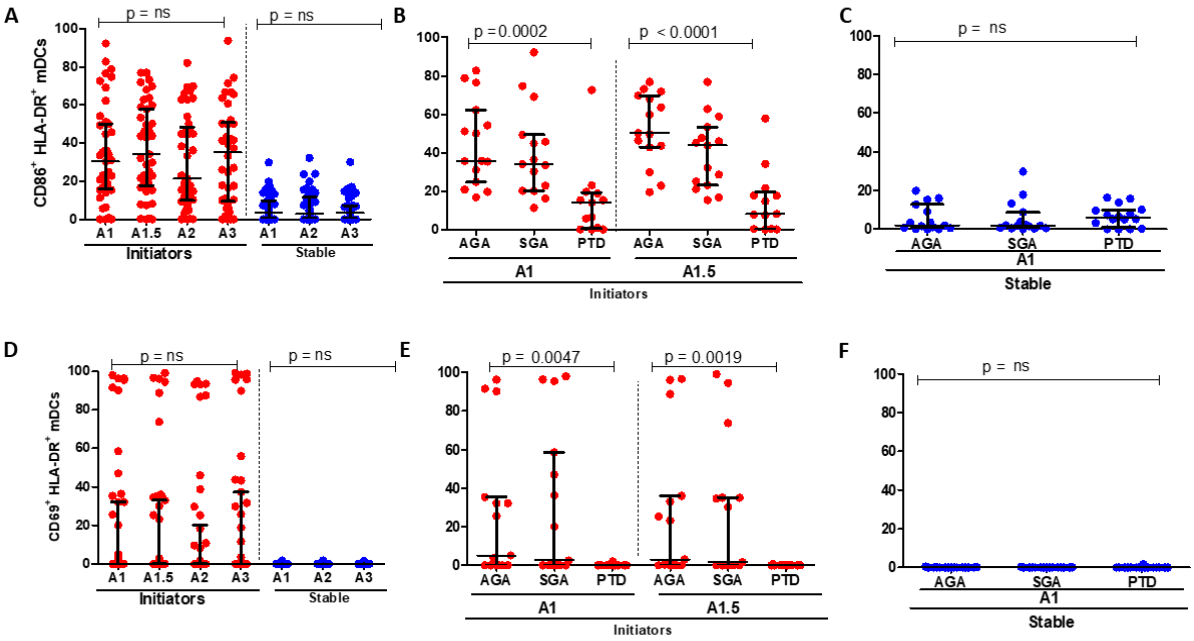


Figure 3.8: mDC activation. A) $CD86^{+}HLA\text{-}DR^{+}$ expression in mDCs in women initiating (red circles) and stable (blue circles) on ART over time and not stratified by birth outcome. B) $CD86^{+}HLA\text{-}DR^{+}$ expression by birth outcomes in mDCs for women initiating ART (in red) at baseline (A1) and two weeks post ART initiation (A1.5). C) $CD86^{+}HLA\text{-}DR^{+}$ expression by birth outcomes in mDCs for women stable on ART (blue) at baseline (A1). (D) $CD69^{+}HLA\text{-}DR^{+}$ expression in mDCs in women initiating (red circles) and stable (blue circles) on ART over time and not stratified by birth outcome. E) $CD69^{+}HLA\text{-}DR^{+}$ expression by birth outcomes in mDCs for women initiating ART (in red) baseline (A1) and two weeks post ART initiation (A1.5). (F) $CD69^{+}HLA\text{-}DR^{+}$ expression by birth outcomes in mDCs for women stable on ART (blue) at baseline (A1).

Expression of both CD86 and CD69 on pDCs did not differ over time (Figure 3.9A & D). Expression of CD86 and CD69 was lowest for PTD cases in initiators; for the ART stable group there were no significant differences in expression by pregnancy outcome (Figure 3.9B, C, E and F). Overall, these data demonstrate low activation in APCs in PTD cases compared to AGA and SGA, especially true for women initiating ART in pregnancy at baseline.

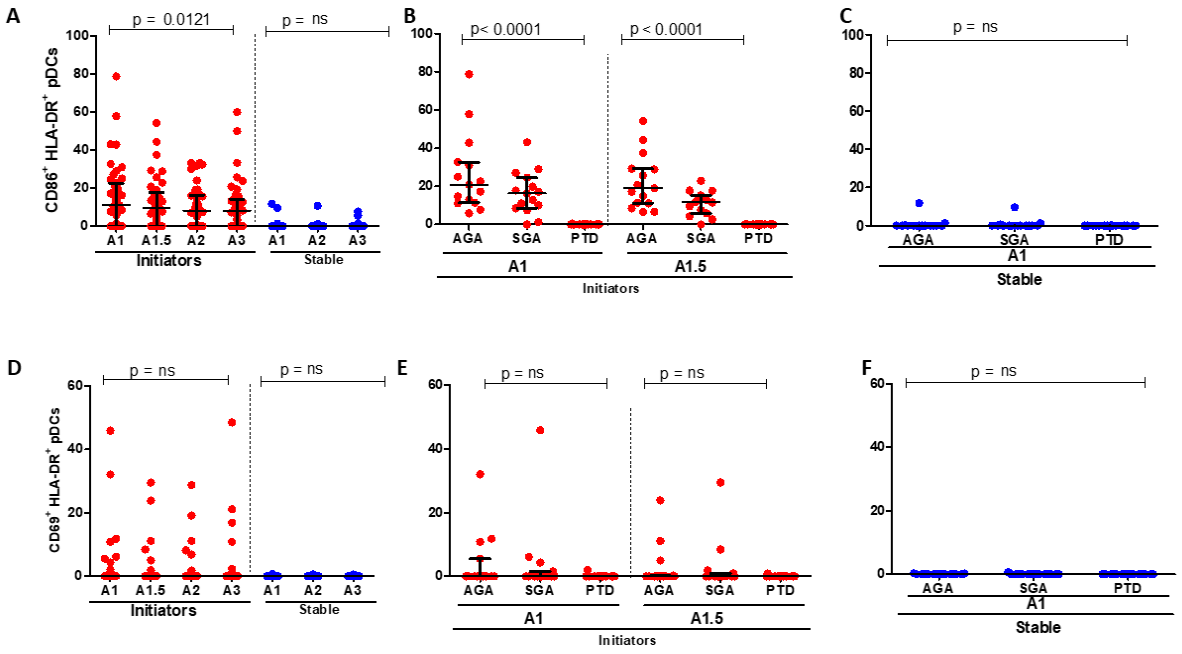


Figure 3.9: **pDC activation**. A) $CD86^{+}HLA\text{-}DR^{+}$ expression in pDCs in women initiating (red circles) and stable (blue circles) on ART over time and not stratified by birth outcome. B) $CD86^{+}HLA\text{-}DR^{+}$ expression by birth outcomes in pDCs for women initiating ART (in red) baseline (A1) and two weeks post ART initiation (A1.5). C) $CD86^{+}HLA\text{-}DR^{+}$ expression by birth outcomes in pDCs for women stable on ART (blue) at baseline (A1). (D) $CD69^{+}HLA\text{-}DR^{+}$ expression in pDCs in women initiating (red circles) and stable (blue circles) on ART over time and not stratifies by birth outcome. E) $CD69^{+}HLA\text{-}DR^{+}$ expression by birth outcomes in pDCs for women initiating ART (in red) baseline (A1) and two weeks post ART initiation (A1.5). (F) $CD69^{+}HLA\text{-}DR^{+}$ expression by birth outcomes in pDCs for women stable on ART (blue) at baseline (A1).

We next explored whether a difference in baseline CD4 count confounded the associations between immune activation levels and PTD (Table 2). SGA cases were not included given the small and insignificant univariate differences between AGA controls and SGA cases (data not shown). For women initiating ART in pregnancy, lower monocyte activation and higher levels of classical and intermediate monocytes remained significantly associated with PTD at baseline (ART-naïve) (Table 3.2), but not for those stable on ART. Thus, we were able to study the confounding effects of CD4 on immune status of the women.

Table 3.2: Logistic regression analysis adjusting for baseline viral load (VL): association between immune activation markers and PTD in women initiating ART at first ANC

		Univariable analysis		Analysis allowing for VL	
		Odds Ratio (95% CI)	**P value**	**Odds Ratio (95% CI)**	**P value**
1	**Bulk CD14$^+$ monocyte activation (HLA-DR/CD86)**	0.89 (0.83-0.96)	0.003	0.89 (0.82-0.97)	0.006
	Baseline Log VL			0.29 (0.03 -2.9)	0.291
2	**mDC activation (HLA-DR/CD86)**	0.92 (0.87-0.98)	0.013	0.93 (0.87-0.99)	0.028
	Baseline Log VL			0.67 (0.21-2.14)	0.502
3	**CD8$^+$ T cell activation (HLA-DR/CD38)**	0.86 (0.76-0.98)	0.026	0.87 (0.76-1.00)	0.045
	Baseline Log VL			0.66 (0.21-2.11)	0.486
4	**Classical and intermediate monocytes**	1.22 (1.03-1.44)	0.021	1.20 (1.02-1.42)	0.031
	Baseline Log VL			0.62 (0.14- 2.74)	0.526

*The models included PTD cases and AGA controls

We next explored whether monocyte activation was a potential biomarker in early pregnancy to identify women at increased risk of PTD. For women initiating ART in pregnancy the area under the ROC curve associated with PTD was 0.905 and 0.931 at and two weeks after ART initiation, respectively. Further, the optimal predictive cut-off for the bulk $CD14^{+}CD86^{+}$ cells that optimizes on both sensitivity and specificity was approximately 20% both at ART initiation and 2 weeks later (Figure 3.10). Overall, these data suggest that monocyte activation is a potential biomarker to identify those at risk of PTD among HIV infected women commencing ART in pregnancy.

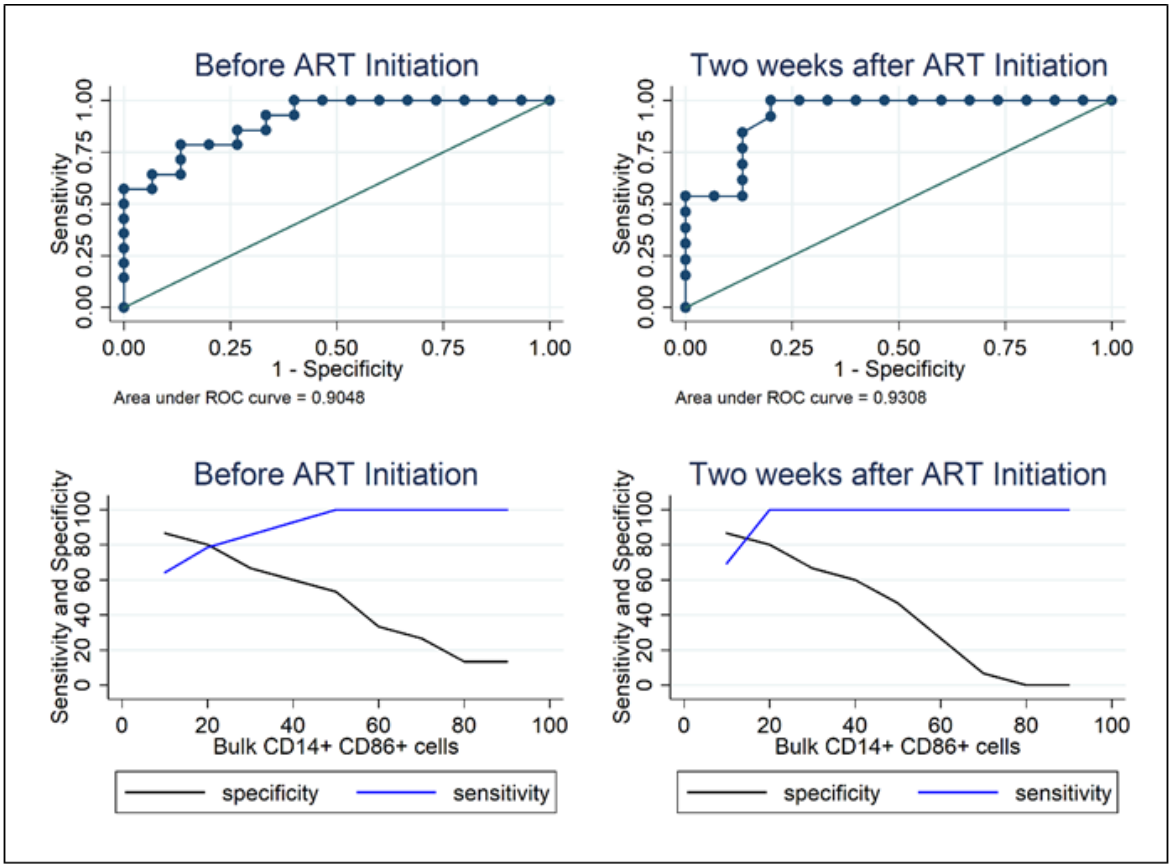


Figure 3.10: Diagnostic accuracy of bulk monocyte activation for predicting PTD. A) Diagnostic accuracy of bulk monocyte activation (Bulk CD14+CD86+) for predicting PTD before ART initiation and 2 weeks after ART initiation. Row 1 shows the ROC curves and row 2 shows the sensitivity (blue) and specificity (black) of monocytes at various predefined cut-off points.

3.3.4 Monocyte TLR ligand induced production of some cytokines is lower in PTD women initiating ART in pregnancy.

In analysis involving all cellular markers of activation and monocyte subsets, bulk monocyte activation and monocyte subsets are associated with PTD. We hypothesized that lower monocyte activation may reflect senescence or refractoriness to stimulation upon microbial exposure. To address this possibility, we performed intracellular cytokine staining (ICS) to quantify the percentage of monocytes producing IFN-α, TNF-α or MIP-1β after stimulation with TLR4 ligand (LPS), TLR7/8 ligand (CL097) or TLR9 ligand ODN2216 (CpG).

The percentage of monocytes producing IFN-α in response to all TLR ligands over time did not differ for both those initiating ART and stable on ART (data not shown). When stratified by outcome; patients with the PTD outcome had lower IFN-α expression among ART initiators compared to AGA or SGA (Figure 3.11A), with no significant difference noted for those stable on ART (Figure 3.11B). Monocytes from PTD women produced significantly lower levels of MIP-1β in response to all three TLR ligands in women initiating ART, with no such differences noted for women stable on ART (Figure 3.11C & D).

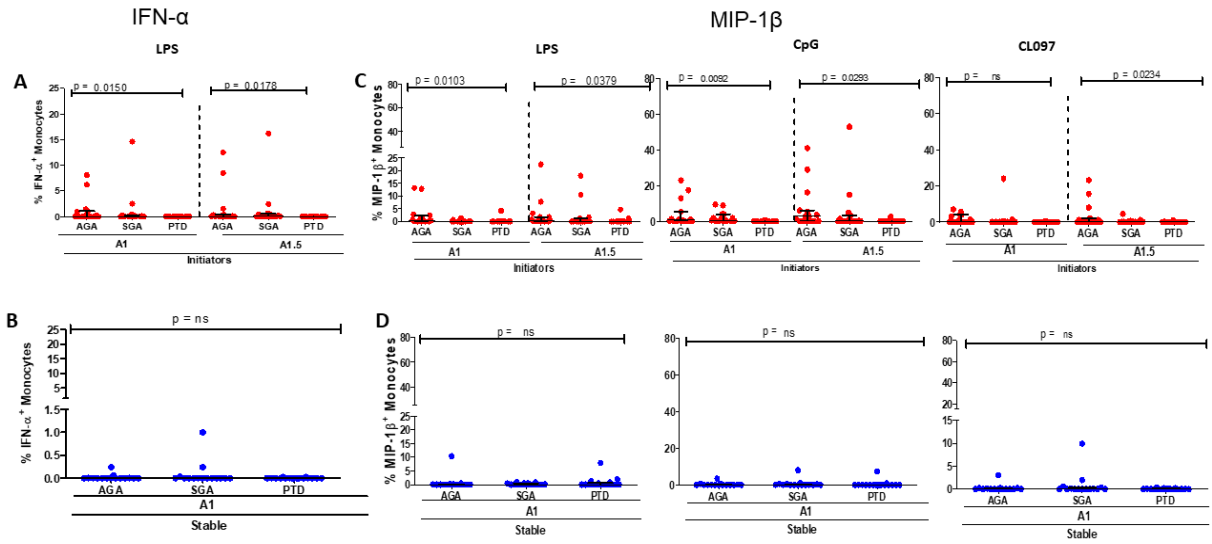


Figure 3.11: Monocyte IFN-α and MIP-1β expression upon TLR stimulation. A) % IFN-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS for patients initiating ART (red circles). B) % IFN-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS for patients stable on ART (blue circles). C) % MIP-1β expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). D) % MIP-1β expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

Although as expected monocytes generally expressed higher levels of TNF-α compared to other cytokines in response to TLR ligand stimulation, there were no significant differences by outcome (Figure 3.12A & B).

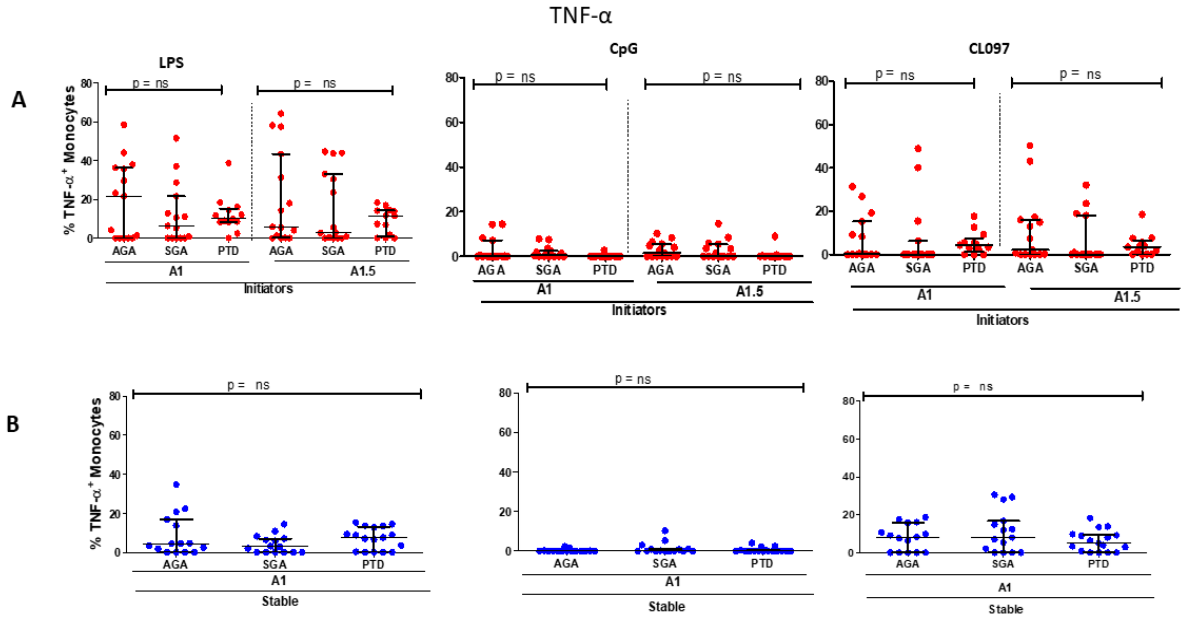


Figure 3.12: Monocyte TNF-α expression upon TLR stimulation. A) % TNF-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). B) % TNF-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

3.4 Summary

We hypothesised that immune activation status, innate immune cell subsets and their phenotypes or functionality modulated by ART status (initiated pre- or during pregnancy) would be associated with PTD or SGA. In our case-control study, lower $CD8^+$ T cell, monocyte, mDC and pDC activation (particularly as defined by CD86 but not CD69 expression for the antigen presenting cells) were all strongly associated with subsequent PTD for women initiating ART in pregnancy but not those who started ART before pregnancy, after allowing for baseline viral load. The association of lower immune activation with PTD in ART initiators was observed before initiation of ART, and at two weeks post-ART initiation. Further, our findings suggest differences in monocyte subsets by pregnancy outcome with inflammatory monocytes frequencies lower (and vice-versa for classical and intermediate monocytes) among ART initiators with subsequent PTD. For most of the immune parameters, women with SGA had similar profiles to control women. ROC curve analysis suggested monocyte activation (CD86 expression) below 20% at approximately 15 weeks of gestation as a potential biomarker to identify women at risk for PTD. Interestingly, TLR4-induced monocyte expression of IFN-α, and TLR4/TLR-7/8/9 induced expression of MIP-1β was decreased in PTD cases in initiators, suggesting that the reduced immune activation may be indicative of reduced responsiveness to antigen stimulation (immune senescence) as an underlying mechanism. Overall, our findings strongly implicate reduced immune activation as an underlying biomarker for PTD but not SGA.

Notably, lower immune activation was associated with PTD mostly in ART-initiating women and not those stable on ART, suggesting that long-term ART may be leading to correction of the underlying immunological dysfunction. This result further suggests that immune activation

is likely only a surrogate for a yet undetermined immunological dysfunction since women stable on ART with overall reduced immune activation did not have higher incidence of PTD. As expected, initiation of ART rapidly lowered immune activation, with noticeable reduction at 2 weeks following ART initiation but lowered immune activation remained associated with PTD even at that stage, suggesting that the immune defect in ART-naïve individuals associated with PTD is not immediately corrected by ART.

Our study could not definitively identify the immunological dysfunction underlying PTD. However, it has also been suggested that increased inflammation may potentiate adverse pregnancy outcomes including PTD (Romero et al., 2006, Cappelletti et al., 2015). Inflammation as an underlying factor for PTD may be indicative of underlying infection as a causative factor for PTD, with the reduced immune activation observed in our study a possible surrogate of reduced potential of immune cells to become activated and respond to infection. A recent transcriptomic study identified adaptive and innate immune genes as risk factors for PTD, although results contrasted when maternal peripheral blood and cord blood, with data from the latter samples (downregulation of innate immune genes) more consistent with our results (Vora et al., 2018). Our findings are consistent with previous reports that PTD infants display a reduced ability to respond to pathogens ex vivo (Goedicke-Fritz et al., 2017, Lavoie et al., 2010), which would be expected if they express lower levels of the costimulatory molecule CD86 on monocytes as we observed. Overall, our findings suggest that reduced immune activation, which may be linked to reduced immune responsiveness to pathogen insult, could precede PTD and indicate an underlying mechanism, particularly in women initiating ART during pregnancy.

Chapter 4 Evaluation of phenotype and function of immune cell subsets in late pregnancy and association with small-for-gestational age and preterm birth outcomes .

4.1 Introduction

During late pregnancy, circulating maternal leukocytes (innate and adaptive) are recruited into reproductive tissues (cervix and myometrium) and to the maternal/fetal interface (decidual tissues) by chemotactic processes (Gomez-Lopez et al., 2009, Gomez-Lopez et al., 2010, Gomez-Lopez et al., 2012, Sivarajasingam et al., 2016, Faas and De Vos, 2018), where a pro-inflammatory state develops and leads to labor and delivery of the baby (Romero et al., 1989, Romero et al., 2006, Gotsch et al., 2009, Berghöfer et al., 2006). It is thought that the premature activation of this pro-inflammatory pathway can lead to a breakdown of fetomaternal tolerance and play a role in the induction of labor, which subsequently can result in preterm birth (Törnblom et al., 2005).

The first line of defense against pathogens is the innate immune system and consists of innate immune cells which are able to respond to infections in a timeous manner through the recognition of pathogen associated molecular patterns (PAMPs) by receptors such as toll-like receptors (TLRs) (Chang et al., 2012). Studies have shown that HIV-1 ssRNA encodes for multiple PAMPs that can be recognized by TLR7 expressed in plasmacytoid dendritic cells (pDC) and TLR8 expressed in both monocytes and myeloid dendritic cells (mDC). As outlined in chapter 1, the level of immune activation in HIV-1-infected individuals is a strong independent predictor for HIV-1 disease progression. Previously, PBMCs derived from women have been shown to produce significantly more IFN-α in response to synthetic TLR7 ligand than PBMCs derived from men (Berghöfer et al., 2006, Ziegler and Altfeld, 2017).

HIV-1 infects several immune cells including dendritic cells (DCs) and monocytes, which contributes to both dissemination of HIV-1 infection and induction of antiviral immunity. These cells produce high amounts of type I IFN and proinflammatory cytokines upon toll-like receptor (TLR) stimulation. During HIV-1 infection, an altered production of proinflammatory

cytokines has been reported (McKenna et al., 2005). However, the mechanisms and specific immune subsets underlying cytokine modulation have not been well described.

Little is known about the immune-related changes necessary for a successful pregnancy in women whose immune status is altered by HIV infection, and how cART initiated during pregnancy affects these immune responses. Reports suggest increased adverse pregnancy outcomes in HIV-1-infected women compared to uninfected controls, but whether that is an effect of HIV per se or of associated disease progression is unclear. Previous studies have looked at pregnant women that had either not been on cART or had taken it for an average of 18 weeks. These studies had not investigated the impact of the exposure to cART on the immunological status for the entire duration of the pregnancies (Hurst et al., 2015). These studies were able to indicate that HIV infection is associated with preterm delivery and low birth weight (Powis et al., 2011, Shapiro et al., 2010) The fetus, a semi-allogeneic graft, grows and develops within the mother without succumbing to immunological rejection, a process that depends on the proper establishment of feto-maternal tolerance (Gomez-Lopez et al., 2014).
This work aimed to investigate the consequences of a sudden decrease in pro-inflammatory immune responses following initiation of ART in pregnant women, compared to those who initiated treatment before pregnancy and therefore have already reduced immune activation.
We hypothesized that HIV or ART modulation of immune cell activation status or alteration of immune cells subsets during pregnancy, particularly the rapid immune recovery experienced following suppression of viremia by ART would be associated with detrimental birth outcomes (PTD or SGA). We focused on T cells, monocytes and dendritic cells over time because these cells play a central immune effector or immunoregulatory role and alterations in their activation status or other perturbations have been reported in HIV infection and pregnancy.

In this chapter, we assessed immune cell surface phenotypic changes over time and functional alterations (cytokine secretion) following stimulation of TLR7/8 (which have been shown to recognize HIV-1 encoded ssRNA), TLR9 (which utilizes the same pathway as TLR7 in inducing IFNα production), and TLR4 (which responds to lipopolysaccharide-LPS, a bacterial component). Intracellular cytokine staining (ICS) using multiparameter flow cytometry was performed to quantify the percentage of APCs producing IFN-α, TNF-α, MIP-1β and IL-12 after stimulation with HIV-1-derived TLR7/8 ligand CL097, TLR9 ligand ODN2216 (CpG), or TLR-4 ligand LPS. These responses were examined over several weeks in late pregnancy in women who conceived on ART versus those who initiated ART during pregnancy. The differences are also delineated according to birth outcome.

4.2 Methods

The Prematurity Immunology in HIV-infected Mothers and their Infants Study (PIMS) was established in Cape Town, South Africa, to investigate the association between timing of ART initiation (pre-conception or during pregnancy), immunological parameters and birth outcomes (Malaba et al., 2020). For the analysis of cell surface activation and intracellular cell staining (ICS); flow cytometric quantification of $CD4^+$ and $CD8^+$ T cells, monocytes, and dendritic cell activation was performed using frozen PBMCs using methods previously described (Mdletshe et al., 2021). The time points studied were as follows: baseline (A1), two weeks post ART initiation (A1.5), at 28 weeks (A2) and finally at birth (A3). The median weeks for gestational age was 15 (11-18).

4.3 Results

The median age of study participants was 32 years (IQR: 26-36) (Table 1). Socioeconomic status (SES) was measured using a composite socioeconomic status score, based on current employment, housing type and access to household assets, and was used to categorize women as ‘high’, ‘mid or ‘low’. There was a total of 28 (31%) women who categorised under the low classification of SES, 30 (33%) under medium and 29 under high accounting for 29% of the total number of women in the study population. There was a total of 3 women who had missing data for this parameter. Of the 90 women, 47 initiated ART pre-pregnancy (stable on ART), and 43 at first ANC (initiators). Most (n=79, 88%) of pregnant women were on TDF-3TC-EFV regimen. The median weight was 70.15 (kg) (IQR:59.4-83.9). Median CD4 count was 436 (IQR: 392-573), at baseline, viral load (VL) median was 21 copies/ml for those stable on ART and 9583 copies/ml for those initiating ART during pregnancy. We measured VL after ART initiation and found a median VL of 198 (IQR:37-431)/ copies/ml.

Table 4.1 Demographic and clinical characteristics of women who initiated ART during or before pregnancy.

	Total	Initiation before pregnancy				Initiation during pregnancy			
	N=90	N=47				N=43			
		AGA	PTD	SGA	P-value	AGA n=15	PTD n=13	SGA n=15	P-value
		N=15	N=17	N=15					
Maternal characteristics									
Age, years: median (IQR)	32 (26-36)	33 (28-35)	36 (32-38)	36 (28-39)	0.205	28 (24-34)	26 (25-31)	31 (25-37)	0.768
Education (finished high school)	32 (35)	5 (33)	5 (29)	5 (33)	0.962	7 (47)	6 (46)	4 (27)	0.450
Employments status: Employed (%)	33 (36)	5 (33)	7 (41)	6 (40)	0.889	6 (40)	3 (23)	6 (40)	0.565
Socioeconomic status (SES)*					0.741				0.860
Lowest	28(31)	3 (20)	6 (35)	5 (33)		4(28)	4(30)	6(40)	
Medium	30(33)	6 (40)	5 (30)	5 (33)		5(35)	5(38)	4(26)	
Highest	29(29)	6 (40)	6 (35)	4 (27)		6(46)	3(23)	4(26)	
Missing	3 (3)	0 (0)	0 (0)	1 (7)		0 (0)	1(7)	2(13)	
Obstetric characteristics									
Gravidity: median (IQR)	3 (2-3)	3 (2-4)	3 (2-3.5)	4 (3-4)	0.420	2 (2-3)	2 (2-3)	2 (1-2)	0.347
Parity: median (IQR)	1(0-2)	1 (1-2)	1 (1-2)	2 (1-2)	0.244	1 (0-1)	1 (0-2)	1 (0-2)	0.315

Previous Preterm*: Yes	9 (10)	0 (0)	2 (12)	5 (33)	0.034	1 (7)	0 (0)	1 (7)	0.635
Gestational age at booking/enrolment: median weeks (IQR)	15 (11-18)	13 (9-15)	16 (9-17)	15 (9-18)		14 (12-17)	19 (14-21)	16 (12-18)	
Height, (cm): median (IQR)	158 (155.5-162.5)	162 (156-166)	158 (155.5-161)	157 (150.5-163)	0.343	160 (156.5-169.5)	158 (155.5-160)	159 (152.5-161.1)	0.161
Haemoglobin (g/dl): median (IQR)	11,4 (10-3-12.4)	11 (11.1-12)	11.8 (11-12.7)	11.6 (10.6-12.9)	0.283	10.5 (10-12)	11.3 (10.2-11.7)	10.9 (10.1-11.6)	0.697
Weight (kg): median (IQR)	70.15 (59.4-83.9)	74.9 (64,30-85,95	76 (67,30-85,45)	61.7 (52,50-74,70)	0.126	80,10 (61,70-98,40)	61,35 (58,28-67,48)	64,00 (56,70-92,00)	0.413
HIV-associated parameters									
Current ART regimen, self-report					0.199				0.353
TDF-3TC-EFV	79 (88)	13 (86)	13	13 (86)		14 (93)	11 (84)	15 (100)	
TDF-3TC-NVP	1 (1)	0 (0)	0	0 (0)		1 (7)	0 (0)	0 (0)	
Other NNRTI-based regimen	3 (3)	0 (0)	3	0 (0)		0 (0)	0 (0)	0 (0)	
PI-based regimen	6 (6)	2 (14)	1	2 (14)		0 (0)	1 (8)	0 (0)	
Missing	1 (1)	0 (0)	0 (0)	0 (0)		0 (0)	1 (8)	0 (0)	
CD4 cell count, (cells/µL)* median (IQR)	436 (392-573)	416 (353-566)	529 (309-638)	485 (333-584)	0.509	368.5 (247-535)	314 (219-457)	493 (283.5-926.5)	0.724

Missing data	14 (16)	2 (13)	2 (11)	0 (0)		3 (20)	4 (30)	3 (20)	
Viral load, copies/ml (baseline A1), median (IQR)	281 (20-80000)	20 (20-27)	25 (20-528)	20 (20-65)	0.342	21900 (6120-59900)	4730 (1300-7950)	2120 (741-18800)	0.681
Viral load, copies/ml (A1.5), median (IQR)	198 (37-431)					431 (275-512)	99 (20-137)	89 (25-307)	0.172

* Study participants are denoted by number with percentages in brackets
*SES was measured using a composite socioeconomic status score, based on current employment, housing type and access to household assets, that was used to categorize women as ‘high’, ‘mid’ or ‘low’ SES. A median of these is shown above.
*CD4 results abstracted from routine records and are the nearest in time to the first ANC visit.
*All study participants were normotensive. Data was missing for 10 patients initiating therapy during pregnancy and 4 patients stable on ART.
*P values denote to the comparison across the three groups (AGA, SGA and PTD

4.4 T cell Immune activation

4.4.1 CD4+ and CD8+ T cell activation over time

The activation level of $CD4^+$ T cells was measured in ART initiators over time, and a significant reduction in activation levels over time was noted for women initiating ART, with no significant change in $CD4^+$ T activation for patients stable on ART (Fig 4.1A). In order to determine the effect of ART initiation on pregnancy outcome, activation levels were further stratified between patients initiating or stable on ART according to pregnancy outcome. The levels of expression of CD38 and HLA-DR on $CD4^+$ T cells was not different according to pregnancy outcome category in patients initiating or stable on ART over time (Fig 4.1B and C). $CD8^+$ T cell activation was higher in initiators prior to ART initiation and progressively decreased over time. No significant differences were noted over time in women stable on ART (Figure 4.1D). Activation levels were further stratified between patients initiating ART or stable on ART according to pregnancy outcome. CD8 activation levels were lowest for the PTD cases in the initiating group both at baseline (ART-naïve) and 2-weeks thereafter (ART-exposed) followed through to birth when compared to the AGA controls and SGA cases (Figure 4.2E). For women stable on ART, $CD8^+$ T cell activation levels did not differ by pregnancy outcomes over time (Figure 4.2F).

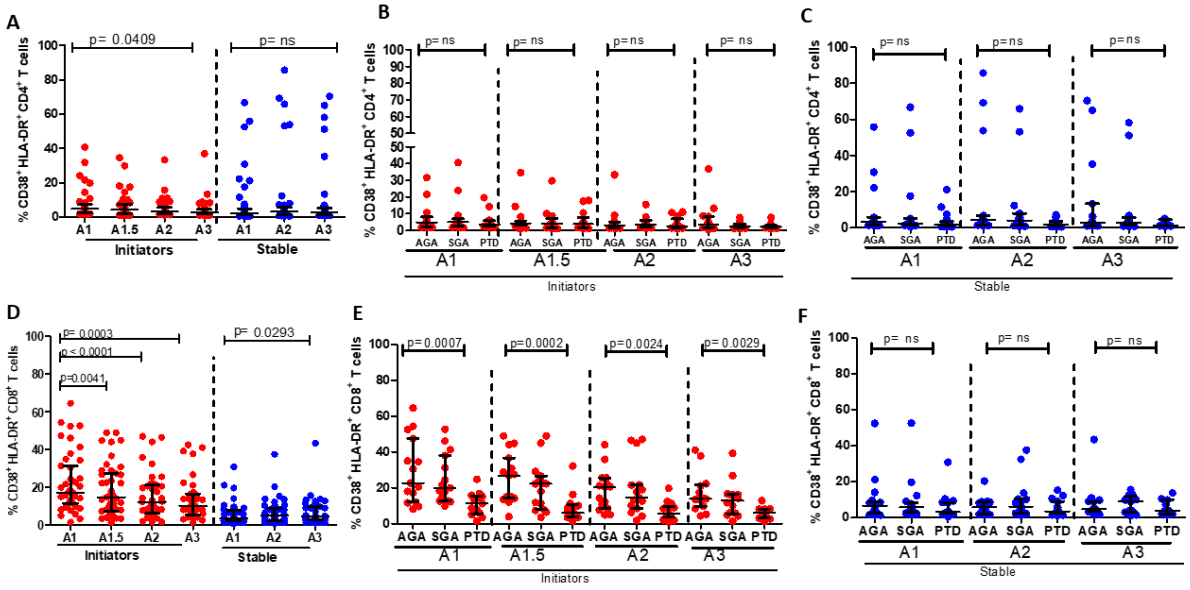


Figure 4.1: CD4$^+$ and CD8$^+$ T cell activation. A) CD4$^+$ T cell activation levels for women initiating (red circles) and stable on cART (blue circles) over time. B) Outcomes for each time point for women initiating cART (in red) and (C) women stable on ART (blue). D) CD8$^+$ T cell activation levels for women initiating (red circles) and stable on cART (blue circles) over time. E) Outcomes for each time point for women initiating cART (in red) and (F) women stable on ART (blue).

4.5 Antigen presenting cell activation and intracellular staining

4.5.1 Monocytes

Measurement of classical monocyte frequencies over time in patients initiating ART compared to those stable on ART showed a significant increase in frequencies over time for both the initiators and for women stable on ART (Figure 4.2A). Stratified by ART timing and birth outcome, frequencies of classical monocytes were consistently higher for PTD cases compared to SGA cases and AGA controls, both for initiators and those stable on ART (Figure 4.2B & C).

Intermediate monocyte frequencies increased significantly 2 weeks post ART initiation, with the significance subsequently lost in the following time points. Frequencies for patients stable on ART were significantly higher at all time points after baseline (Figure 4.2D). Stratified by birth outcome and ART timing, intermediate monocyte frequencies were higher in PTD than in SGA cases and AGA controls for initiators over time (Figure 4.2E), with no significant differences noted for those stable on ART (Figure 4.2F).

Accounting for 5-10% of the total circulating monocyte population, inflammatory monocytes have a pro-inflammatory phenotype and express markers which allow them to migrate into tissues in response to CX3CL1. These cells are also known to express pro-inflammatory TNF-α. Inflammatory monocyte frequencies significantly decreased over time for patients initiating ART with no difference for those stable on ART over time (Figure 4.2G). Among initiators, inflammatory monocytes frequency was lower in PTD than SGA cases and AGA controls at the time point when patients were ART naïve and 2 weeks post initiation only, with no significant differences in subsequent time points (Figure 4.2H). No significant differences were noted for those stable on ART (Figure 4.2I).

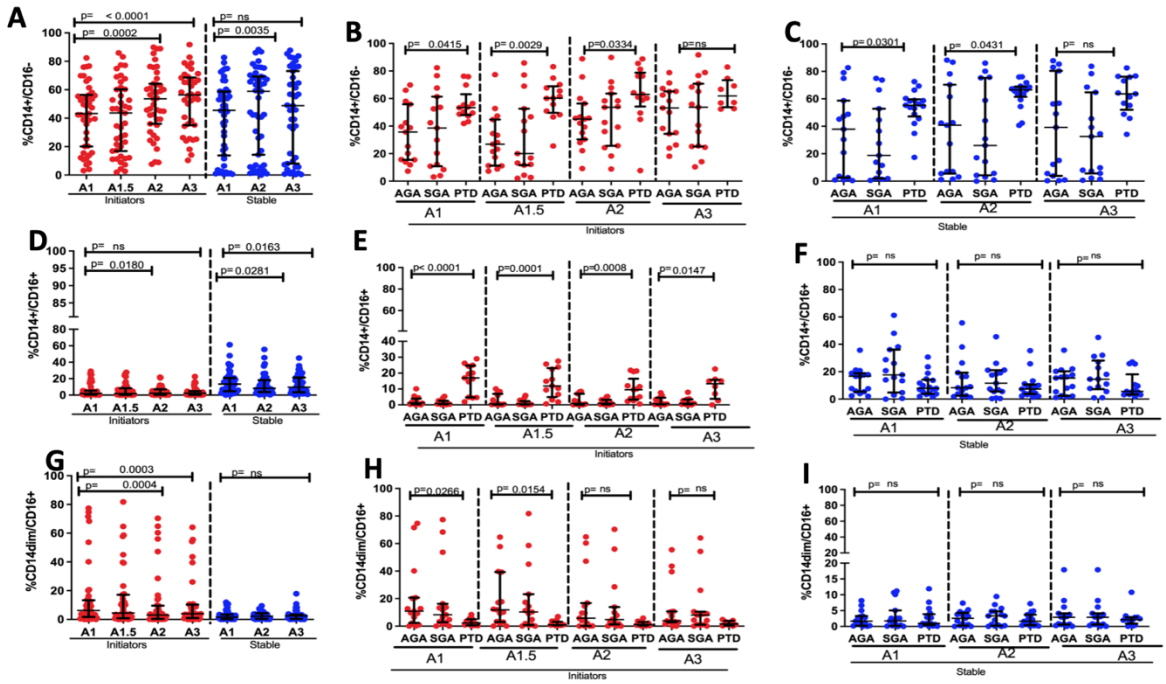


Figure 4.2: Classical (CD14+CD16-), intermediate (CD14+CD16+) and inflammatory (CD14dimCD16+) monocyte frequencies: A) Classical monocytes for women initiating (red circles) and stable on cART (blue circles) over time. B) Outcomes for each time point for women initiating cART (in red) and (C) women stable on ART (blue). (D) Intermediate monocytes for women initiating (red circles) and stable on cART (blue circles) over time. E) Outcomes for each time point for women initiating cART (in red) and (F) women stable on ART (blue). (G) Inflammatory monocytes for women initiating (red circles) and stable on cART (blue circles) over time. (H) Outcomes for each time point for women initiating cART (in red) and (I) women stable on ART (blue).

4.5.2 Monocyte activation

Expression of CD86 in bulk monocytes was not significantly different over time for both patients initiating, as well as those stable on ART (Figure 4.3A). Stratified by outcome and treatment group, expression of CD86 on monocytes was significantly lower in PTD cases in both ART groups over time (Figure 4.3B & C). Levels of CD69 expression in bulk monocytes were not different over time for both initiators and those stable on ART (Figure 4.3D). Stratified by outcome, expression of CD69 on monocytes was significantly lower in PTD cases in both ART groups over time (Figure 4.3E & F).

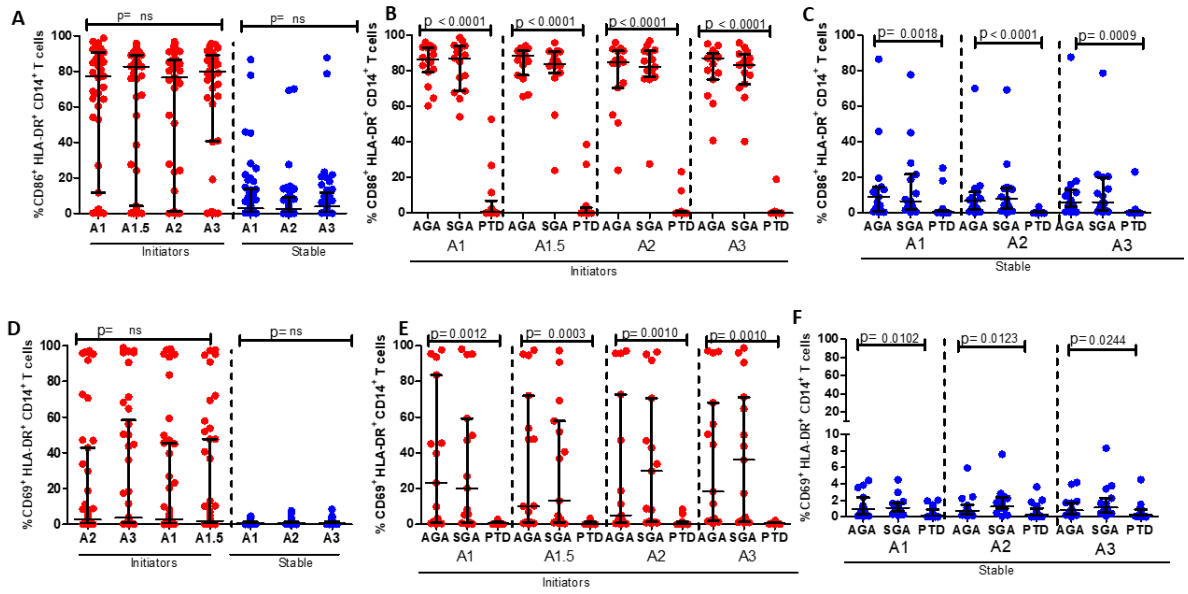


Figure 4.3: Bulk monocyte activation in study participants. A) $CD86^{+}HLA\text{-}DR^{+}$ expression in bulk monocytes in women initiating (red circles) and stable (blue circles) on ART over time. B) Bulk monocyte activation frequencies. Outcomes for each time point for women initiating cART (in red). C) Bulk monocyte activation frequencies. Outcomes for each time point for women stable on ART (blue). (D) Bulk monocyte activation in study participants. C) $CD69^{+}HLA\text{-}DR^{+}$ expression in bulk monocytes in women initiating (red circles) and stable (blue circles) on ART over time. B) Bulk monocyte activation frequencies comparing women initiating (red circles) and stable on cART (blue circles) at each time point. D) Outcomes for each time point for women initiating cART (in red) and (D) women stable on ART (blue).

4.5.3 Stimulation of monocytes with TLR ligands in HIV positive patients initiating and stable on ART.

We first assessed IFN-α production in monocytes in response to LPS, CpG and CL097 and we noted a significant difference between baseline and A3 for patients initiating ART in response to TLR9 (CpG). However, no significant differences in responses were seen over time in patients initiating and those stable on ART for all other stimulations (Figure 4.4A). We next examined only patients initiating ART and grouped them according to outcome (Figure 4.4B). There was a significantly higher response to LPS stimulation over time with PTD having the least expression of IFN-α in initiators. At 28 weeks of gestation (A3) there was a significant difference in outcomes for both TLR7/8 and TLR9 agonists (CpG and CL097) in women initiating ART. There were no significant differences in responses in patients who conceived on ART in response to TLR4 and TLR9. There was however, a significantly lower amount of IFN-α produced by patients with the PTD outcome compared to SGA and AGA but only during the 28 (A2) and 34 (A3) weeks gestation time points (Figure 4.4C).

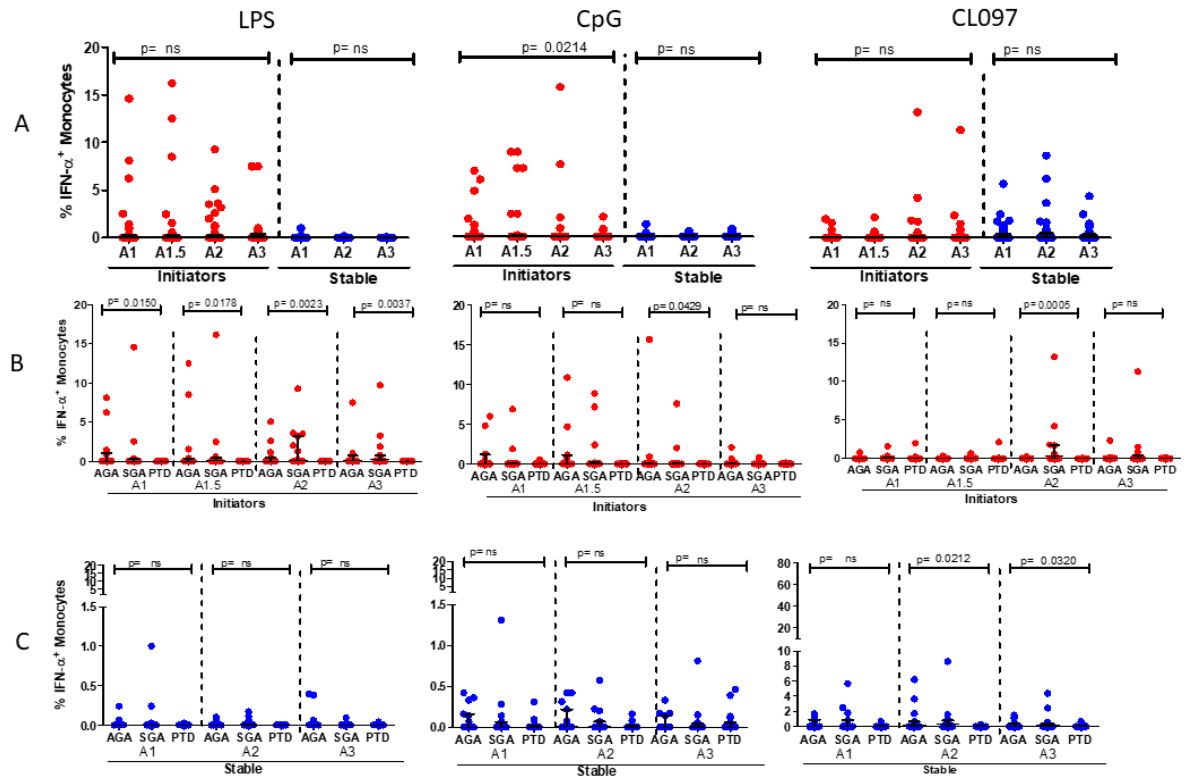


Figure 4.4: **Monocyte IFN-α expression upon TLR stimulation.** A) IFN-α expression over time following stimulation with LPS, CpG and CL097 for patients initiating ART (red circles) and stable on ART (blue circles). B) IFN-α expression for each outcome (AGA, SGA, PTD) after stimulation with LPS, CpG and CL097 for patients initiating ART (red circles). C) IFN-α expression for each outcome (AGA, SGA, PTD) following stimulation with LPS, CpG and CL097 for patients stable on ART (blue circles).

Analysis of monocytes for MIP-1β production in response to LPS, CpG and CL097 did not show any significant responses over time in patients initiating and those stable on ART for all stimulations. (Figure 4.5A). We assessed only patients initiating ART and grouped them according to birth outcome (Figure 4.5B); we here show significant response to LPS, CpG and CL097 stimulation over time, although there was no consistent pattern on the expression of MIP-1β. There were no significant responses in patients who conceived on ART (Figure 4.5C).

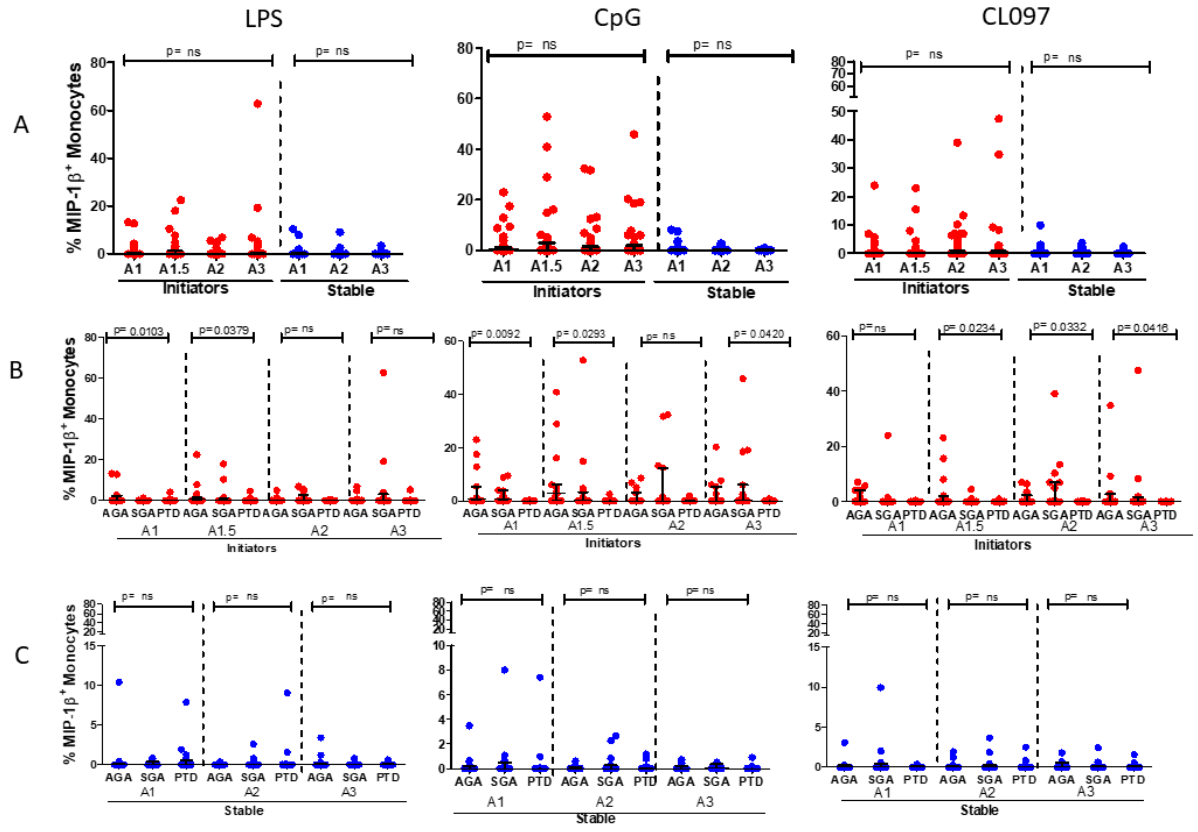


Figure 4.5: Monocyte MIP-1β expression upon TLR stimulation. A) MIP-1β expression over time when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles) and stable on ART (blue circles). B) MIP-1β expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). C) MIP-1β expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

TNF-α expression in monocytes in response to LPS, CpG and CL097 did not show any significant differences in responses over time when comparing patients initiating ART; a significant increase in TNF-α at time point 2 was noted for women stable on ART upon stimulation with LPS (Figure 4.6A). LPS stimulation did not have a significant effect on the expression of TNF-α expression in women initiating ART regardless of their birth outcome over time (Figure 4.6B). Monocytes showed significantly lower expression of TNF-α in the PTD outcome at 28 weeks when stimulated with CpG and CL097 (Figure 4.6B). Analysis of patients stable on ART and grouped according to outcome showed no significant responses to stimulation over time for LPS, CpG and CL097 (Figure 4.6C).

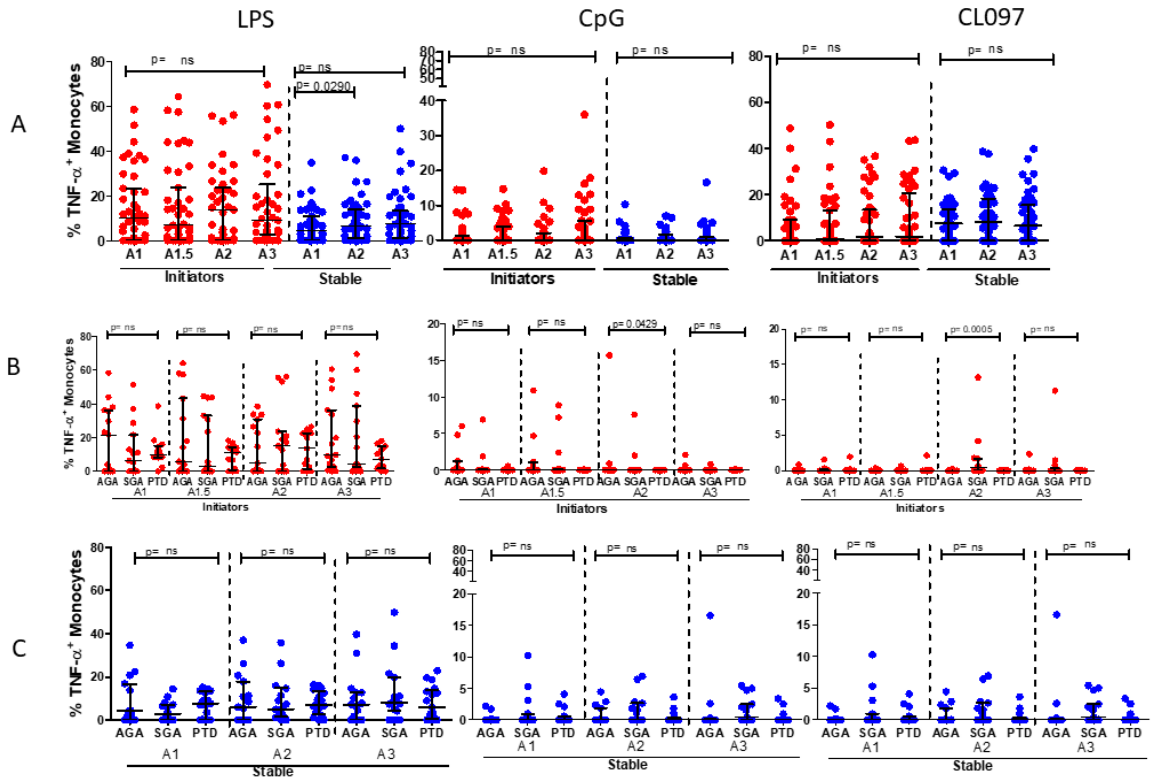


Figure 4.6: **Monocyte TNF-α expression upon TLR stimulation**. A) TNF-α expression over time when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles) and stable on ART (blue circles). B) TNF-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). C) TNF-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

4.5.4 Dendritic cell surface activation analysis in HIV positive patients initiating and stable on ART.

4.5.4.1 Myeloid dendritic cells

When levels of activation were measured in mDCs as measured by co-expression of CD86 and HLA-DR, no significant significant difference were found in patients initiating or stable on ART over time (Figure 4.7A). When stratified according to birth outcome, the PTD group had a significantly lower expression of CD86 when compared to AGA and SGA for the initiators (Figure 4.7B). No significant difference was noted for patients stable on ART when stratified by outcome (Figure 4.7C). When assessing expression of CD69; no significant difference was noted in the treatment groups over time (Figure 4.7D). CD69 expression was consistently lower in the PTD group over time for patients initiating ART with no significant difference noted for women stable on ART (Figure 4.7E and F).

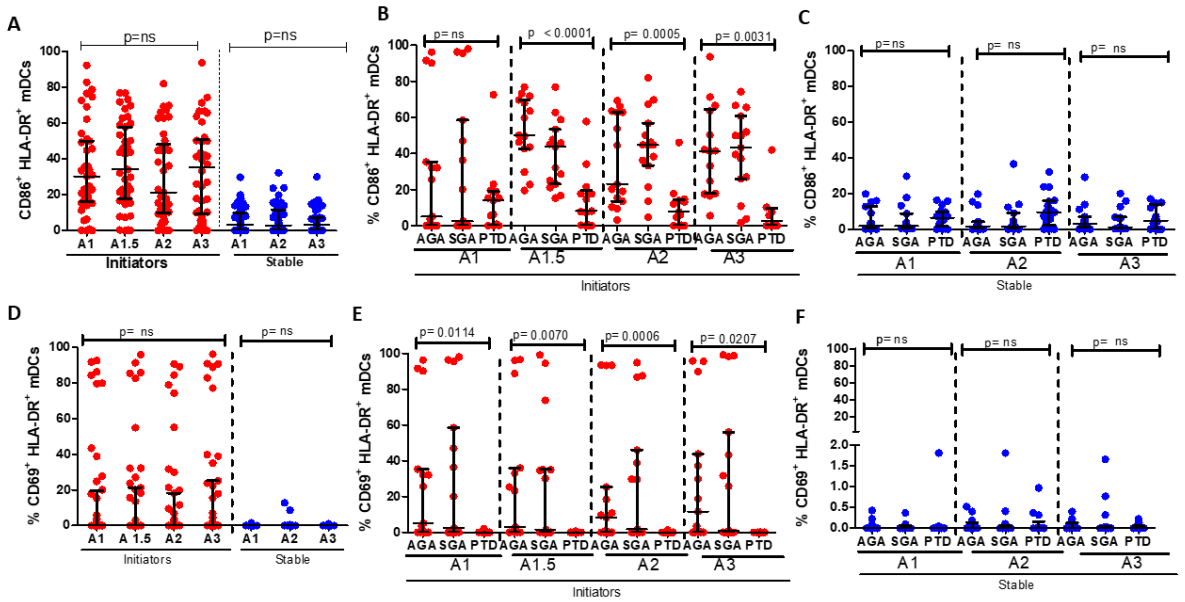


Figure 4.7: **mDC activation in study participants**. A) $CD86^{+}HLA\text{-}DR^{+}$ expression in bulk mDCs in women initiating (red circles) and stable (blue circles) on ART over time. B) Outcomes for each time point for women initiating ART (in red) and (C) women stable on ART (blue). D) $CD69^{+}HLA\text{-}DR^{+}$ expression in bulk mDCs in women initiating (red circles) and stable (blue circles) on ART over time. E) Outcomes for each time point for women initiating ART (in red) and (F) women stable on ART (blue).

4.5.4.2 Stimulation of mDCs with TLR ligands in HIV positive patients initiating and stable on ART

Assessment of IFN-α production in mDCs did not show any significant responses over time when comparing patients initiating and those stable on ART for all stimulations (LPS, CpG and CL097) (Figure 4.8A). A significant response was observed upon LPS at baseline, CpG and CL097 at later time points in patients initiating ART upon stratification by birth outcome (Figure 4.8B). In all instances the PTD group had lower responses across all stimulations. mDC IFN- α were consistently lower in patients stable on ART across all stimulations and across all time points (Figure 4.8C).

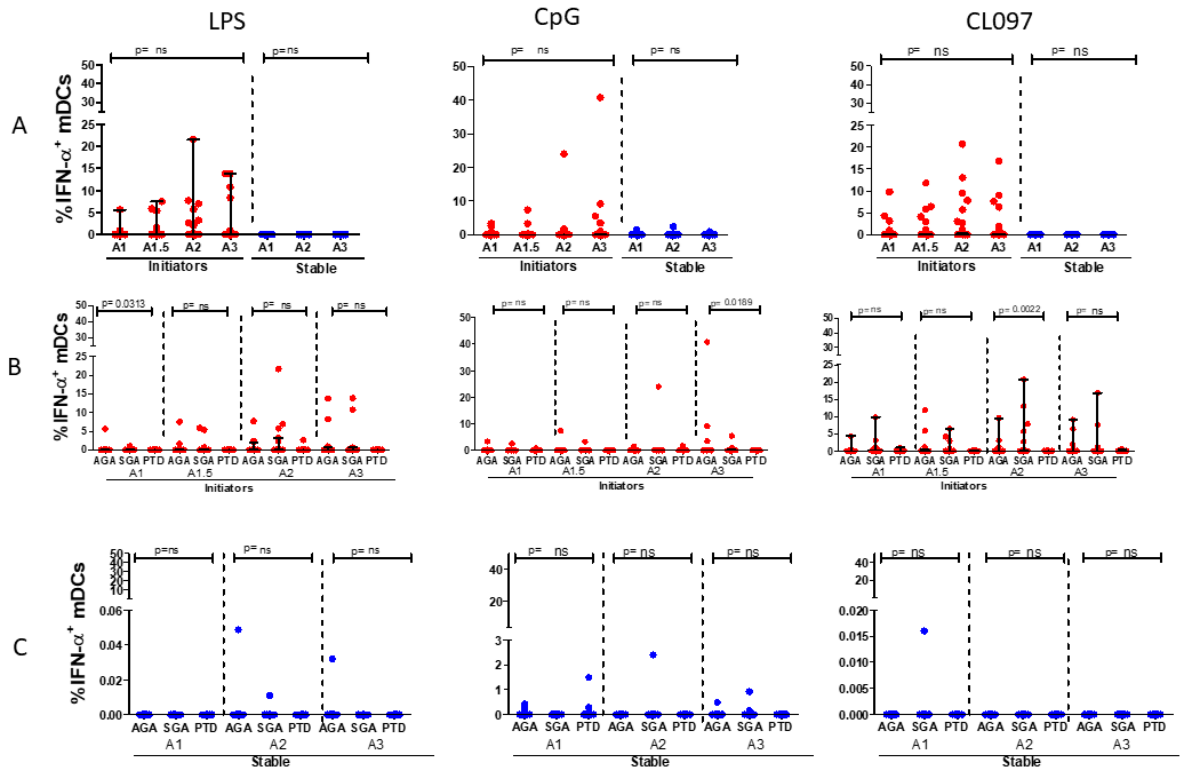


Figure 4.8 mDC IFN-α expression upon TLR stimulation. A) IFN-α expression over time when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles) and stable on ART (blue circles). B) IFN-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). C) IFN-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

Upon analysis of MIP-1β production in mDCs in response to LPS, CpG and CL097, we noted a significant decrease in IFN-α expression 2 weeks post ART initiation and this significance was lost over subsequent time points. There was no significant difference in responses over time for all other stimulations when comparing patients initiating and those stable on ART. (Figure 4.9A). We next examined only patients initiating ART and grouped them according to outcome (Figure 4.9B). There was no significant response to LPS and CL097 stimulation in patients initiating ART. MIP-1β expression was lower for the PTD outcome for all time points post ART initiation when stimulated with CpG. There was also no significant differences over time for stimulation with CL097 for patients stable on ART (Figure 4.9C).

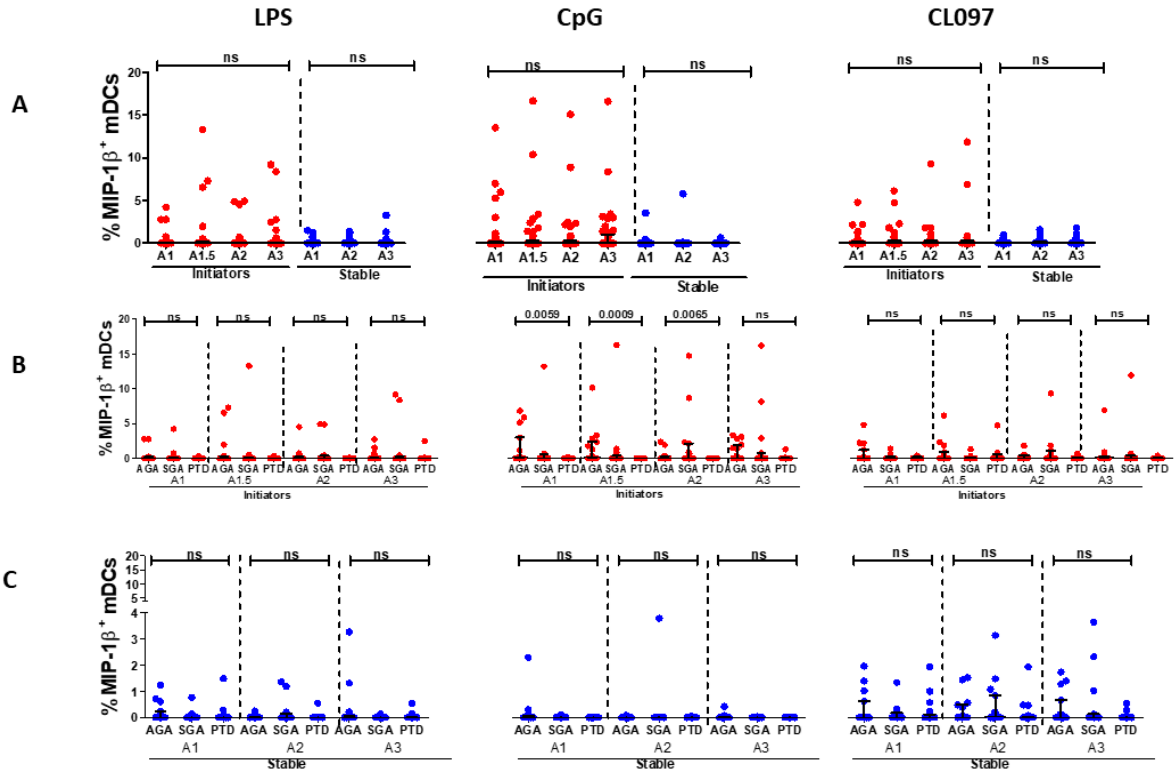


Figure 4.9: **mDC MIP-1β expression upon TLR stimulation**. A) MIP-1β expression over time when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles) and stable on ART (blue circles). B) MIP-1β expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). C) MIP-1β expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

We investigated the expression of TNF-α by mDCs in response to LPS, CpG and CL097, over time. There was no significant difference in responses over time when comparing patients initiating and those stable on ART for all stimulations (Figure 4.10A). When patients initiating ART were grouped according to birth outcome, we noted no significant response to LPS and CL097 stimulation (Figure 4.10B). TNF-α expression was lower for the PTD outcome for the first three time points when stimulated with CpG. No significant differences over time were noted for patients stable on ART (Figure 4.10C).

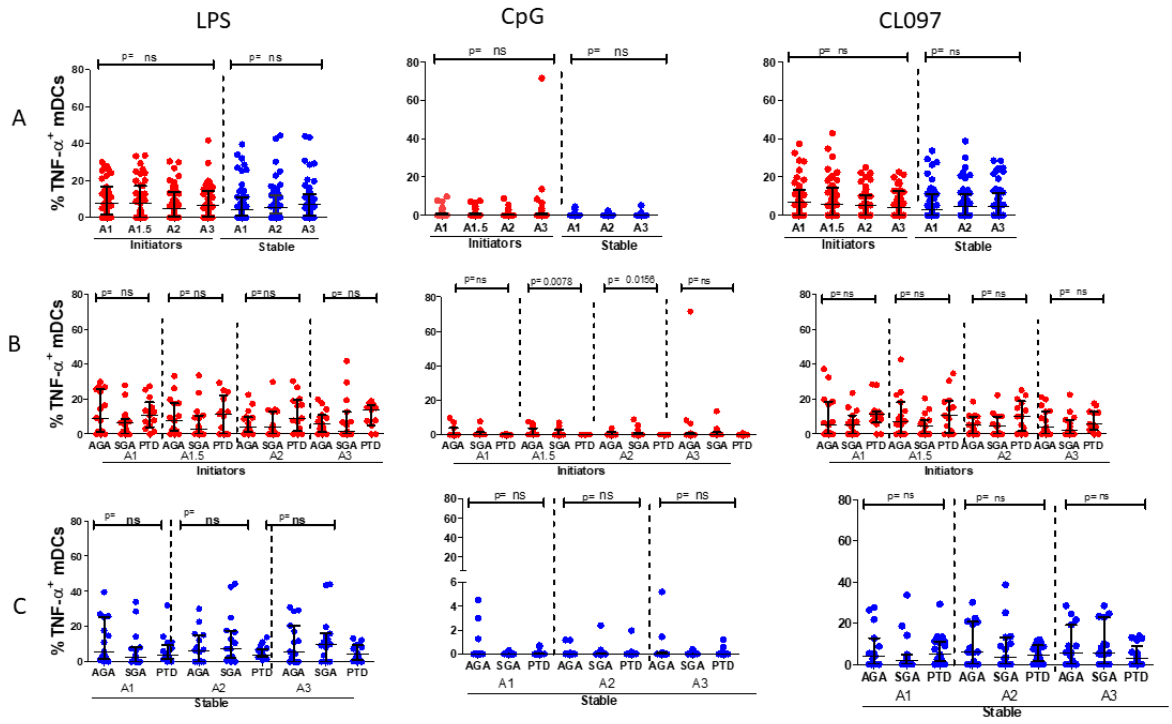


Figure 4.10: mDC TNF-α expression upon TLR stimulation. A) TNF-α expression over time when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles) and stable on ART (blue circles). B) TNF-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). C) TNF-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

4.5.4.3 Plasmacytoid dendritic cells

There was a significant decrease in the expression of CD86 levels on pDCs over time among patients initiating and no change to those stable on ART (Figure 4.11A). When stratified according to outcome, there were significantly lower CD86 expressing patients with a PTD outcome than in AGA or SGA for ART initiators (Figure 4.11B). There were no significant differences in the expression of CD86 on pDCs when stratified by pregnancy outcome for the ART stable group (Figure 4.11C). The expression of CD69 on pDCs was not different for both patients initiating and those stable on ART (Figure 4.11D) and this was the case irrespective of treatment group when the patients were stratified by outcome (Figure 4.11E&F).

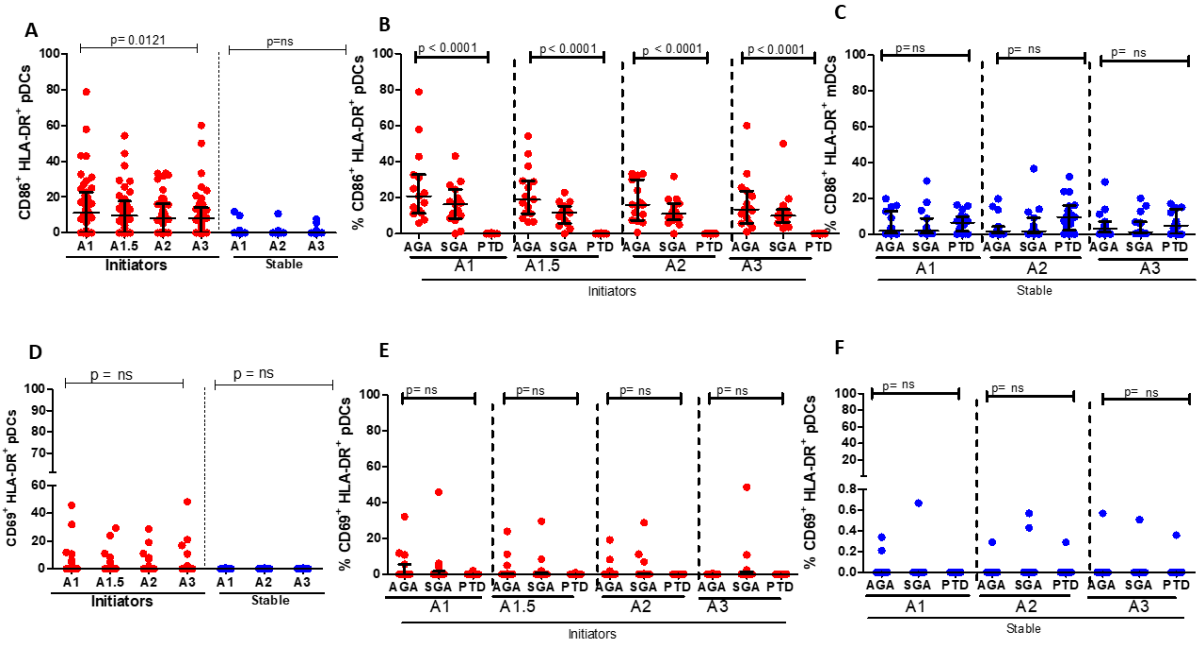


Figure 4.11: pDC activation in study participants. A) $CD86^{+}HLA\text{-}DR^{+}$ expression in bulk mDCs in women initiating (red circles) and stable (blue circles) on ART over time. B) Outcomes for each time point for women initiating ART (in red) and (C) women stable on ART (blue). D) $CD69^{+}HLA\text{-}DR^{+}$ expression in bulk mDCs in women initiating (red circles) and stable (blue circles) on ART over time. E) Outcomes for each time point for women initiating ART (in red) and (F) women stable on ART (blue).

4.5.5 Stimulation of pDCs with TLR ligands in HIV positive patients initiating and stable on ART

When we assessed IFN-α production by pDCs in response to LPS, CpG and CL097; there was a significantly lower difference in pDCs from patients initiating ART under LPS stimulation only. We did not find any significant differences in responses over time across all other stimulations when patients initiating and those stable on ART were compared (Figure 4.12A). We next studied only patients initiating ART grouped according to outcome and we noted a significant response to LPS and CL097 stimulation (Figure 4.12 B). IFN-α expression was lower for the PTD group at different time points over time but not consistently. There was a significantly lower expression of IFN-α in patients stable on ART over time for CL097 and this was specifically for the PTD outcome. No change was seen for LPS and CpG stimulation over time (Figure 4.12 C).

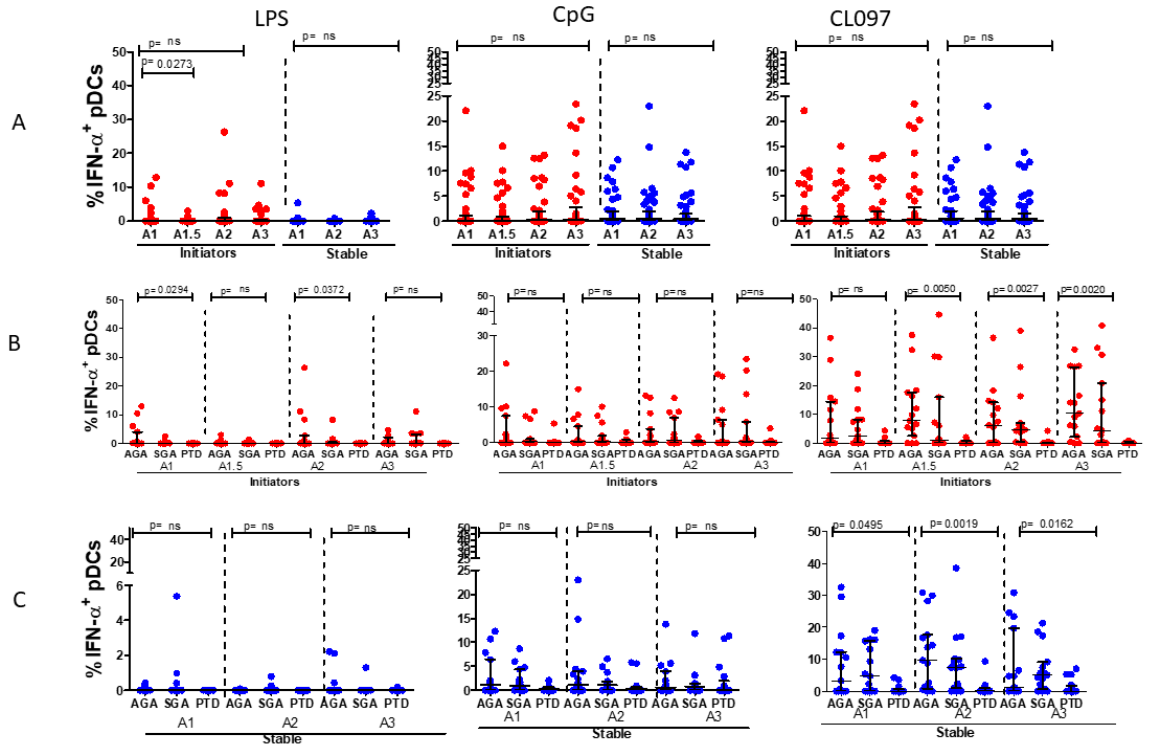


Figure 4.12: pDC IFN-α expression upon TLR stimulation. A) IFN-α expression over time when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles) and stable on ART (blue circles). B) IFN-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). C) IFN-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

MIP-1β production in pDCs did not change over time in response to LPS, CpG and CL097 when comparing patients initiating and those stable on ART (Figure 4.13A). We next examined only patients initiating ART and grouped them according to outcome (Figure 4.13B). There was no significant MIP-1β response to LPS, CpG and CL097 stimulation over time, regardless of outcome. We found no expression of MIP-1β in response to stimulation over time and by outcome in patients who conceived on ART (Figure 4.13C).

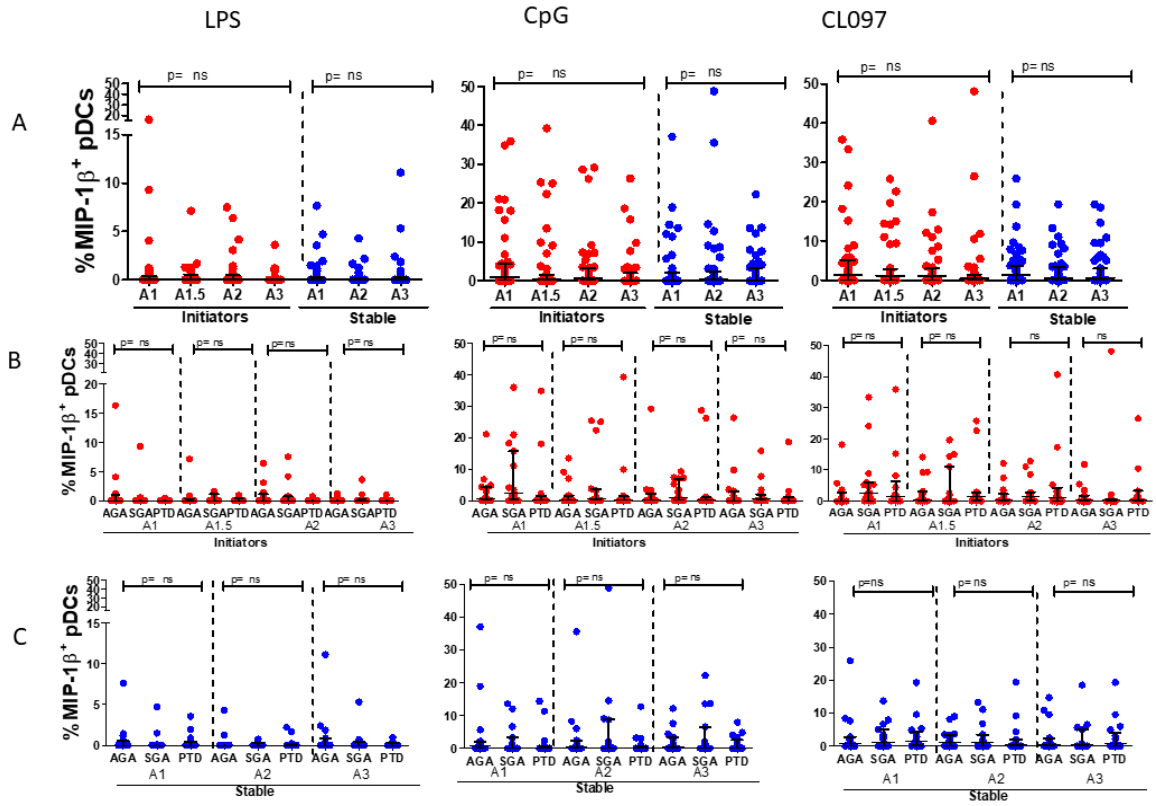


Figure 4.13.: **pDC MIP-1β expression upon TLR stimulation**. A) MIP-1β expression over time when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles) and stable on ART (blue circles). B) MIP-1β expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). C) MIP-1β expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

There was no significant TNF-α expression in response to LPS, CpG and CL097 in pDCs over time when comparing patients initiating and those stable on ART (Figure 4.14A). There was an expression of TNF-α in response to TLR9 (CpG) at time point A.15 (two weeks post ART initiation) and the time point after (A2). Here we found that the expression was lower in the PTD outcome, at 2 weeks post initiation followed by a significantly higher expression for this outcome. This was maintained even to the last timepoint (A3) although not statistically significant. No significant differences were noted in response to TLR4 and 7/8 in women initiating ART by outcome overtime (Figure 4.14B). There were also no differences seen in women stable on ART for all stimulations (Figure 4.14C).

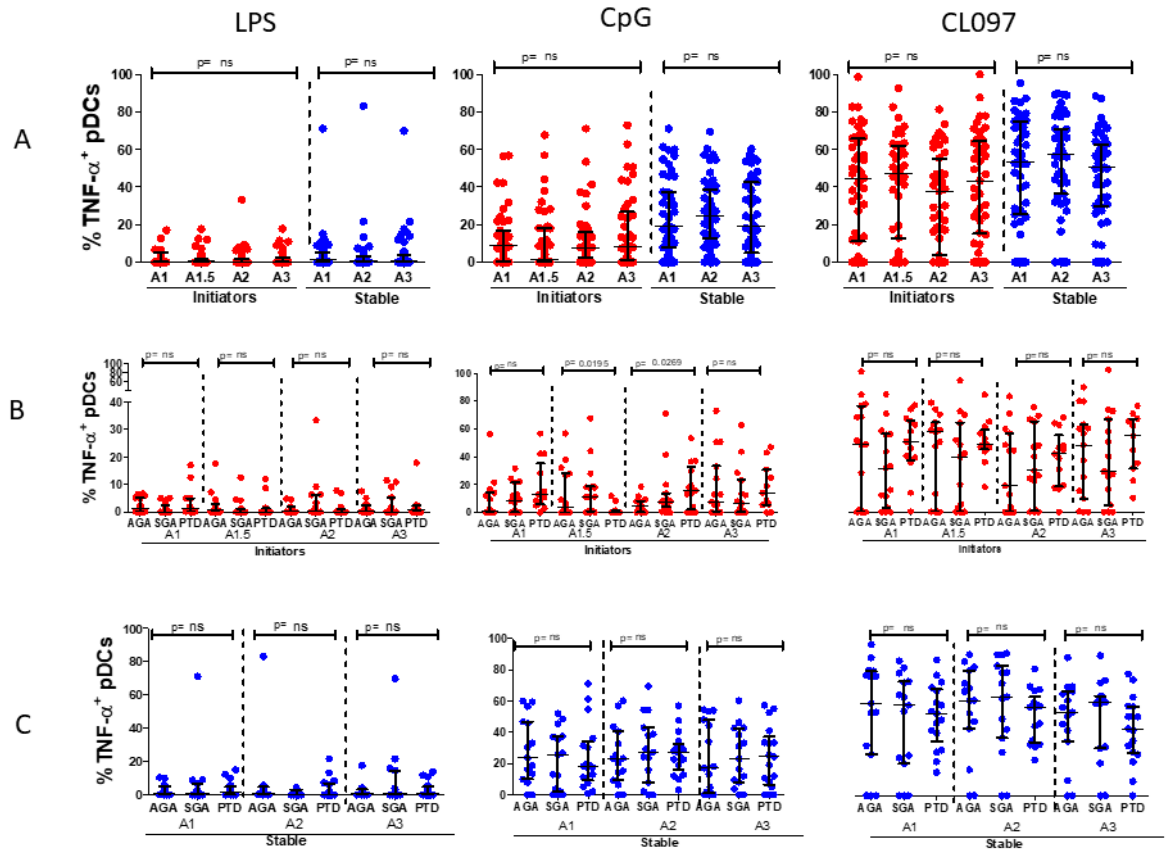


Figure 4.14: **pDC TNF-α expression upon TLR stimulation**. A) TNF-α expression over time when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles) and stable on ART (blue circles). B) TNF-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients initiating ART (red circles). C) TNF-α expression for each outcome (AGA, SGA, PTD) when stimulated with LPS, CpG and CL097 for patients stable on ART (blue circles).

4.6 Summary

The role of TLRs during HIV infection is still not clearly defined. Reports have suggested that microbial products activate HIV-1 replication through the stimulation of TLRs (Scagnolari et al., 2009). Such stimulation induces the activation of NF-κB and the production of pro-inflammatory cytokines involved in HIV-1 replication through transactivation of viral promoters (Equils et al., 2003). One report showed an increase of TLR expression in monocytes from HIV-1-infected patients, which favors both an increase in viral replication and secretion of TNF-α (Giraldo et al., 2015). Markers of immune activation, particularly in $CD8^+$ (HLA-DR and $CD38^+$) are indicative of HIV disease progression. $CD8^+$ cellular activation, as opposed to $CD4^+$ activation, has been said to be a more predictive of long-term immunologic responses and this is because $CD4^+$ cells are infected by HIV and are more likely to be removed through apoptosis (Zhang. Z et al., 2011). During pregnancy, studies have found that infection activates CD8 T cells resulting in their expansion and expression of HLA-DR antigens (Musyoki et al., 2014). We looked at activation marker CD86 as well which is a costimulatory molecules used as molecular markers for activation/maturation of antigen presenting cells. (Giraldo et al., 2015)

We hypothesized that the immune activation status on innate immune cell subsets and their phenotypes or functionality would be modulated by ART status (initiated pre- or during pregnancy) and associated with PTD or SGA. We anticipated antigen presenting cell function would be altered in HIV infected women who initiate ART during pregnancy contributing to differential birth outcomes.

We previously performed a baseline analysis and reported lower $CD8^+$ T cell, monocyte, mDC and pDC activation (particularly as defined by CD86 but not CD69 expression for the antigen

presenting cells) were all strongly associated with subsequent PTD for women initiating ART in pregnancy but not those who started ART before pregnancy, after allowing for baseline viral load (Mdletshe et al., 2021). There was an association of lower immune activation with PTD in ART initiators before initiation of ART, and at two weeks post-ART initiation. Further, our findings suggested differences in monocyte subsets by pregnancy outcome with inflammatory monocytes frequencies lower (and vice-versa for classical and intermediate monocytes) among ART initiators with subsequent PTD at baseline. For most of the immune parameters, women with SGA had similar profiles to control women.

As previously mentioned, we next wanted to interrogate this dataset over time. In our case-control study over time, lower $CD8^+$ T cell, monocyte, mDC and pDC activation as defined by CD86 and CD69 expression were strongly associated with subsequent PTD for women initiating ART in pregnancy and in some cases those who started ART before pregnancy, after adjusting for baseline viral load. The association of lower immune activation with PTD in ART initiators was observed before initiation of ART, at two weeks post-ART initiation up until the last time point, at birth.

Next, we studied monocyte populations over time and noted that there was an increase in frequencies of classical monocytes irrespective of treatment status. The $CD16^+$ subset are expanded in numerous viral infections like HIV (Han et al., 2009). In a study performed in HIV positive men, monocyte subsets were not significantly altered in individuals with HIV-1 infection receiving antiretroviral therapy but the proportion of CD16+ monocytes was increased in therapy-naive patients (Jaworowski et al., 2007). This result was consistent with our findings that monocyte subsets in HIV positive women initiating therapy had an increase in the frequency of the $CD16^+$ population (Figure 4.2F). In a previous study, the researchers

conducted a study on HIV^+ or HIV- pregnant women in Malawi and found higher proportions of CD16+ monocytes in the peripheral blood of healthy pregnant and postpartum Malawian women which suggested that the monocyte subset was expanded irrespective of pregnancy status (Jaworowski et al., 2007).

In our study, we simulated an HIV environment by using TLR stimulants on antigen presenting cells. APC stimulation with TLR agonists LPS, CpG and CL097 was performed for monocytes. These cell types reacted differently to stimulus and the data shows a reduced expression in inflammatory cytokines IFN-α, TNF-α and MIP-1β mostly in patients initiating ART. There was some expression of cytokines in patients stable on ART as well. Specifically, the induction of IFN-α expression in monocytes was TLR-4 induced and was prominent in the initiators with the PTD outcome displaying the lowest expression. In the same cells, among those stable on ART, we found differential cytokine expression when cells were stimulated with TLR 7/8. In the mDCs all stimulations resulted in lowest cytokine expression for those with PTD, however, the differences were only significant for patients initiating ART. For the pDCs, TLR4 stimulation was resulted in significantly lower cytokine production for the PTD cases. TLR7/8 stimulation was associated with the expression of IFN-α at later pregnancy time points, with the PTD outcome displaying the lowest expression both for initiators and for those stable on ART. Overall, our findings strongly implicate reduced immune activation as an underlying biomarker for PTD but not SGA.

Lower immune activation was associated with PTD mostly in ART-initiating women and not those stable on ART, suggesting that long-term ART may be leading to correction of the underlying immunological dysfunction (Mdletshe et al., 2021). This result further suggests that immune activation is likely only a surrogate for a yet undetermined immunological dysfunction

since women stable on ART with overall reduced immune activation did not have higher incidence of PTD. As expected, initiation of ART rapidly lowered immune activation, with noticeable reduction at 2 weeks following ART initiation but lowered immune activation remained associated with PTD even at that stage, suggesting that the immune defect in ART-naïve individuals associated with PTD is not immediately corrected by ART.

Our study could not definitively identify the immunological dysfunction underlying PTD. However, it has also been suggested that increased inflammation may potentiate adverse pregnancy outcomes including PTD (Romero et al., 2006, Cappelletti et al., 2015). Inflammation as an underlying factor for PTD may be indicative of underlying infection as a causative factor for PTD, with the reduced immune activation observed in our study a possible surrogate of reduced potential of immune cells to become activated and respond to infection. A recent transcriptomic study identified adaptive and innate immune genes as risk factors for PTD, although results contrasted when maternal peripheral blood and cord blood, with data from the latter samples (downregulation of innate immune genes) more consistent with our results (Vora et al., 2018). Our findings are consistent with previous reports that PTD infants display a reduced ability to respond to pathogens ex vivo (Goedicke-Fritz et al., 2017, Lavoie et al., 2010), which would be expected if they express lower levels of the costimulatory molecule CD86 on monocytes as we observed. Overall, our findings suggest that reduced immune activation, which may be linked to reduced immune responsiveness to pathogen insult, could precede PTD and indicate an underlying mechanism, particularly in women initiating ART during pregnancy.

Chapter 5 Investigation of regulatory T cell frequencies during pregnancy and association with preterm birth and small for gestational age births.

5.1 Introduction

The adoption of the Global Plan towards elimination of new HIV infections among children has had a transformative impact on the reduction of new HIV infections among children in sub Saharan Africa (UNAIDS, 2012, WHO, 2012). Under the Global Plan, there has been increased advocacy for the increased roll out and uptake of Option B+ in which all HIV-infected individuals including pregnant women initiate lifelong antiretroviral therapy (ART), regardless of CD4 count (Chersich et al., 2018). Consequently, there has been a significant decline in the number of AIDS-related deaths among women of reproductive age and in the number of new infections among HIV-exposed uninfected children (Haroz et al., 2017, Slogrove et al., 2020). Despite the successes there is limited data on the effect of ART use during pregnancy especially when comparing ART started before conception and continued during pregnancy versus ART started during pregnancy. In this regard, recent evidence suggests that women who initiate ART before pregnancy are more likely to have very-preterm, preterm, or low birth weight deliveries in comparison to women who initiate ART during pregnancy (Uthman et al., 2017). However, the limitation of these findings is that very few studies included pregnant women on Option B+ regimens, which is what has now been widely implemented since 2013.

Taking into consideration that pregnancy represents a unique immunological paradox, where a mother has to tolerate her semi-allogeneic fetus, it is clear that immunological pathways are involved in the regulation of pregnancy. HIV and antiretroviral therapy are also important modulators of immune responses, and it is therefore important to elucidate immune pathways that may be altered in cases of pregnancy, HIV infection and antiretroviral use and whether these pathways play a role in HIV or ART associated adverse birth outcomes. In this regard, regulatory T cells (Tregs), have been shown to play a key role in the establishment and

maintenance of fetal-maternal (FM) tolerance (Guerin et al., 2009, Jørgensen et al., 2019). Evidence from animal models, demonstrating insufficient production of pregnancy induced Tregs in abortion prone mice, and data from human placentas demonstrating higher proportions of Tregs in the maternal decidua all support the important role of Tregs in pregnancy (Salvany-Celades et al., 2019, Woidacki et al., 2015, Zenclussen et al., 2005, Mjösberg et al., 2009). One of the aspects mentioned is the clonal expansion of Tregs in the 2nd and 3rd trimester for normal pregnancy and how this expansion is decreased in pre-eclamptic patients (Tsuda et al., 2019). In addition, women with spontaneous preterm delivery have been reported to have diminished Tregs in their systemic circulation and studies on the depletion of Tregs at various time points demonstrate that the pre- and peri-implantation phase is the most vulnerable; impairment of Treg suppressive function shortly after mating causes complete implantation failure (Zenclussen et al., 2005, Shima et al., 2010, Koucký et al., 2014, Schober et al., 2012a, Kisielewicz et al., 2010b).

It is likely that adverse birth outcomes in HIV-infected women may be associated with altered Tregs frequency and functioning in maternal circulation, resulting in a perturbance of FM tolerance. HIV infection has been shown to impair Treg activity (López-Abente et al., 2016). During chronic HIV infection, Treg numbers in peripheral blood decline and direct HIV infection of Tregs has been shown to impair Treg suppressive capacity (Thorborn et al., 2010, Pion et al., 2013). In addition, immune reconstitution disease after ART initiation has been associated with Tregs with reduced immunosuppressive capacity (Seddiki et al., 2009). Working within a large birth cohort, the Prematurity Immunology in HIV-infected Mothers and their infants Study (PIMS) in Cape Town, South Africa (Malaba et al., 2020) we report on Treg subsets in the maternal circulation of HIV-infected women initiating ART before or during pregnancy, in a case-control study including women with preterm deliveries (PTD),

small for gestational age deliveries (SGA) as cases and appropriate for gestational age (AGA) as controls.

We aimed to assess frequency and characterise the phenotype and functional regulatory T cells (Tregs) changes associated with initiation of ART during pregnancy and pre-term delivery. We hypothesised that there would be reconstitution of immune responses under ART in pregnant women which would enhance immunity against the fetus and increased preterm birth.

5.2 Methods

5.2.1 Study participants

HIV infected women ≤24 weeks gestation, as assessed by ultrasound, were enrolled and followed with two study visits for those on ART pre-conception (stable on ART) at <20 weeks (baseline), 28 weeks of pregnancy, plus an additional study visit two weeks after ART initiation for women newly identified as HIV infected and initiated on ART at their first ANC visit. At each visit, blood was drawn into sodium heparin tubes (BD Vacutainer, New Jersey, USA) and peripheral blood mononuclear cells (PBMCs) isolated within 4 hours of blood collection by density gradient centrifugation, counted by the trypan blue method and stored in liquid nitrogen.

For the section of the study presented here, 25 cases of PTD, 25 SGA cases and 25 appropriate-for-gestation age (AGA)/term controls were selected for analysis. Controls and cases were matched on timing of ART initiation and analysed blinded. The median gestational age at enrolment was 15 weeks both for women initiating and stable on ART. PTD was defined as delivery <37 weeks, SGA as weight for gestational age ≤10^{th} centile, AGA controls were term, with weight for gestational age ≥25^{th} centile (Malaba et al., 2020). Baseline information was collected by trained study nurses. CD4 cell counts and viral load were closest to the visit on which the sample was taken.

5.2.2 Flow cytometry analysis

For this analysis, surface staining and ICS of regulatory T cells using flow cytometry method is outlined in detail in in chapter 2.6. Briefly, for the quantification of percentages of Tregs,

cryopreserved PBMCS cells were thawed, prepared and stained for surface markers using a cocktail containing CD8 (Clone: SK1, BioLegend (BL)), CD39 (Clone: A1, BL), CD25 (Clone: 2A3, BD), CD127 (Clone: A019D5, BL), CD14 (Clone: MHCD1417, Life Technologies), CD4 (Clone: SK3, BL), CD3 (Clone: UCHT1, BL), TIGIT (Clone: MBSA43, eBiosciences), CD45 (Clone: HI30, BD), CD28 (Clone: CD28.2, BL), CD45RA (Clone: H100, BL), PD-1 (Clone: EH12.1, BD). The cells were then incubated in the dark, washed and fixed and permeabilised preparing them for intracellular staining labelling of FoxP3 and CTLA4. Stained cells were fixed using BD Stabilizing Fixative (BD Biosciences) and stored at 4°C until flow cytometry recording (within 24 hours). Data on Tregs was acquired on an LSRFortessa (BD), using BD FACSDiva software (BD Biosciences) and analyzed with FlowJo software Version 9.8.5 (TreeStar).

5.2.3 Statistical analysis

Boolean gating, which is useful when sorting cells stained with multiple fluorescent probes was used to analyse multifunctionality in Tregs. We did coupled analysis in Pestle/simplified presentation of incredibly complex evaluations (SPICE) which provided the most user-friendly manipulations and readable output, evaluating molecules expressed by Tregs. These softwares are from Mario Roederer, Chief, ImmunoTechnology Section, Vaccine Research Centre, National Institutes of Health / NIAID.

5.3 Results

5.3.1 Gating strategy

To begin the analysis, we first defined the Treg cell populations as shown in Figure 5.1. Using forward scatter height and area, single cells were identified. This was followed by gating on T

cells that are CD45+. We then gated for live cells using vivid dye as a viability marker. We excluded monocytes by gating on a $CD14^-$ population. Lymphocytes were identified based on their forward- and side-scatter properties. We further identified T cells by gating on CD3 among the previously selected viable lymphocytes. CD4 T cells and CD8 T cells were identified as uniquely expressing CD4 or CD8 antigens. Conventional Tregs were defined as $CD4^+$ T cells co-expressing CD25, dim CD127 expression and expressing transcription factor forkhead box P3 (FOXP3).

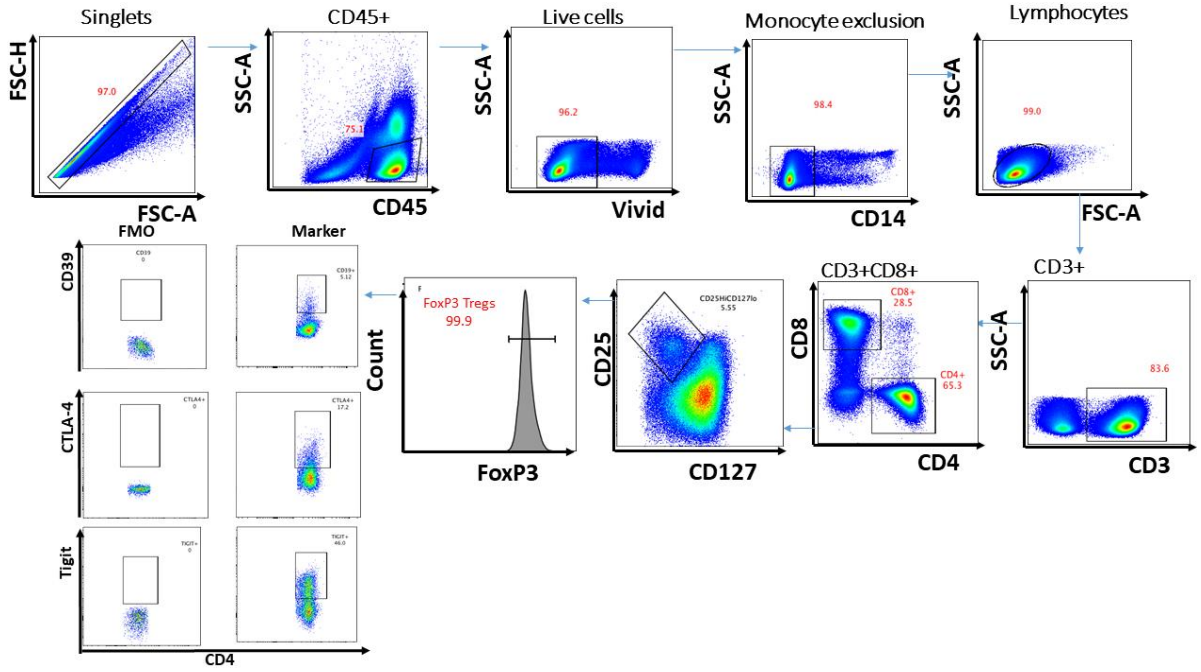


Figure 5.1: Identification of CD4+CD25brightCD127dimFOXP3+ Treg cells along with the co-inhibitory markers. Representative gating strategy for the identification of Tregs by flow cytometry. Initial gating was on singlets, followed by gating on CD45+ cells, this was then followed by exclusion of dead cells using vivid dye. Thereafter, monocytes were excluded, followed by gating on lymphocytes. This was followed by gating on $CD3^+$ cells and subsequently $CD4^+$ and $CD8^+$ T cells. The next gate measured CD25 and CD127 expression which then led to the identification of FoxP3 expression. The subsequent plots show expression of the co-inhibitory markers CD39, CTLA-4 and Tigit respectively. Included are the FMO plot for these markers.

5.3.2 CD4+ T cell frequencies

$CD4^+$ and $CD8^+$ T cell subsets were gated from a parent population of $CD45^+CD14^-CD3^+$ using the gating strategy shown in Figure 5.1. The frequency of systemic $CD4^+$ T cells stratified by ART exposure and birth outcomes is shown in Figure 5.2. Overall, there was a median $CD4^+$ T cell frequency of 41.3% (IQR: 30.9% - 53.9%) in the stable group at baseline 45.5% (IQR: 35.2% - 54.9%) and at follow up compared to 36.4% (IQR: 27.2% - 46.9%) at baseline (ART naïve) in the initiating group and 45.4% (IQR: 37.4% - 51.8%) at follow up (Figure 5.2A). We did not find any significant differences in $CD4^+$ T cells at follow up (A2) in both the initiating and stable groups (Figure 5.2A). When we looked at $CD4^+$ T cell proportions according to birth outcomes in the initiating and stable groups over time, we observed no significant differences in the patients initiating ART (Figure 5.2B) and those stable on ART (Figure 5.2C). An inverse association was found for the $CD8^+$ T cells, also gated from a parent population of $CD45^+CD14^-CD3^+$ (Figure 5.1). There was a median CD8 T cell frequency of 50.2% (IQR: 40.6% - 61.5%) in the stable group at baseline and 46.4% (IQR: 39.2% - 57.4%) at follow up compared to 54.4% (IQR: 44.4% - 60.7%) at baseline in the initiating group and 46.5% (IQR: 40.2% - 55.7%) at follow up (A2) (p=0.0068) (Figure 5.2D). There were lower proportions of CD8 T cells at A2 in the initiating group. The same trend was seen in the stable group, this association was however not significant. There were no differences in the proportions of $CD8^+$ T cells, over time when stratified by birth outcomes for those initiating ART (Figure 5.2E) and those stale on ART (Figure 5.2F). The stable outcome for patients initiating ART had lower $CD8^+$ T cell frequencies at both time points, although not significant. In summary, these data show a decreased $CD8^+$ T cell frequency after ART initiation with no differences by outcome with no notable differences seen for $CD4^+$ T cell frequencies.

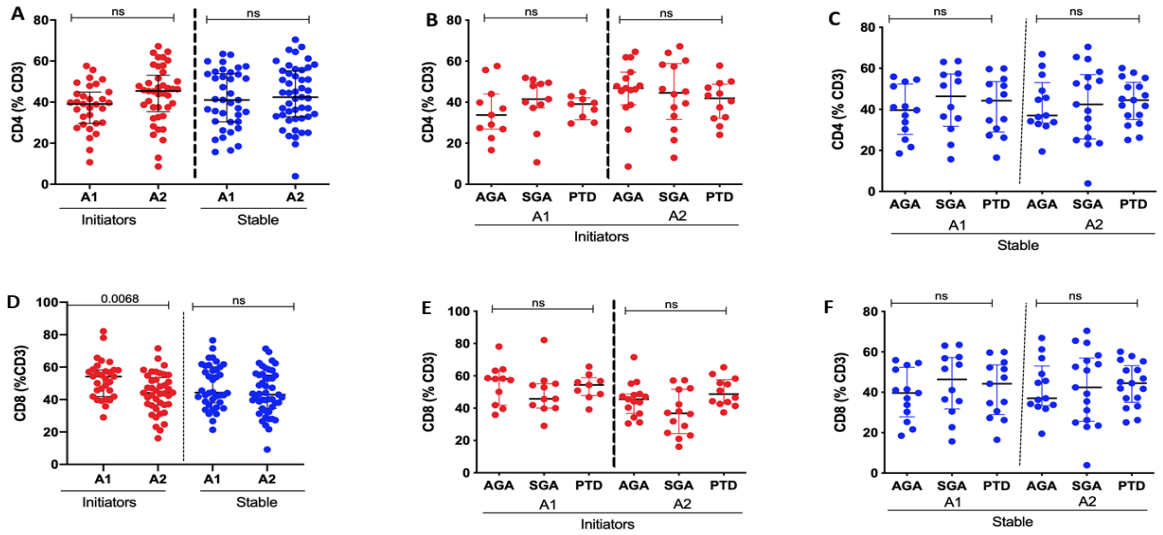


Figure 5.2: CD4⁺ and CD8⁺ T cell frequencies. (A) CD4⁺ T cell frequencies for women initiating (red circles) and stable on ART (blue circles) over time and not stratified by birth outcome. B) CD4⁺ T cell frequencies by birth outcomes for women initiating ART (in red) at baseline (A1), 28 weeks gestation (A2). (C) CD4⁺ T cell activation levels by birth outcomes for women stable on ART (blue) baseline (A1) and 28 weeks gestation (A2). D) CD8⁺ T cell frequencies for women initiating (red circles) and stable on ART (blue circles) over time and not stratified by birth outcome. B) CD8⁺ T cell frequencies by birth outcomes for women initiating ART (in red) at baseline (A1), 28 weeks gestation (A2). (C) CD8+ T cell activation levels by birth outcomes for women stable on ART (blue) baseline (A1) and 28 weeks gestation (A2).

5.3.3 Treg frequencies are increased in patients initiating ART.

The median FoxP3^{+} Treg cell frequency was 4,2% (IQR 3,6% - 5,44%), in the initiating group at baseline and 5,2% (IQR: 3,4% - 6,6%) at follow up compared to 4,8 (IQR: 3,2-5,7) at baseline in the stable group and 4,4 (IQR: 3,4-5,1) at follow up (Figure 5.3). Patients initiating ART had significantly higher proportions of Tregs at the A2 time point (Figure 5.3A, p=0.0003), no significant differences were observed in Treg frequencies by birth outcome (Figure 5.3B and 5.3C). There was also no correlation with viral load data at baseline (data not shown). Overall, treatment initiation increased Treg frequencies although this difference was not translated to birth outcomes.

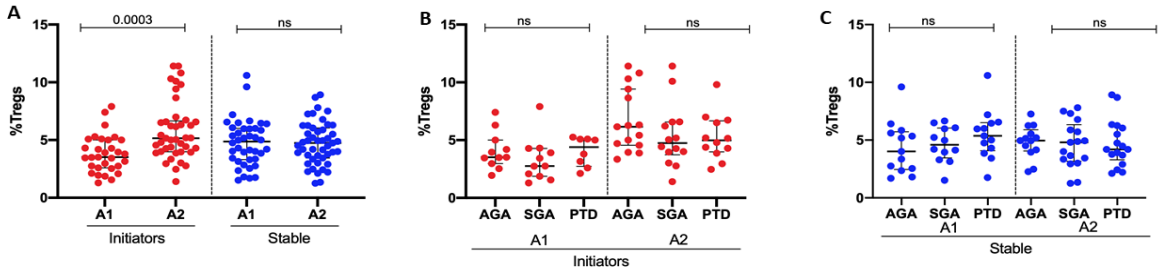


Figure 5.3: Treg frequencies defined as CD4^{+}CD25^{bright}CD127^{dim}FoxP3^{+}Tregs: A) Treg frequencies of women initiating and stable on ART at each time point over time. (B) Treg cell frequencies by birth outcomes for women initiating ART (in red) at baseline (A1), 28 weeks gestation (A2).(C) Treg cell frequencies by birth outcomes for women stable on ART (blue) baseline (A1) and 28 weeks gestation (A2).

5.3.4 Expression of Treg co-inhibitory receptors over time.

Previous research has shown that surface receptors play a critical role in controlling the adaptive arm of immune responses and are critical for Treg function. These include two members of the CD28 family of receptors, the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) as well as the inducible costimulator (ICOS), and CD39, a member of the E-NTPDases (ectonucleoside triphosphate diphosphohydrolases) (Schulze zur Wiesch et al., 2011, Rocco et al., 2018, Takahashi et al., 2000, Borsellino et al., 2007, Mandapathil et al., 2009, Mandapathil et al., 2010). Another co-inhibitory receptor that we explored is TIGIT which is known to be highly expressed on Treg cells. TIGIT expression defines a functionally distinct Treg cell subset with an activated phenotype (Joller et al., 2014).

These three receptors were measured over two time points exploring the differences in expression between women who conceived on ART and those stable on ART. We then further delineated expression to look at differences within birth outcomes. The purpose of this analysis was to ascertain if there would be any differences associated with treatment initiation as well as birth outcomes.

Firstly, CD39 expression on Tregs was measured and no significant differences were found in women who initiated ART during pregnancy or those who conceived on ART at both time points (Figure 5.4A). Upon stratification by birth outcome, initiators showed no differences in CD39 expression (Figure 5.4B) whereas a significantly higher CD39 expression was observed in the SGA and AGA outcomes at baseline and follow-up timepoints in women stable on ART (Figure 5.4C). There were no associations with the PTD outcome.

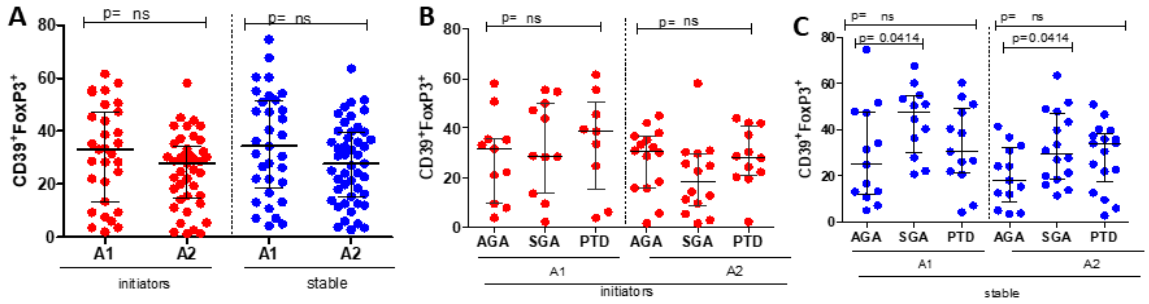


Figure 5.4: CD39 expression on Tregs: A) $CD39^+$ Treg frequencies of women initiating and stable on ART at each time point over time. (B) $CD39^+$ Treg frequencies by birth outcomes for women initiating ART (in red) at baseline (A1), 28 weeks gestation (A2). (C) $CD39^+$ Treg frequencies by birth outcomes for women stable on ART (blue) baseline (A1) and 28 weeks gestation (A2).

Analysis of CTLA-4 expression demonstrated a significantly higher expression of CTLA-4 in patients stable on ART at the second time point with no differences over time for patients initiating ART (Figure 5.5A). There were no notable differences in the expression of CTLA-4 by birth outcomes for both those initiating (Figure 5.5B) and those stable on ART (Figure 5.5C).

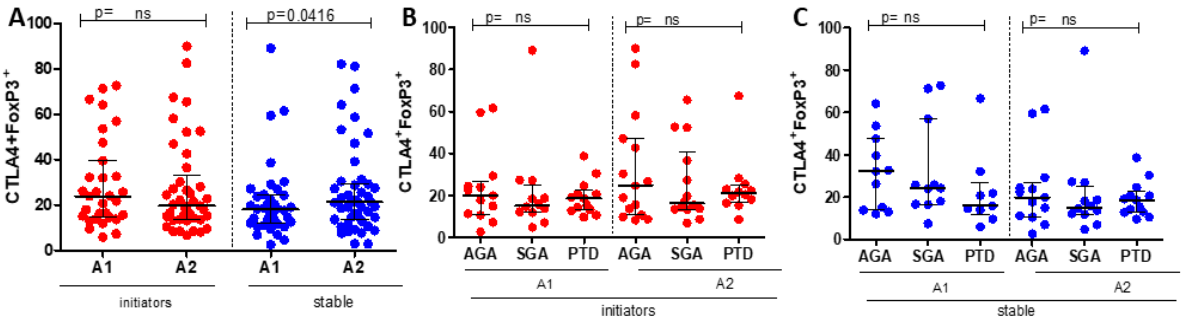


Figure 5.5: CTL-A expression on Tregs: A) CTLA-4^{+} Treg frequencies of women initiating and stable on ART at each time point over time. (B) CTLA-4^{+} Treg frequencies by birth outcomes for women initiating ART (in red) at baseline (A1), 28 weeks gestation (A2). (C) CTL-4^{+} Treg frequencies by birth outcomes for women stable on ART (blue) baseline (A1) and 28 weeks gestation (A2).

Lastly, Tigit showed significantly lower expression levels in women initiating and stable on ART at time point A2 (Figure 5.6A). There were no differences in birth outcomes for both initiators (Figure 5.6B) and those stable on ART (Figure 5.6C). Taken together, these data show us that there is differential expression of the molecules on Tregs but there is no distinct pattern of expression when stratified by birth outcome.

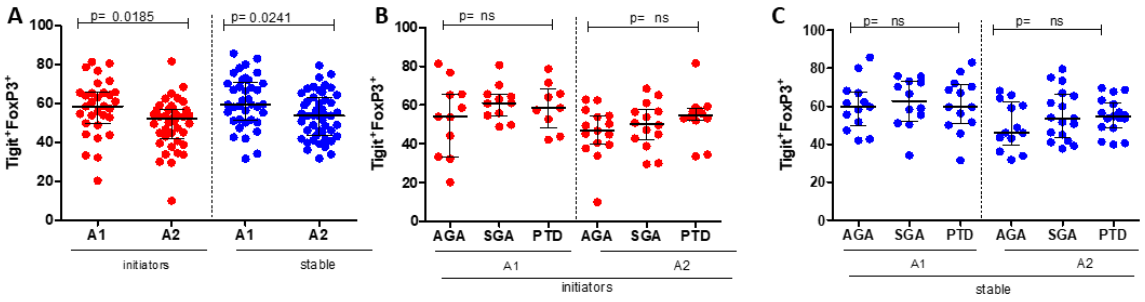


Figure 5.6: Tigit expression on Tregs: A) Tigit$^+$ Treg frequencies of women initiating and stable on ART at each time point over time. (B) Tigit$^+$ Treg frequencies by birth outcomes for women initiating ART (in red) at baseline (A1), 28 weeks gestation (A2). (C) Tigit$^+$ Treg frequencies by birth outcomes for women stable on ART (blue) baseline (A1) and 28 weeks gestation (A2).

5.4 Assessment of correlation of co-inhibitory receptors on Tregs

Next, we assessed whether the 3 co-inhibitory receptors correlated with each other at baseline (A1) and follow-up (A2) timepoints. We noted a significant negative correlation in the expression of Tigit and CD39 in patients initiating ART at baseline visit (Figure 5.7A). No relationship was found between Tigit and CD39 in patients initiating ART at follow up (Figure 5.7B). There were no significant correlations observed in patients stable on ART both at baseline and at follow up (Figure 5.7C and D). This could mean these co-inhibitory molecules are co-dependent in these patients before ART initiation.

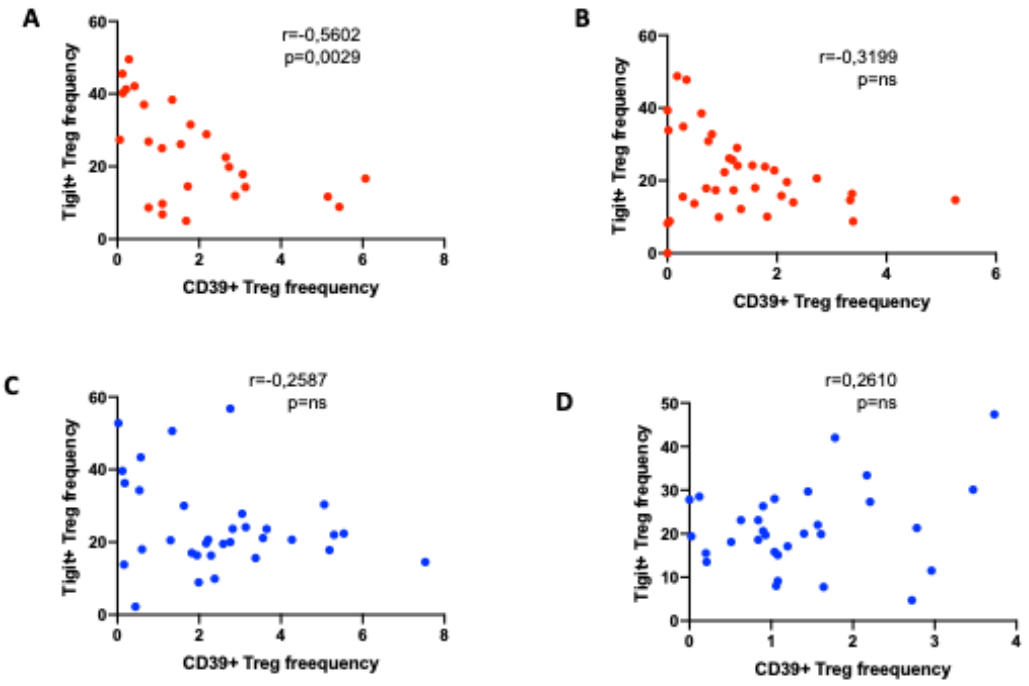


Figure 5.7: Scatter plots of correlations between Treg co-inhibitory recptors Tigit and CD39. Scatter plots showing the relationship at baseline and follow up between (A) Tigit$^+$ vs CD39$^+$ Treg expression frequencies for patients initiating ART (red circles) at A1 (B) Tigit$^+$ vs CD39$^+$ Treg expression frequencies and CD39 expression for patients initiating ART (red circles) at A2(C) Tigit$^+$ vs CD39$^+$ Treg expression frequencies for patients stable on ART (blue circles) at A1 (D) Tigit$^+$ vs CD39$^+$ Treg expression frequencies for patients stable ART (blue circles) A2.

Assessment of Tigit and CTLA-4 expression showed a negative correlation in patients initiating (Figure 5.8A) and stable on ART (Figure 5.8C) at baseline. This was also seen at follow up (A2) for both those initiating (Figure 5.8B) and stable on ART (Figure 5.8D).

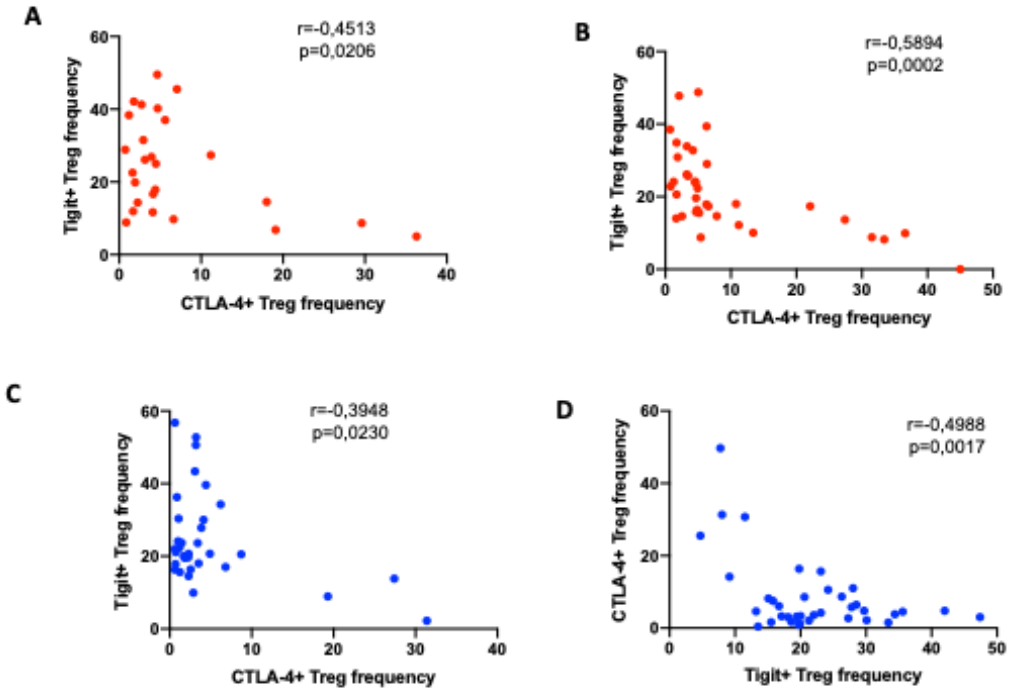


Figure 5.8: Scatter plots of correlations between Treg co-inhibitory receptors Tigit and CTLA-4. Scatter plots showing the relationship at baseline and follow up between (A) Tigit$^+$ vs CTLA-4$^+$ Treg expression frequencies for patients initiating ART (red circles) at A1 (B)) Tigit$^+$ vs CTLA-4$^+$ Treg expression frequencies for patients initiating ART (red circles) at A2 (C) Tigit$^+$ vs CTLA-4$^+$ Treg expression frequencies for patients stable on ART (blue circles) at A1 (D) Tigit$^+$ vs CTLA-4$^+$ Treg expression frequencies for patients stable on ART (blue circles) at A2.

For CTLA-4 and CD39 expression in Tregs, we found no significant correlation in patients initiating ART both at baseline (Figure 5.9A) and follow up (Figure 5.9B). There was a negative correlation in the patients stable on ART for both time points respectively (Figure 5.9C and D). Taken together these results suggested a negative correlation between the measured co-stimulatory molecules, which was specific to those stable on ART.

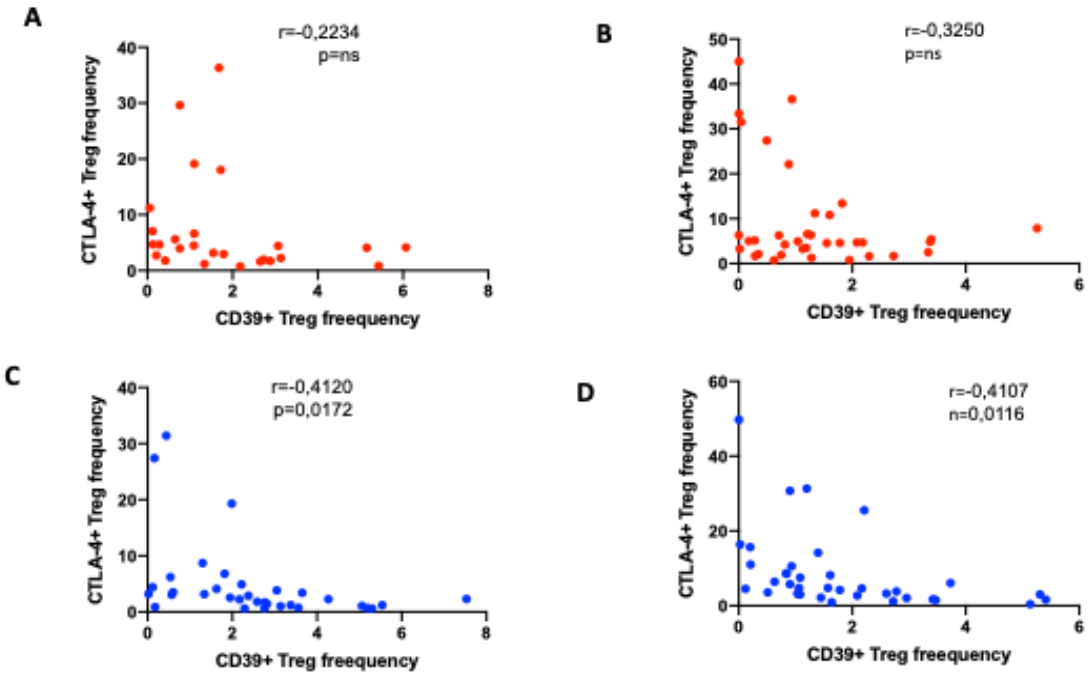


Figure 5.9: Scatter plots of correlations between Treg co-inhibitory receptors CTLA-4 and CD39. Scatter plots showing the relationship at baseline and follow up between (A) CTLA-4$^+$ vsCD39$^+$ Treg expression frequencies for patients initiating ART (red circles) at A1 (B) CTLA-4$^+$ vs CD39$^+$ Treg expression frequencies for patients initiating ART (red circles) at A2(C) CTLA-4$^+$ vsCD39$^+$ Treg expression frequencies for patients stable on ART (blue circles) at A1 (D) CTLA-4$^+$ vs CD39$^+$ Treg expression frequencies for patients stable on ART (blue circles) at A2.

5.5 Assessment of co-expression of TIGIT, CTLA-4 and CD39 by Tregs

Co-inhibitory or immune checkpoint receptors have a critical role in the maintenance of immune homeostasis: and their expression on regulatory T (Treg) cells guarantees the proper function of Treg cells in their role to control effector T cells (Anderson et al., 2016). We next measured co-expression of the three receptors using Boolean gating and SPICE. At baseline (Figure 5.10), our data shows that the majority of the Tregs are monofunctional as shown by the expression of one of the three receptors, followed by a subset that expresses 2 receptors in different combinations and a minority subset that express all 3 receptors (CD39, CTLA4 and TIGIT). The patients stable on ART as indicated by the red bars, has significantly lower

combined expression of Tigit and CTLA-4 ($p<0.0001$) at this time point when compared to those initiating ART during pregnancy (yellow bars). This could suggest that ART may affect the expression of these co-inhobitory factors.

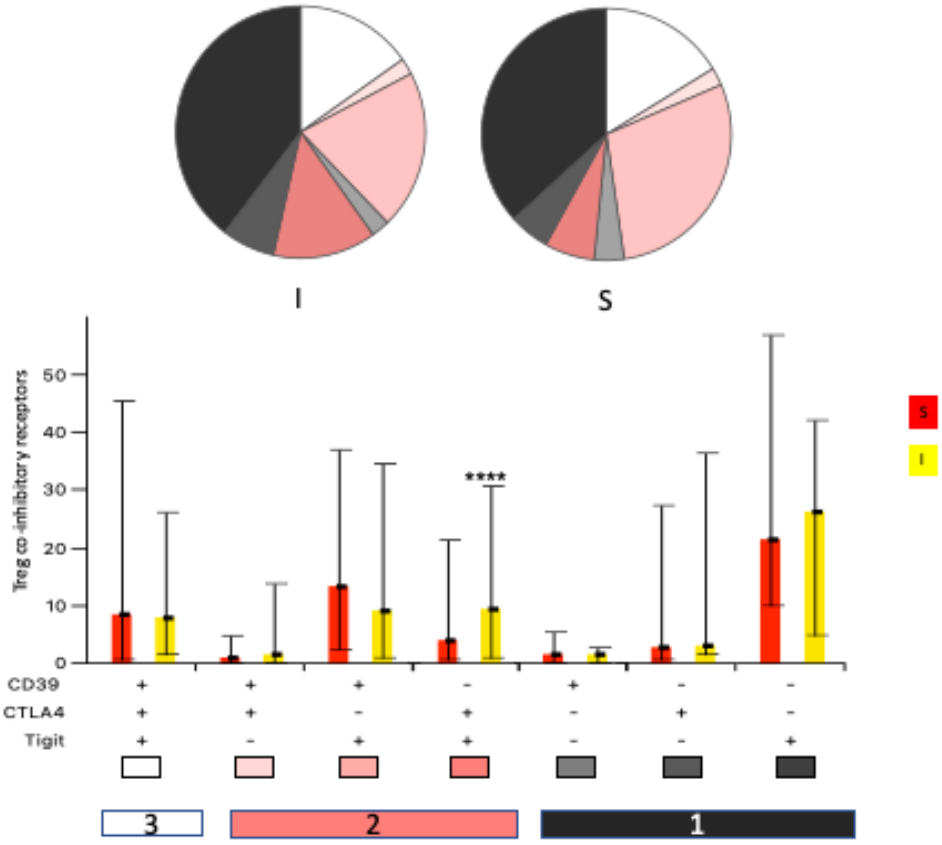


Figure 5.10: Treg expression of co-inhibitory receptors at baseline (A1). The cytokines studied are CD39, CTLA4 and Tigit. On the pie charts, white represents 3 functions, pink 2 and black 1 function. Boolean gating was performed in order to allow for the creation of possible combinations of response patterns. Positive responses were reported after background subtraction and the percentage of Tregs expressing the markers. Pestle (V2.0) and SPICE 6.0 (were used to were used to analyse the polyfunctional data. The letter I denotes initiators and the letter S denotes those stable on ART.

At follow up (A2), no significant associations were found as was observed at baseline (Figure 5.11). In summary, our all the possible response combinations, Tregs co-expressing Tigit and CTLA-4 were mostly significant and lower in patients stable on ART at both timepoints.

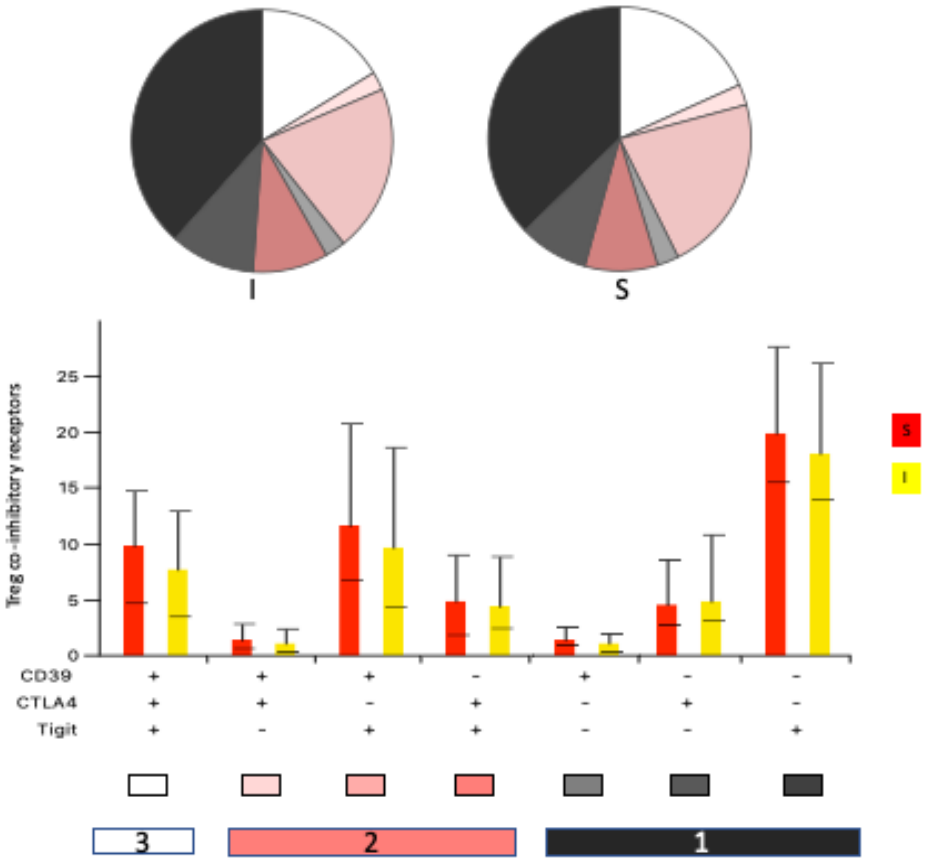


Figure 5.11: Treg expression of co-inhibitory receptors at follow up. (A2). The cytokines studied are CD39, CTLA4 and Tigit. On the pie charts, white represents 3 functions, pink 2 and black 1 function. Boolean gating was performed in order to allow for the creation of possible combinations of response patterns. Positive responses were reported after background subtraction and the percentage of cytokine producing Tregs. Pestle (V2.0) and SPICE 6.0 were used to were used to analyse the polyfunctional data. The letter I denotes initiators and the letter S denotes those stable on ART .

The next analysis was to delineate these responses by outcome for both timepoints i.e., baseline (A1) and at follow up (A2) in those initiating ART. Here we found no significant differences in the co-expression of the co-inhibitory molecules for any of the outcomes at baseline (Figure 5.12A) and at follow up visit (Figure 5.12B) . Patients with AGA and PTD outcomes had high proportion of Tigit only expressing cells whereas SGA patients had high levels of CD39 and Tigit co-expressing Tregs at baseline. At follow up, there was expression of Tigit in PTD and SGA and AGA had a higher expression of either one molecule. Although not significant, the initiation of ART may have changed these expression patterns as shown by differences in the proportions on cells expressing two moleculs at follow-up visit compared to baseline.

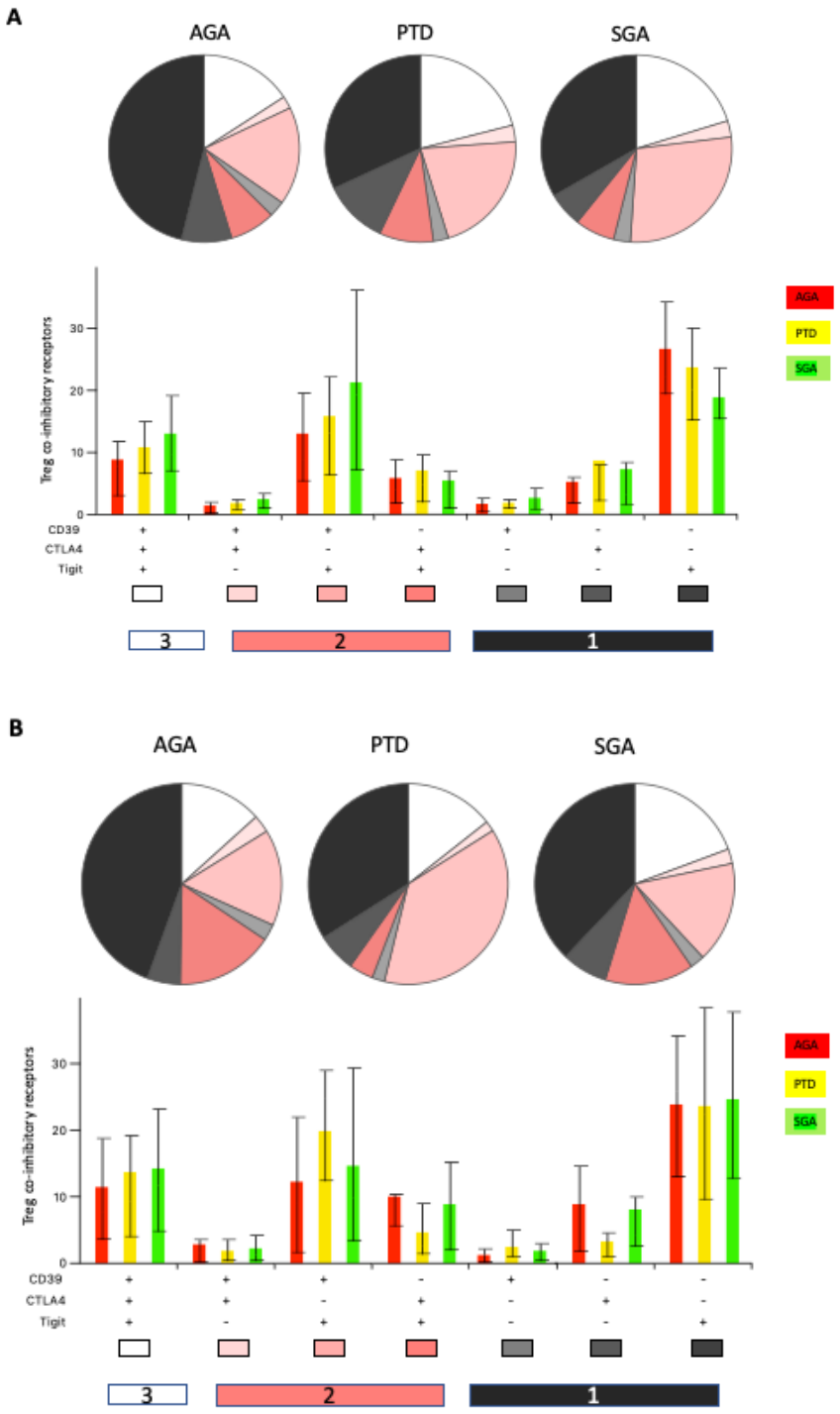
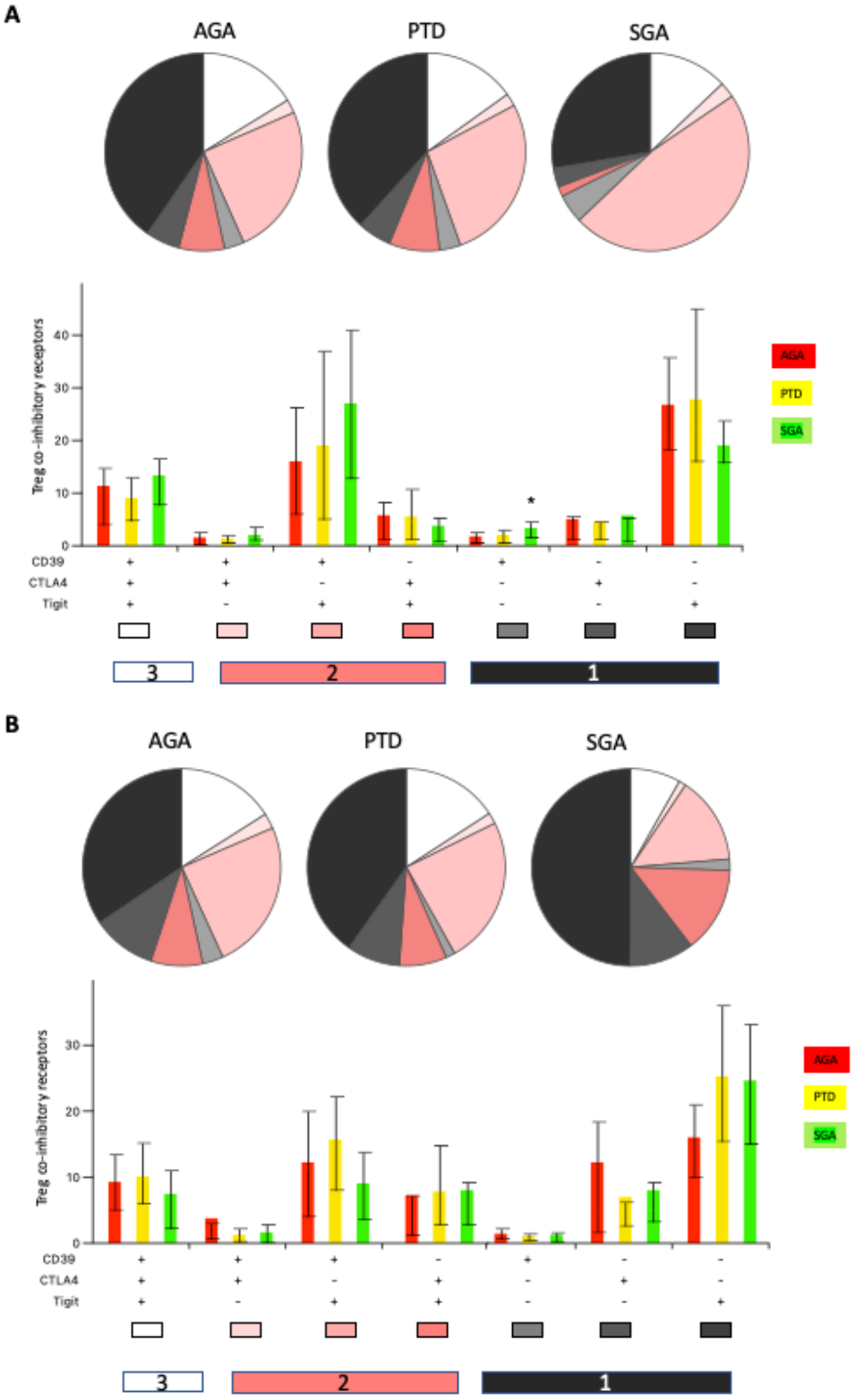


Figure 5.12: Treg expression of co-inhibitory receptors by outcome at at baseline (A1) and follow up (A2) for patients initiating ART. A) Data for baseline (A1) and B) results for and follow up (A2) time points. The cytokines studied are CD39, CTLA4 and Tigit. On the pie charts, white represents 3 functions, pink 2 and black 1 function. Boolean gating was performed in order to allow for the creation of possible combinations of response patterns. Positive responses were reported after background subtraction and the percentage of cytokine producing Tregs. Pestle (V2.0) and SPICE 6.0 were used to were used to analyse the polyfunctional data.

Lastly, assessment of patients stable on ART showed a significantly higher expression of CD39 ($p=0.0346$) in the SGA outcome compared to AGA and PTD (Figure 5.13A). In general, AGA and PTD outcomes expressed more Tigit, while the SGA outcome expressed a combination of Tigit and CD39. At follow up (A2), Figure 5.13B, there were no significant differences in the outcomes. Overall for all three outcomes, Tigit was mostly expressed; Tregs co-expressed Tigit with either CTLA4 or CD39 in this cohort.



5.13: Treg expression of co-inhibitory receptors by outcome at baseline (A1) and follow up (A2) for patients stable on ART. A) Data for baseline (A1) and B) results for and follow up (A2) time point. The cytokines studied are CD39, CTLA4 and Tigit. On the pie charts, white represents 3 functions, pink 2 and black 1 function. Boolean gating was performed in order to allow for the creation of possible combinations of response patterns. Positive responses were reported after background subtraction and the percentage of cytokine producing Tregs. Pestle (V2.0) and SPICE 6.0 were used to were used to analyse the polyfunctional data.

5.6 Summary

The aim of this work was to assess Tregs at baseline; analyze them in relation to birth outcome and to identify whether the initiation of ART would have an impact on the Treg balance and determine the relation to birth outcomes. To carry out this aim, samples from HIV infected women who were either ART naïve or conceived on ART were used. Our findings show that there are no significant differences in $CD4^+$ T cell frequencies in treatment groups as well as outcomes. There was a decrease in $CD8^+$ T cell frequencies in patients initiating ART at the time point post ART initiation, but this was not seen by birth outcome. Treg frequencies in this cohort were expanded in patients initiating ART which was not seen at follow-up time points. There was differential expression of co-inhibitory molecules on Tregs, however these were not detected when assessed by birth outcome. Lastly, co-expression data showed that Tregs mostly expressed Tigit with either CTLA4 or CD39 respectively.

During pregnancy, $CD4+CD25^{hi}FOXP3+$ regulatory T cells (Tregs) are found at high levels in decidual tissue and have the ability to suppress fetus-specific and nonspecific responses (Salvany-Celades et al., 2019, Aluvihare et al., 2004). Tregs have been confirmed to play a major role in preventing autoimmunity and tolerating allogenic organ grafts even outside the context of pregnancy. Normal pregnancy has also been associated with increased inflammatory biomarkers along with the higher Treg proportions (Zenclussen, 2005). Studies done on abortion prone mice were the first indicators of an insufficient generation of Tregs during pregnancy (Zenclussen et al., 2005). Data from human studies also found that Tregs have reduced functional abilities in patients experiencing miscarriages (Sasaki et al., 2004). Studies assessing the impact of ART on Treg function have have been controversial. Some have found that when preventing viral replication for example in the case of HBV infection, Treg

frequencies diminished (Nan et al., 2012). A study that was looking at HIV specifically found that the cell population was normalised upon treatment initiation (Presicce. P et al., 2011). In contrast, other studies reported Treg frequency levels remained significantly higher in ART-treated HIV patients compared to those in healthy subjects (Nikolova et al., 2011, Schulze zur Wiesch et al., 2011).

The data here showed that SGA outcome in patients stable on ART expressed higher levels on CD39 at both time points while no correlations could be made in patients initiating ART during pregnancy. Previous research has shown that the expansion of the $CD39^{+}$ Tregs subset correlates positively with the level of immune activation and negatively with CD4 T-cell counts in HIV-infected subjects (Nikolova et al., 2011). The data further suggests that ART initiation may disrupt the function of these Tregs, as co-expression of these molecules is indicative of Treg effector function (Anderson et al., 2016), this is at least true for CTLA-4 and CD39 whereas Tigit may be minimally affected.

There was a negative correlation between Tigit vs CD39 at baseline (A1) before the commencement of treatment which was lost upon follow-up. This result could suggest that the expression of these receptors on Tregs might be co-dependant and that ART may influence this expression. Co-expression analysis showed that the Tregs produced are suppressive because they express either one of the surface molecules that aid the cells in their function (Schulze zur Wiesch et al., 2011).

Tregs significantly co-express CTLA-4 and Tigit at baseline i.e., time point before ART and not at follow up with no significant differences in the stable on ART patients again suggesting that ART may have an effect of expression of these receptors (Anderson et al., 2016).

The SGA outcome of patients stable on ART had significantly higher levels of CD39. The expression of CD39 could indicate an immune checkpoint mediator which can be used to identify naïve Tregs that are prone to apoptosis. CTLA-4 is a molecule expressed on T lymphocytes capable to down-regulate cytotoxic T cells and is known to be constitutively expressed on Tregs (Osinska et al., 2015). It was also interesting that most Tregs expressed Tigit indicating that the cells form part of the distinct Treg cell subset that specifically suppresses pro-inflammatory T helper 1 (Th1) and Th17 cells, but not Th2 cell responses (Joller et al., 2014).

The study was limited in that it failed to analyse major differences in the frequencies of circulating Treg cells. It would be worthwhile to further reliably distinguish Tregs from activated effector $CD4^+$ T cells. To do this additional quantification of the overall frequency of functional Tregs will need to be performed based on the methylation status of the Treg-specific demethylated region (TSDR) of the FOXP3 gene using qPCR. (Angin et al., 2012). Another limitation of the study is the lack identification of Treg phenotypic populations as the data in these cells would have complemented the co-stimulatory receptor data and confirmed the type of Tregs that are expressed in this cohort (Shevyrev and Tereshchenko, 2020).

In conclusion, we had hypothesised that the reconstitution of immune responses under ART in pregnant women would result in enhanced immunity against the fetus resulting in increased

rates of preterm birth. Our data suggest that that the Tregs produced are high in patients initiating ART and subsequently altered by ART, however this alteration did not predict birth outcome. It is highly plausible that Tregs measured here are naïve and immune-suppressive $Foxp3^{+}$ regulatory T (Treg) cells owing to their expression of CD39 (Borsellino et al., 2007, Zhao et al., 2017), however further research is required as the direct functionality of Tregs was not measured in this study. Nonetheless, the modulation of co-inhibitory molecules and Tregs function is undoubtedly important as a therapeutic potential for treatment of a wide variety of inflammatory disorders, including cancer, chronic infection, autoimmune disease, and pregnancy complications.

Chapter 6 Plasma cytokine profiles in HIV positive pregnant women initiating and stable on ART

6.1 Introduction

The homeostasis of the immune system is mediated by cytokines and infection with Human Immunodeficiency virus (HIV) has been shown to lead to a deregulation in the production of both pro- and anti-inflammatory cytokines (Osuji et al., 2018, Kedzierska and Crowe, 2001). T cells play a role in the regulation and stimulation of the immune system. T-helper (Th) cells are classified into Th1 cells, which produce interleukin (IL)-2 and interferon (IFN) γ and are involved in cellular immunity, and Th2 cells, produce IL-4, IL-5 and IL-13 and are involved in humoral immunity (Byrnes et al., 2008, Saito et al., 2010). The success or failure of pregnancy can be attributed to a cytokine shift. Maternal tolerance toward fetal alloantigens has historically been explained by a predominant Th2-type immunity during pregnancy, which overrules Th1-type immunity, therefore protecting the fetus from maternal Th1-cell attack (Saito et al., 2010, Sykes et al., 2012). Pregnancy is further defined as a process that is both pro and anti-inflammatory depending on the stage of gestation. During the progression of HIV infection, a Th1 (pro-inflammatory) to Th2 (eosinophilic/anti-inflammatory) cytokine shift has been observed. This change has also been associated with HIV disease progression but has been associated with unsuccessful pregnancy outcome and preterm delivery (Sutton et al., 2004, Osuji et al., 2018, Kedzierska and Crowe, 2001). It has also been described that ART causes a shift and counteracts this balance (Fiore et al., 2006, Maharaj et al., 2017). It has been shown that these two cell types play critical roles in defensive immune responses and in immunopathological disorders such as allergic reactions and autoimmune diseases, and the methods of detecting Th1 and Th2 cells have become more important. We therefore hypothesized that an ART mediated cytokine shift in HIV infected women who initiate ART could be responsible for an increase in preterm deliveries. To carry this out we quantified plasma cytokine profiles associated with the initiation of ART during pregnancy and assed their impact on pregnancy outcomes.

6.2 Methods

6.2.1 Study participants and study design

HIV infected women ≤24 weeks gestation, as assessed by ultrasound, were enrolled, and followed with three study visits for those on ART pre-conception (stable on ART) at <20 weeks (baseline), 28 and 34 weeks of pregnancy, plus an additional study visit two weeks after ART initiation for women newly identified as HIV infected and initiated on ART at their first ANC visit. At each visit, blood was drawn into sodium heparin tubes (BD Vacutainer, New Jersey, USA) and peripheral blood mononuclear cells (PBMCs) and plasma isolated within 4 hours of blood collection by density gradient centrifugation.

For the data presented here, 30 cases of PTD, 30 SGA cases and 30 appropriate-for-gestation age (AGA)/term controls were selected (Figure 6.1). Controls and cases were matched on timing of ART initiation, and analysis carried out blinded. The median gestational age at enrolment was 15 weeks both for women initiating and stable on ART. PTD was defined as delivery <37 weeks, SGA as weight for gestational age ≤10^{th} centile, AGA controls were term, with weight for gestational age ≥25^{th} centile (Malaba et al., 2020).

As previously shown in chapter 2, patient's plasma was taken at four time points for patients initiating therapy and three for patients stable on ART. We first looked at the expression levels of each cytokine in the initiating and the stable groups and further examined the outcome for each longitudinally over the time points. To understand the pathways induced during ART initiation in HIV infected pregnant women, we assessed systemic levels of 15 soluble biomarkers (IFN-γ, IL-1α/ß, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p40 and p70), IFN-α, TNFα, MIP1β, and IP-10) longitudinally in individuals who initiated ART during pregnancy

compared to those who conceived on ART. These biomarkers were measured at baseline, 2 weeks post ART initiation, at 28 weeks and at 34 weeks of gestation.

6.2.2 Statistical analysis

Univariate analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Mann-Whitney U and Wilcoxon Signed Rank tests were used for unmatched and matched comparisons, respectively. Spearman Rank tests were used to test for correlations. P-values<0.05 were considered significant. Heat maps were generated using STATA™ (StataCorp, Texas, USA) e cut-off of +0.5 and -0.5 for a significant positive and negative correlation, respectively.

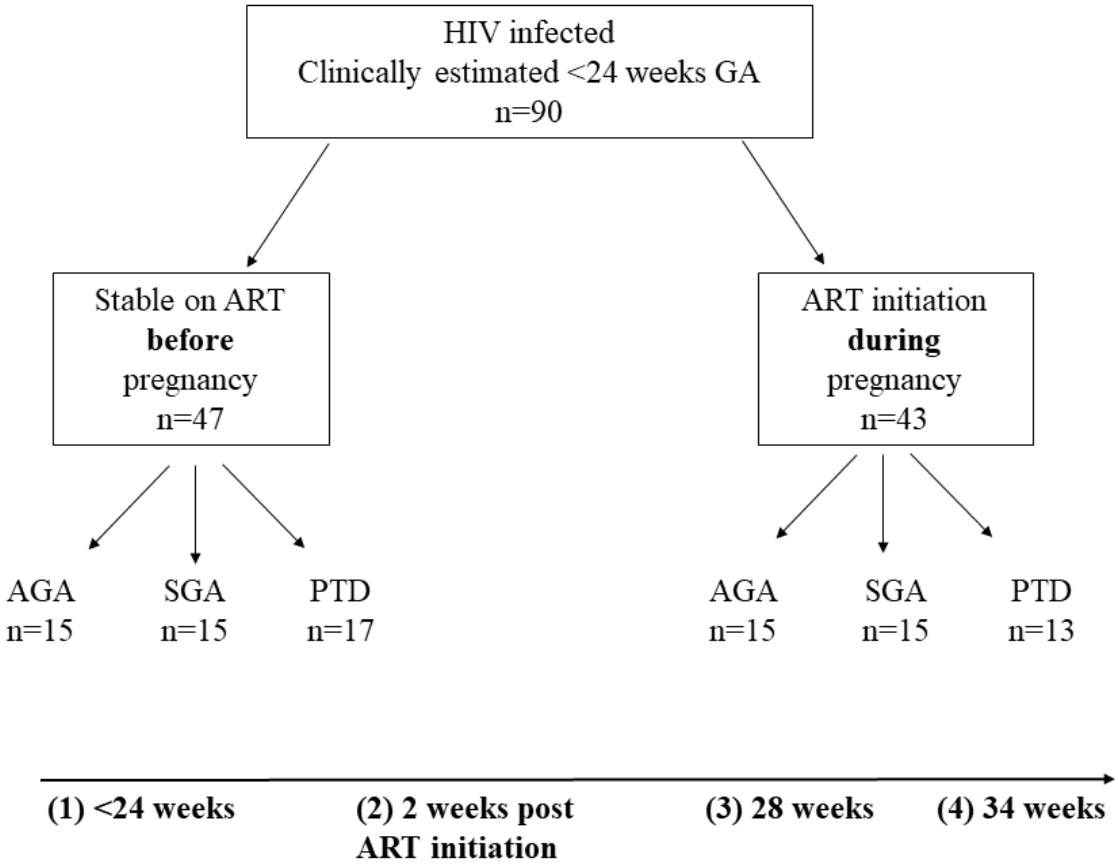


Figure 6.1: Patient layout.

6.3 Results

Median age of study participants was 32 years (IQR: 26-36) (Table 6.1). Socio economic status (SES) was measured using a composite socioeconomic status score, based on current employment, housing type and access to household assets, that was used to categorize women as ‘high’, ‘mid’ or ‘low’. There was a total of 28 women who categorised under low, 30 medium and 29 under high accounting for 29% of the total number of women in the study population. Of the 90 women, 47 initiated ART pre-pregnancy (stable on ART), and 43 at first ANC (initiators). Most (n=79, 88%) of pregnant women were on TDF-3TC-EFV regimen. The median weight was 70.15 (kg) (IQR:59.4-83.9). Median CD4 count is 436 (IQR: 392-573), at baseline, viral load median is 281 copies/ml.

Table 6.1 Demographic and clinical characteristics of women who initiated ART during or before pregnancy.

	Total	Initiation before pregnancy				Initiation during pregnancy			
	N=90	N=47				N=43			
		AGA	PTD	SGA	P-value	AGA n=15	PTD n=13	SGA n=15	P-value
		N=15	N=17	N=15					
Maternal characteristics									
Age, years: median (IQR)	32 (26-36)	33 (28-35)	36 (32-38)	36 (28-39)	0.205	28 (24-34)	26 (25-31)	31 (25-37)	0.768
Education (finished high school)	32 (35)	5 (33)	5 (29)	5 (33)	0.962	7 (47)	6 (46)	4 (27)	0.450
Employments status: Employed (%)	33 (36)	5 (33)	7 (41)	6 (40)	0.889	6 (40)	3 (23)	6 (40)	0.565
Socioeconomic status (SES)*					0.741				0.860
Lowest	28(31)	3 (20)	6 (35)	5 (33)		4(28)	4(30)	6(40)	
Medium	30(33)	6 (40)	5 (30)	5 (33)		5(35)	5(38)	4(26)	
Highest	29(29)	6 (40)	6 (35)	4 (27)		6(46)	3(23)	4(26)	
Missing	3 (3)	0 (0)	0 (0)	1 (7)		0 (0)	1(7)	2(13)	
Obstetric characteristics									
Gravidity: median (IQR)	3 (2-3)	3 (2-4)	3 (2-3.5)	4 (3-4)	0.420	2 (2-3)	2 (2-3)	2 (1-2)	0.347
Parity: median (IQR)	1(0-2)	1 (1-2)	1 (1-2)	2 (1-2)	0.244	1 (0-1)	1 (0-2)	1 (0-2)	0.315
Previous Preterm*: Yes	9 (10)	0 (0)	2 (12)	5 (33)	0.034	1 (7)	0 (0)	1 (7)	0.635

Gestational age at booking/enrolment: median weeks (IQR)	15 (11-18)	13 (9-15)	16 (9-17)	15 (9-18)		14 (12-17)	19 (14-21)	16 (12-18)	
Height, (cm): median (IQR)	158 (155.5-162.5)	162 (156-166)	158 (155.5-161)	157 (150.5-163)	0.343	160 (156.5-169.5)	158 (155.5-160)	159 (152.5-161.1)	0.161
Haemoglobin (g/dl): median (IQR)	11,4 (10-3-12.4)	11 (11.1-12)	11.8 (11-12.7)	11.6 (10.6-12.9)	0.283	10.5 (10-12)	11.3 (10.2-11.7)	10.9 (10.1-11.6)	0.697
Weight (kg): median (IQR)	70.15 (59.4-83.9)	74.9 (64,30-85,95	76 (67,30-85,45)	61.7 (52,50-74,70)	0.126	80,10 (61,70-98,40)	61,35 (58,28-67,48)	64,00 (56,70-92,00)	0.413
HIV-associated parameters									
Current ART regimen, self-report					0.199				0.353
TDF-3TC-EFV	79 (88)	13 (86)	13	13 (86)		14 (93)	11 (84)	15 (100)	
TDF-3TC-NVP	1 (1)	0 (0)	0	0 (0)		1 (7)	0 (0)	0 (0)	
Other NNRTI-based regimen	3 (3)	0 (0)	3	0 (0)		0 (0)	0 (0)	0 (0)	
PI-based regimen	6 (6)	2 (14)	1	2 (14)		0 (0)	1 (8)	0 (0)	
Missing	1 (1)	0 (0)	0 (0)	0 (0)		0 (0)	1 (8)	0 (0)	
CD4 cell count, (cells/µL)* median (IQR)	436 (392-573)	416 (353-566)	529 (309-638)	485 (333-584)	0.509	368.5 (247-535)	314 (219-457)	493 (283.5-926.5)	0.724
Missing	14 (16)	2 (13)	2 (11)	0 (0)		3 (20)	4 (30)	3 (20)	
Viral load, copies/ml (baseline A1), median (IQR)	281 (20-80000)	20 (20-27)	25 (20-528)	20 (20-65)	0.342	21900 (6120-59900)	4730 (1300-7950)	2120 (741-18800)	0.681

Viral load, copies/ml (A1.5), median (IQR)	198 (37-431)	431 (275-512)	99 (20-137)	89 (25-307)	0.172

* Study participants are denoted by number with percentages in brackets

*SES was measured using a composite socioeconomic status score, based on current employment, housing type and access to household assets, that was used to categorize women as ‘high’, ‘mid’ or ‘low’ SES. A median of these is shown above.

*CD4 results abstracted from routine records and are the nearest in time to the first ANC visit.

*All study participants were normotensive. Data was missing for 10 patients initiating therapy during pregnancy and 4 patients stable on ART.

*P values denote to the comparison across the three groups (AGA, SGA and PTD

6.4 Cytokine quantification

TH1-related cytokines are involved in cell mediated immunity, and typically comprise of cytokines Interleukin (IL)-1, IL-2, IL-6, IL-12, IFN-γ, TNF-α, interferon (IFN) -γ, and tumour necrosis factor (TNF)-α, MIP-1β, and IP-10. The Th2 cells secrete anti-inflammatory cytokines such as interleukin 4 (IL-4), IL-10, and IL-13.

6.4.1 IFN-α expression profile over time.

Upon analysis, we found no significant differences in the expression of IFN-α over time when looking at patients initiating and those stable on ART as shown in Figure 6.2A. When we compared patients initiating and stable on ART at each time point, not having delineated them according to outcome, there was a significantly higher expression of IFN-α in patients initiating ART than those stable on ART at baseline and at week 34 (Figure 6.2B). When we looked at the outcome in patients initiating ART and those stable; we found no significant differences over time (Figure 6.2C and D).

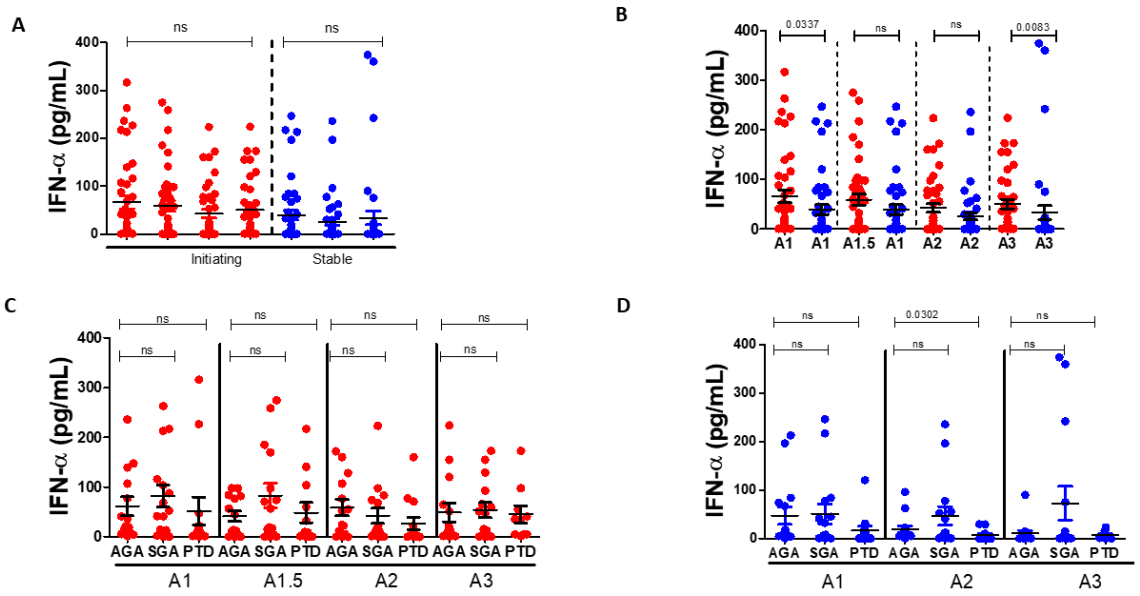


Figure 6.2: IFN-α expression profile over time. A) IFN-α expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IFN-α expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.2 IFN-γ expression profile over time

We did not find any significant difference in the expression of IFN-γ over time as show in Figure 6.3A. When we compared patients initiating and stable on ART over time, not having delineated them according to outcome, there was a significantly higher expression of IFN-γ in patients initiating ART than those stable on ART over time (Figure 6.3B). When we looked at the outcome in patients initiating ART and those stable we found no significant differences in expression over time (Figure 6.3C and D).

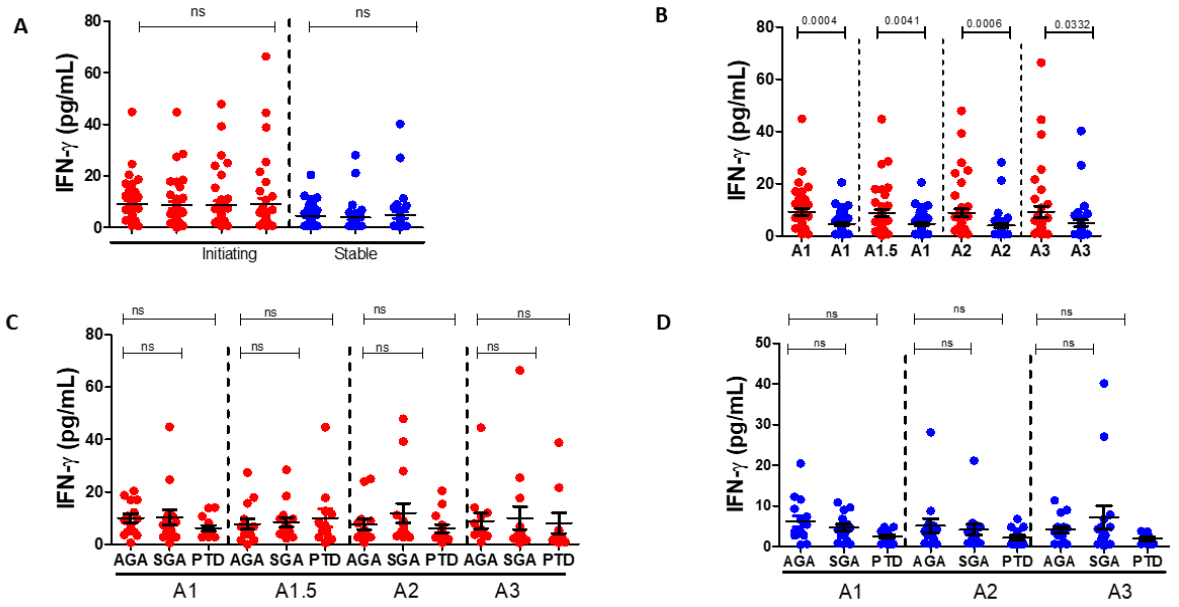


Figure 6.3: IFN-γ expression profile over time. A) IFN-γ expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IFN-γ expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.3 Expression profile of interleukin 12 (p40) and p70

There was no significant expression of IL-12 (p40) regardless of the comparisons made (Figure 6.4A-D).

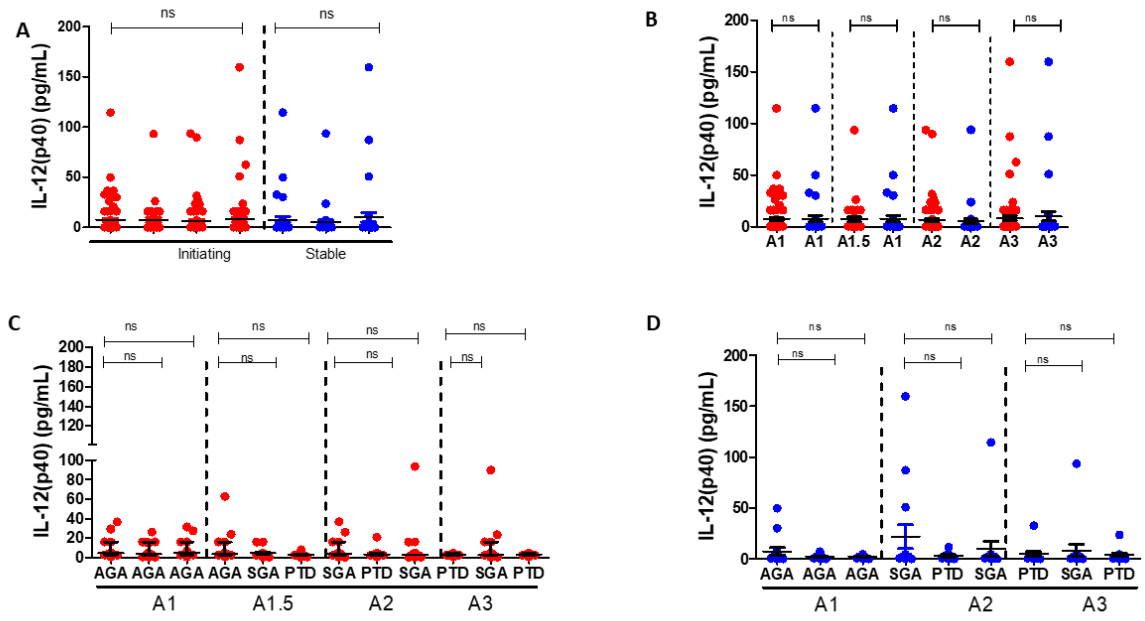


Figure 6.4: IL-12 (p40) expression profile over time. A) IL-12 (p40) expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-12 (p40) expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.4 Expression profile of interleukin 12 (p70)

There was no significant expression of IL-12 (p70) regardless of the comparisons made (Figure 6.5A-D).

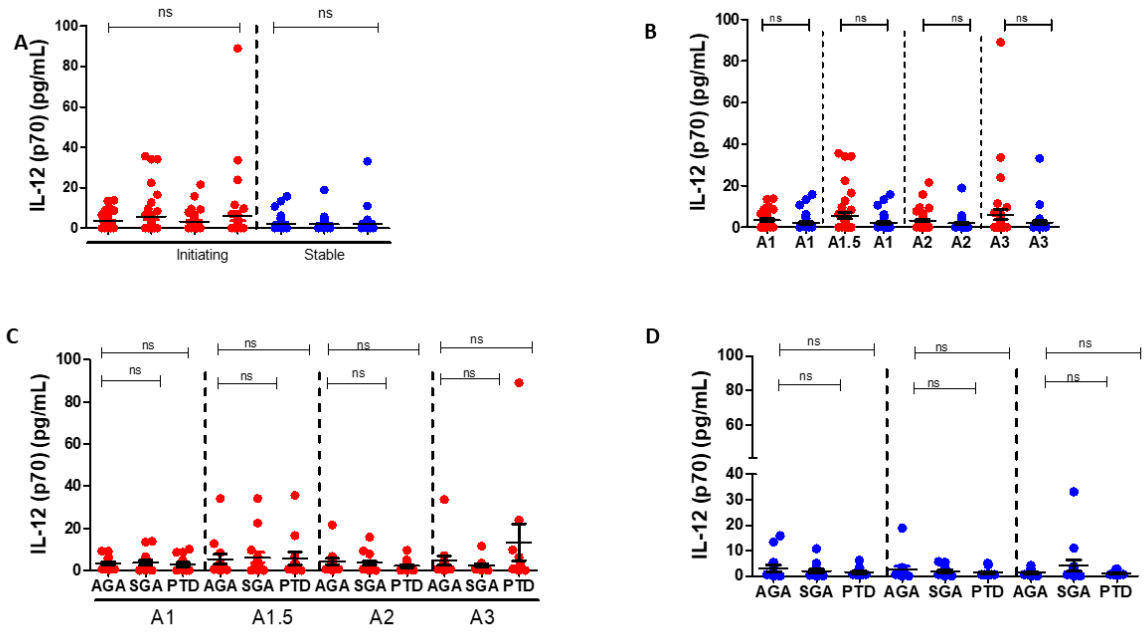


Figure 6.5: IL-12 (p70) expression profile over time. A) IL-12 (p70) expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-12 (p70) expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.5 Expression profile of IP-10

We found high amounts of IP-10 and these did not differ over time, irrespective of the treatment status (Figure 6.6A). When we compared IP-10 levels at each time point between those initiating and those stable, the was a significantly lower expression at baseline and two weeks post ART initiation in those stable compared to patients initiating (Figure 6.6B). No differences were seen in patients when they were delineated by treatment and outcome for both those initiating (Figure 6.6C) and those stable on ART (Figure 6.6D).

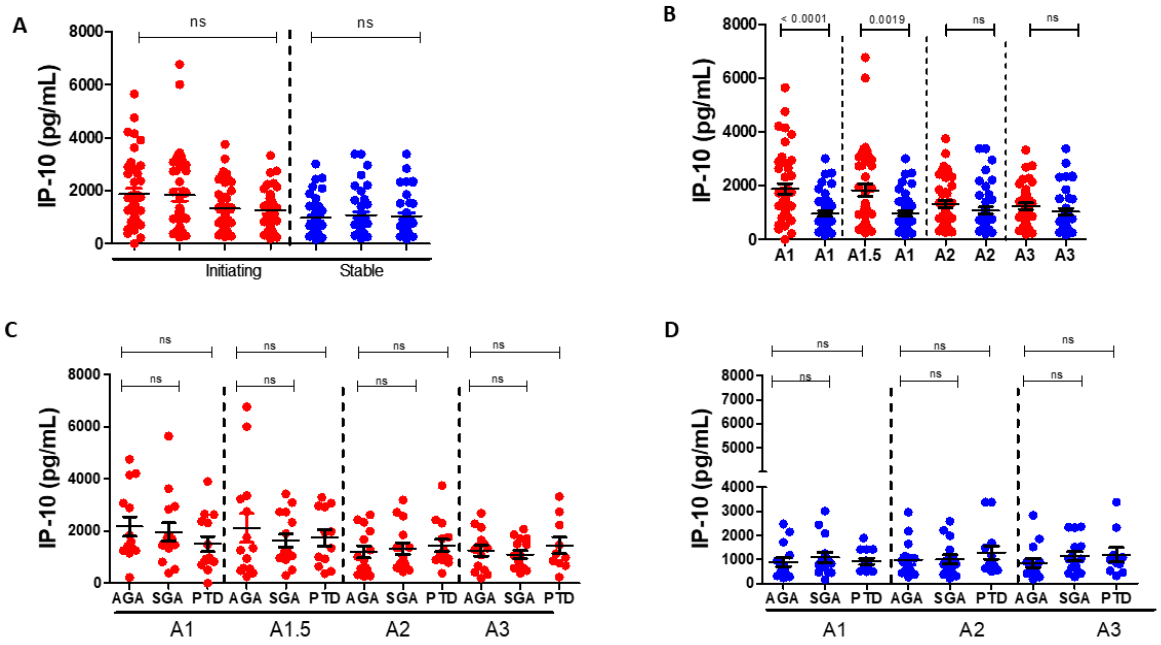


Figure 6.6: IP-10 expression profile over time. A) IP-10 expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IP-10 expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.6 Expression profiles of interleukin-1α and β

There was no significant difference in the expression of both IL-1α and IL-1β in patients initiating or those stable on ART over time (Figure 6.7A and 6.8A). When we compared patients initiating and those stable, we found that for IL-1α there were significantly lower levels of the cytokine in patients who were stable on ART compared to those who were ART naïve at baseline (Figure 6.7B). Whereas for IL-1β there was consistently, lower levels of expression for patients stable on ART compared to those initiating ART during pregnancy (Figure 6.8B). No dereferences were seen in patients when they were delineated by treatment and outcome for both those initiating (Figure 6.7C and 6.8C) and those stable on ART (Figure 6.7D and 6.8D).

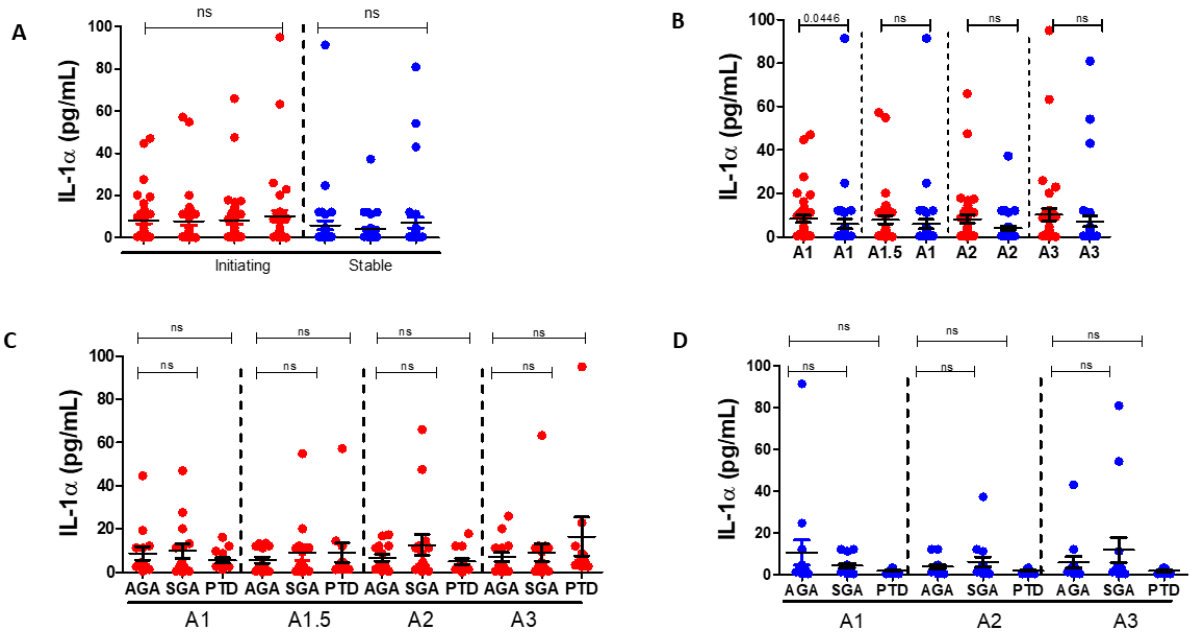


Figure 6.7: IL-1α expression profile over time. A) IL-1α expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-1α expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

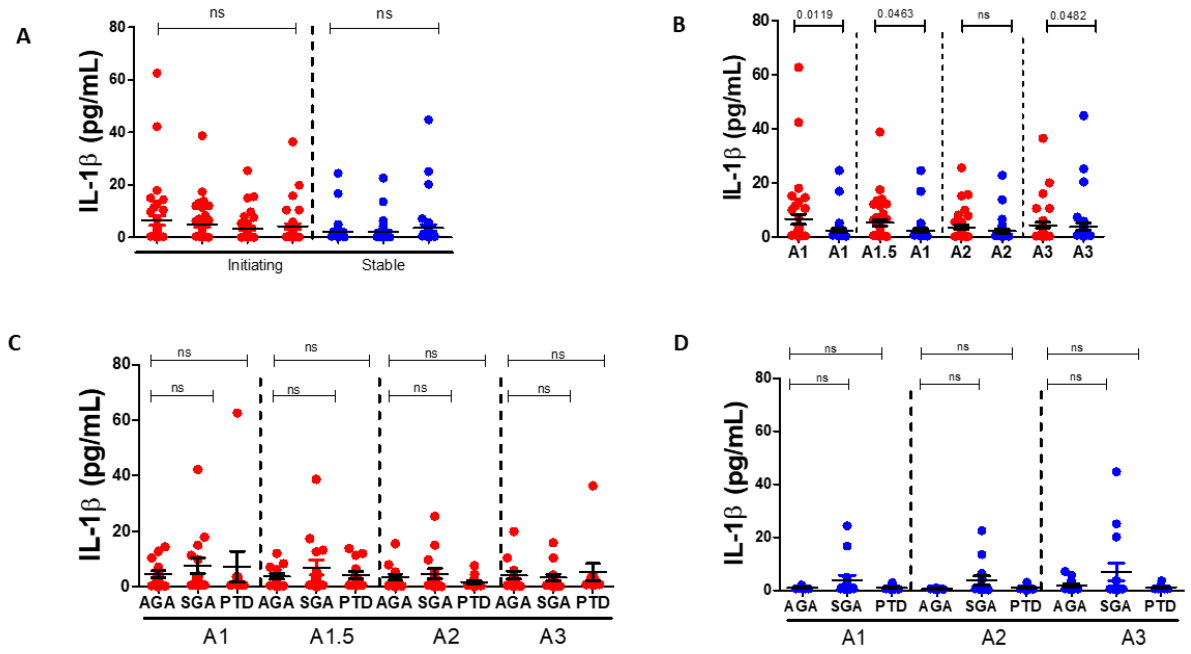


Figure 6.8: IL-1β expression profile over time. A) IL-1β expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-1β expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.7 Expression profile of MIP-1β

Macrophage inflammatory protein-1α (MIP-1α) and MIP-1β play an important role in modulating immune responses. Upon analysis, we did not find any significant difference in MIP-1β expression over time in patients initiating and stable on ART (Figure 6.9A). We then compared patients initiating and stable on ART at each time point and found a significantly higher expression at 2 weeks post initiation versus stable on ART (Figure 6.9B). There were no differences in outcomes over time in patients initiating ART (Figure 6.9C). We found a higher expression of MIP-1β in SGA compared to AGA at the second time point in patients stable on ART (Figure 6.9D).

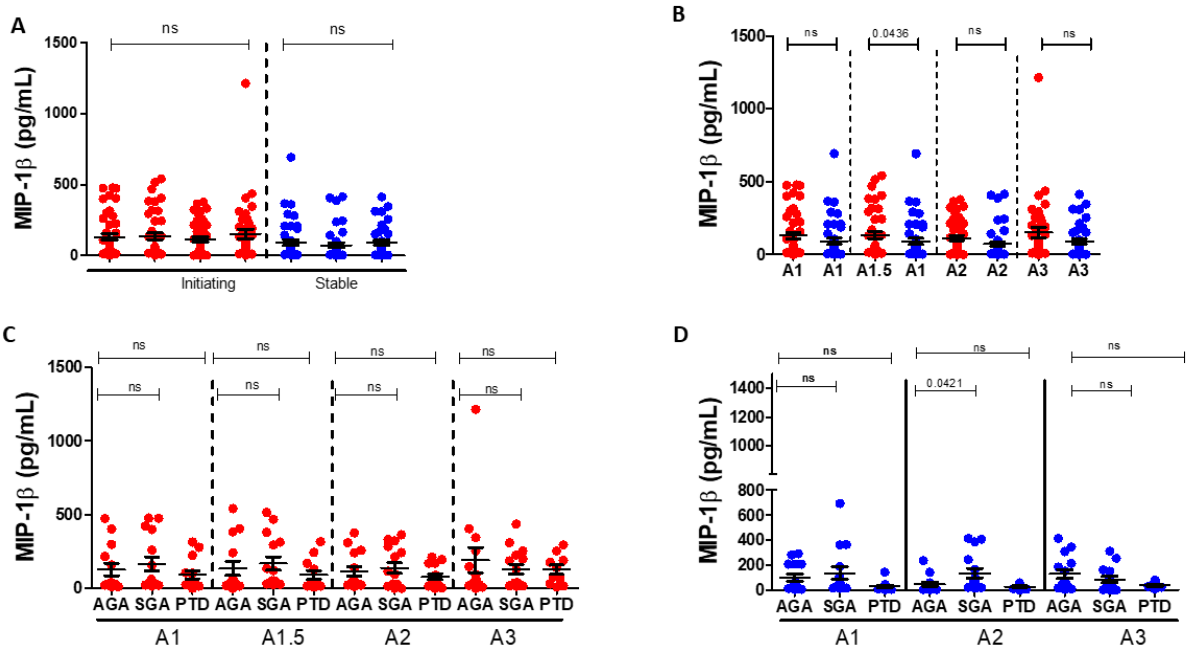


Figure 6.9: IL-1α expression profile over time. A) IL-1α expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-1α expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.8 Expression profile of interleukin-4

We did not see any differences in the expression of IL-4 over time when comparing patients initiating and those stable on ART (Figure 6.10A). We also did not see any differences when comparing patients initiating and stable on ART at each time point (Figure 6.10B). Again, no differences were seen in patients initiating (Figure 6.10C) and those stable on ART (Figure 6.10D) when differentiated by outcome.

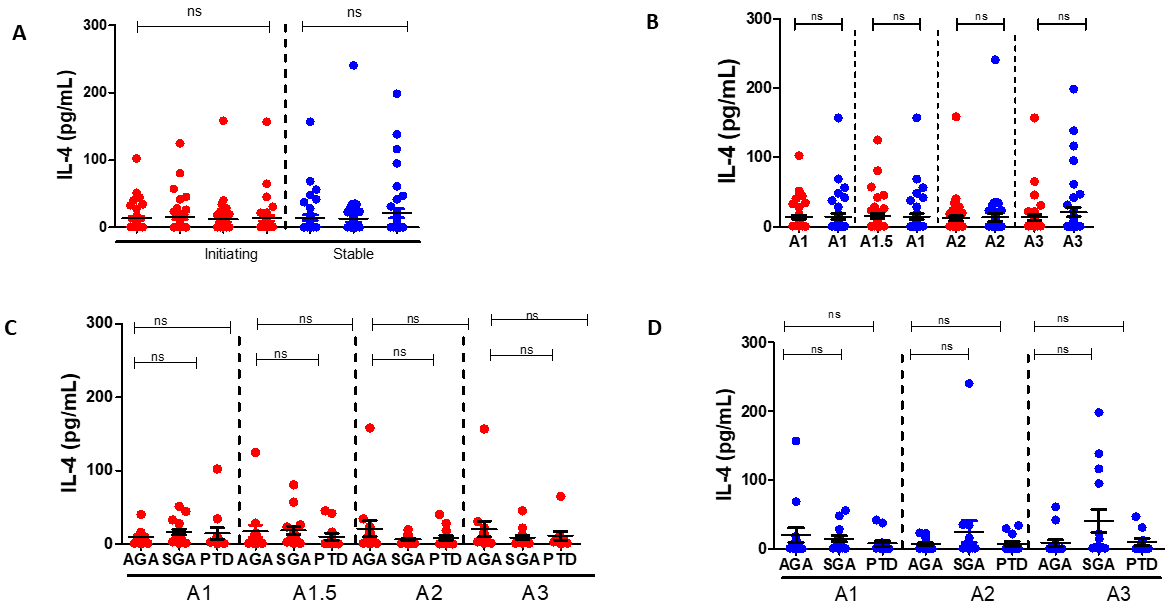


Figure 6.10: IL-4 expression profile over time. A) IL-4 expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-4 expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.9 Expression profile of interleukin-10

There were no significant differences in the expression of IL-10 over time in patients initiating and stable on ART (Figure 6.11A). In line of what was reported in literature, we saw lower IL-10 levels in patients stable on ART when compared to those initiating (Osuji et al., 2018) (Figure 6.11B). We found no significant differences in outcomes when we looked at on patients initiating ART during pregnancy (Figure 6.11C) and those stable on ART (Figure 6.11D).

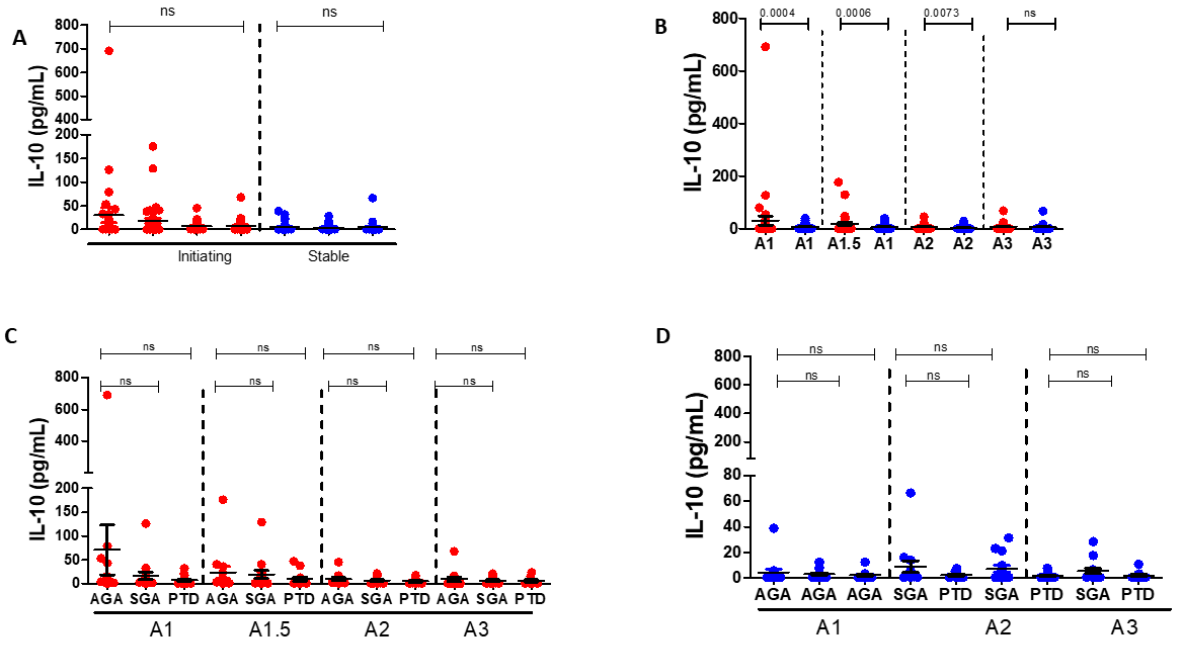


Figure 6.11: IL-10 expression profile over time. A) IL-10 expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-10 expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.10 Expression profile of interleukin-6

We did not see any differences in the expression of IL-6 over time when comparing patients initiating and those stable on ART (Figure 6.12A). We saw a difference at one time point with a higher expression in patients initiating ART compared to those stable on ART (Figure 6.12B). No differences were seen in patients initiating (Figure 6.12C) and those stable on ART (Figure 6.12D) when differentiated by outcome.

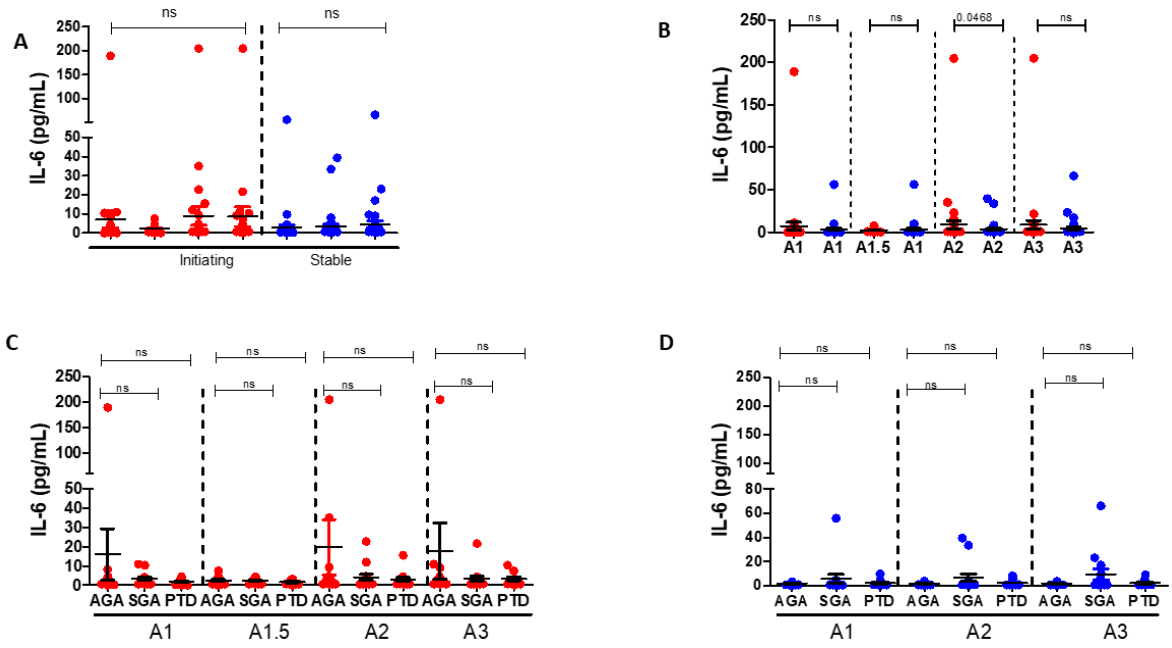


Figure 6.12: IL-6 expression profile over time. A) IL-6 expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-6 expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.11 Expression profile of interleukin-5

Upon analysis, we did not find any significant difference in IL-5 expression over time in patients initiating and stable on ART (Figure 6.13A). We then compared patients initiating and stable on ART at each time point and found a significantly higher expression at baseline and weeks 28 and 34 of gestation in patients initiating versus stable on ART (Figure 6.13B). There was significantly lower levels of IL-5 for the PTD outcome at 28 weeks of gestation in patients initiating ART (Figure 6.13C). We found lowered expression of IL-5 in PTD compared to AGA at the baseline patients stable on ART (Figure 6.13D).

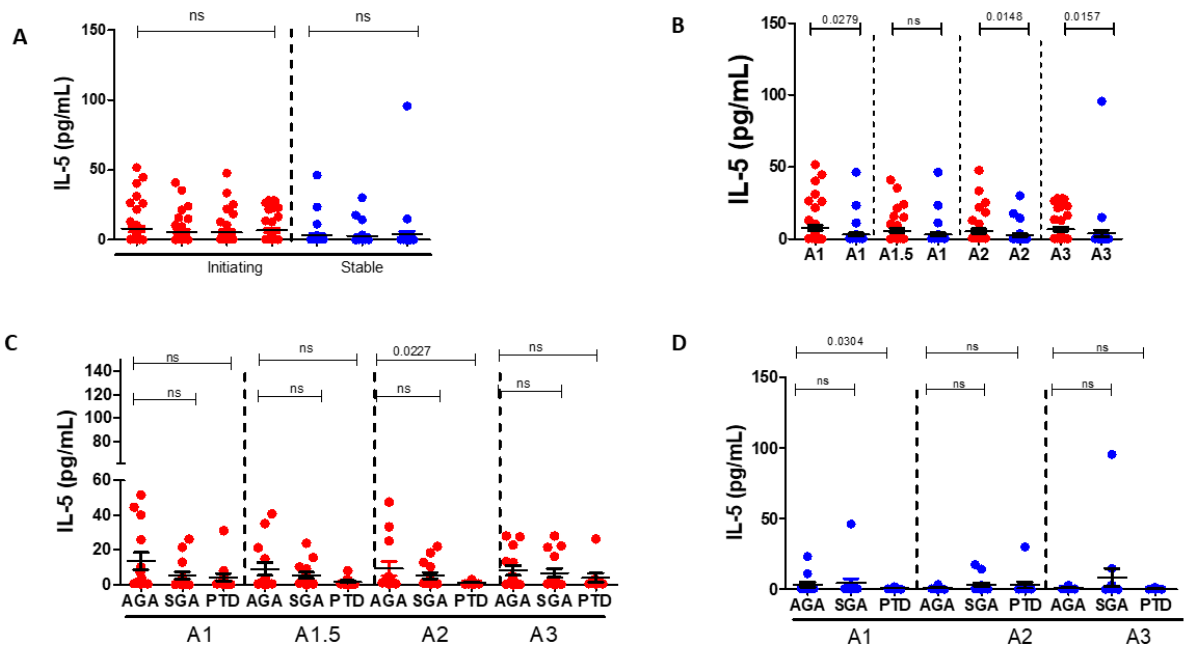


Figure 6.13: **IL-5 expression profile over time.** A) IL-5 expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-5 expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.12 Expression profile of interleukin-2

Upon analysis, we did not find any significant difference in IL-2 expression over time in patients initiating and stable on ART (Figure 6.14A). We then compared patients initiating and stable we found no significant differences in the expression in patients initiating versus those stable on ART (Figure 6.14B). There were no differences in outcomes over time in patients initiating ART (Figure 6.14C). We found a lower expression of IL-2 in PTD compared to AGA at the second time point in patients stable on ART (Figure 6.14D).

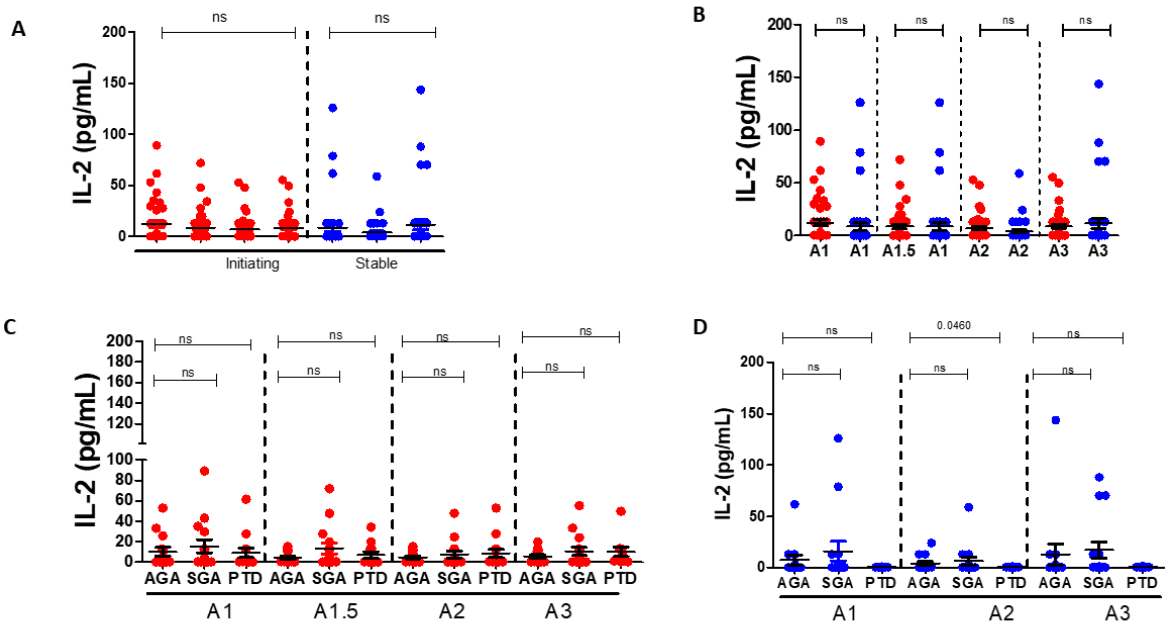


Figure 6.14: **IL-2 expression profile over time.** A) IL-2 expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-2 expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.13 Expression profile of interleukin-7

IL-7, a key factor in the generation, activation, and homeostasis of the T cell compartment of the immune system (Vassena et al., 2007). We found no significant difference in IL-7 expression over time in patients initiating and stable on ART (Figure 6.15A). We then compared patients initiating and stable on ART at each time point and found a significantly higher expression when patients initiating ART were naïve versus those stable on ART (Figure 6.15B). There were no differences in outcomes over time in patients initiating ART (Figure 6.15C) or stable on ART (Figure 6.15D).

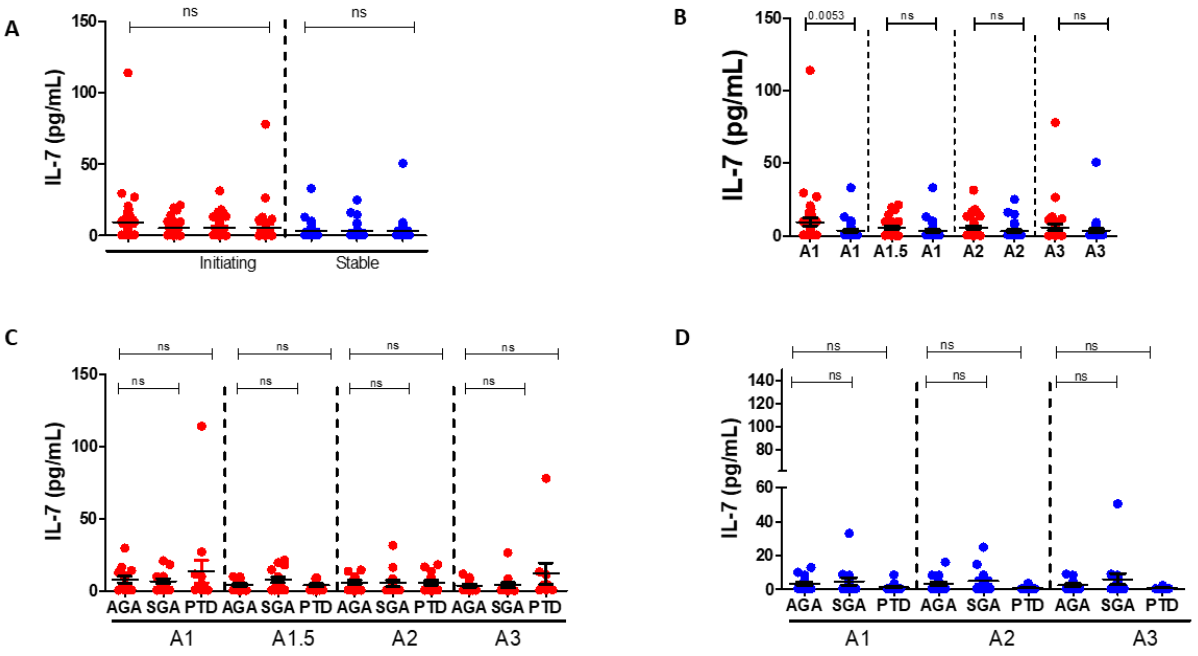


Figure 6.15: IL-7 expression profile over time. A) IL-7 expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) IL-7 expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.4.14 Expression profile of TNF-α

No significant differences in TNF-α expression over time in patients initiating and stable on ART were found (Figure 6.16A). in line with previous studies (Osuji et al., 2018), We then compared patients initiating and stable on ART at each time point and found a significantly higher expression at baseline (ART naïve) in those initiating versus stable on ART (Figure 6.16B). There were no differences in outcomes over time in patients initiating ART (Figure 6.16C) and those stable on ART (Figure 6.16D).

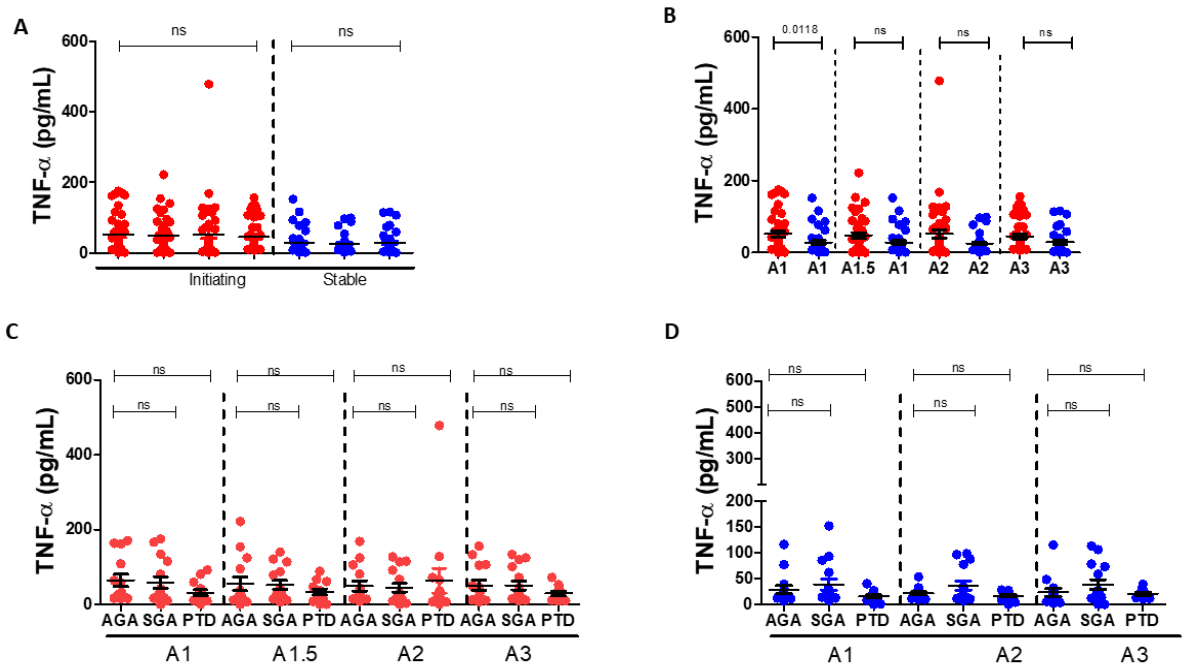
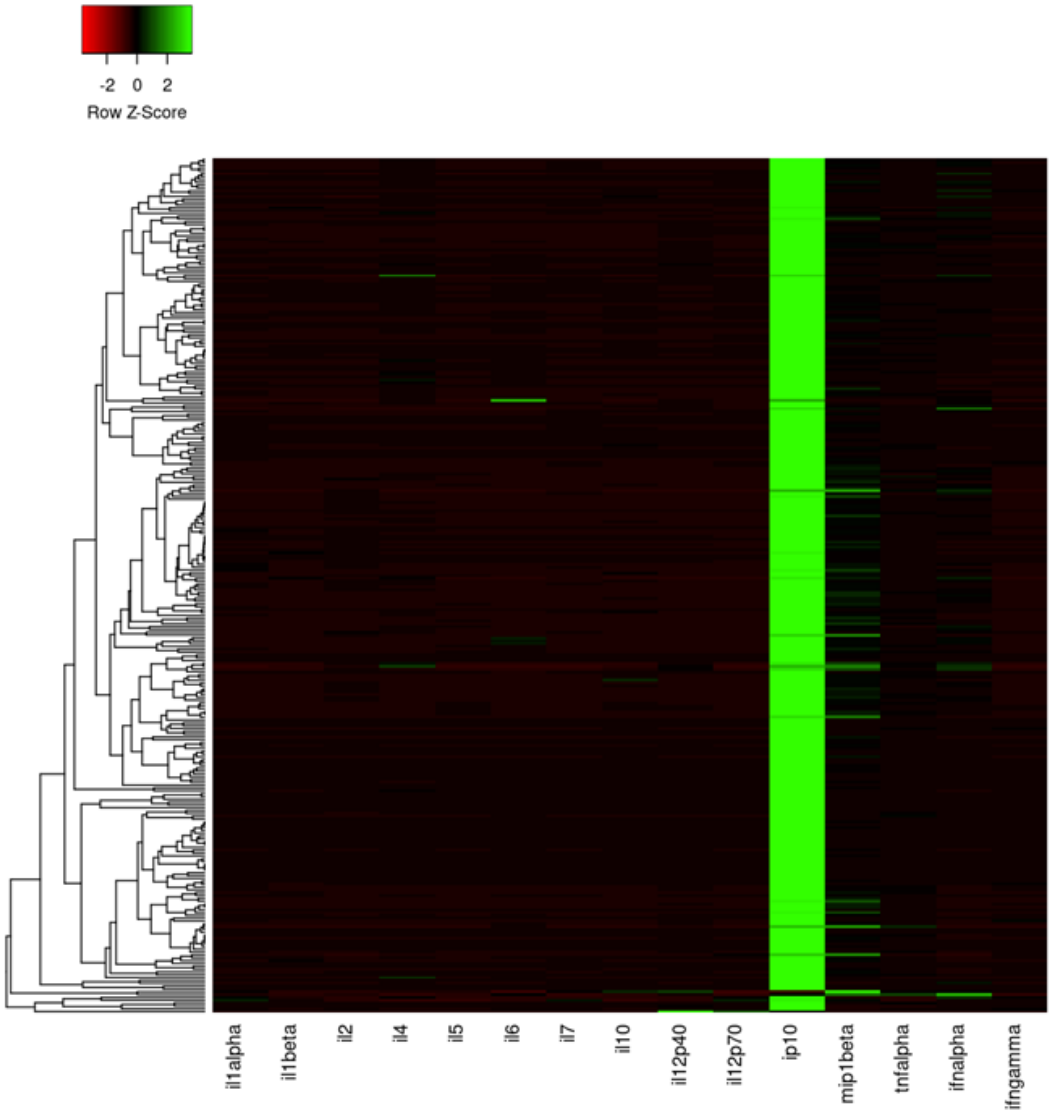


Figure 6.16: TNF-α expression profile over time. A) TNF-α expression levels for women initiating (red circles) and stable on ART (blue circles) over time. B) TNF-α expression levels comparing women initiating (red circles) and stable on ART (blue circles) at each time point. C) Outcomes for each time point for women initiating ART (in red) and (D) women stable on ART (blue).

6.5 Cumulative cytokine responses

Upon analysis of the plasma samples using the luminex platform, over time, IP-10 was highly expressed in all patients regardless of ART timing or outcome (Figure 6.17A-B). The second most expressed cytokine was MIP-1β followed by TNF-α and IL-6. These cytokines fall into the Th1 cytokine profile.

A


-2 0 2
Row Z-Score
il1alpha
il1beta
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ip10
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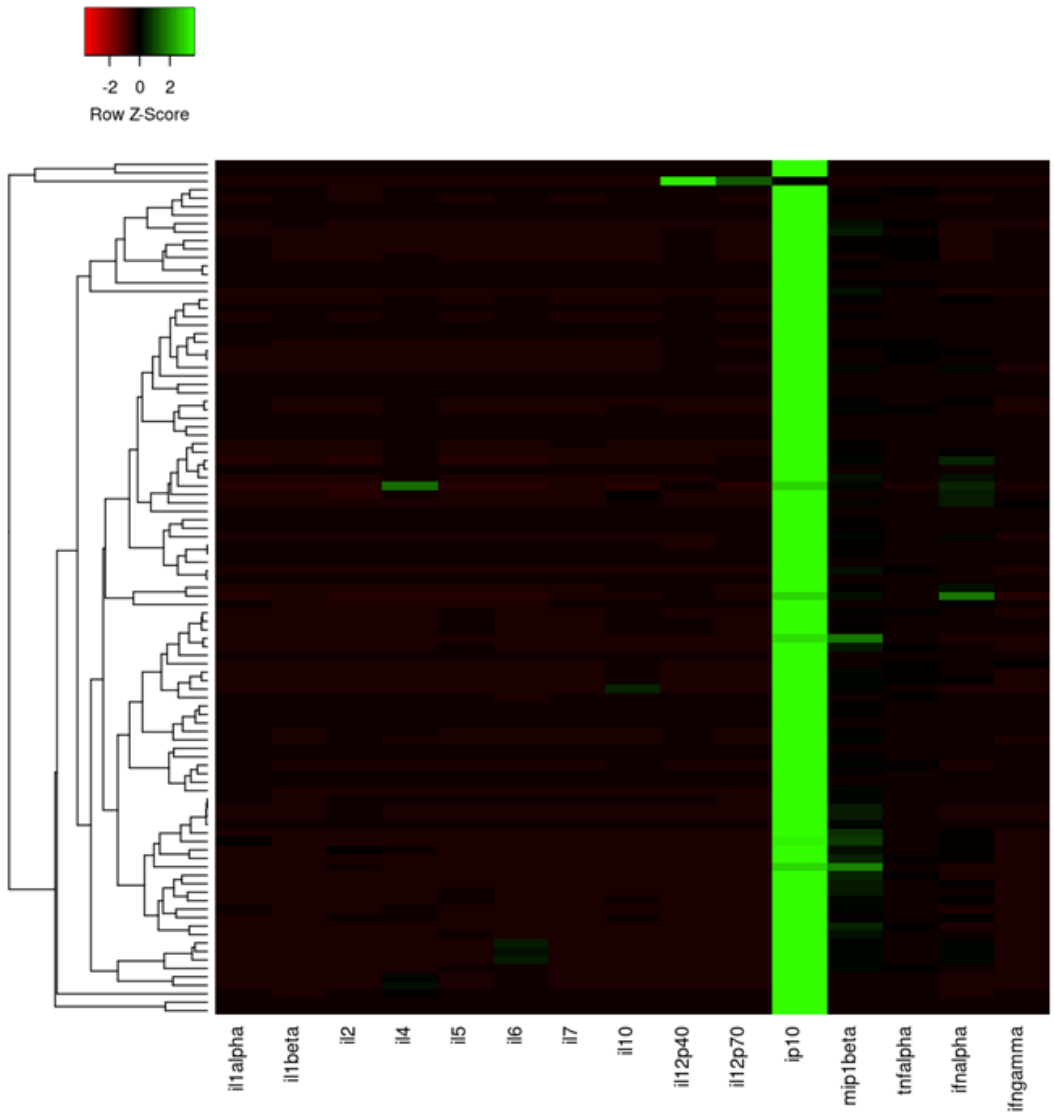
tnfalpha
ifnalpha
ifngamma

B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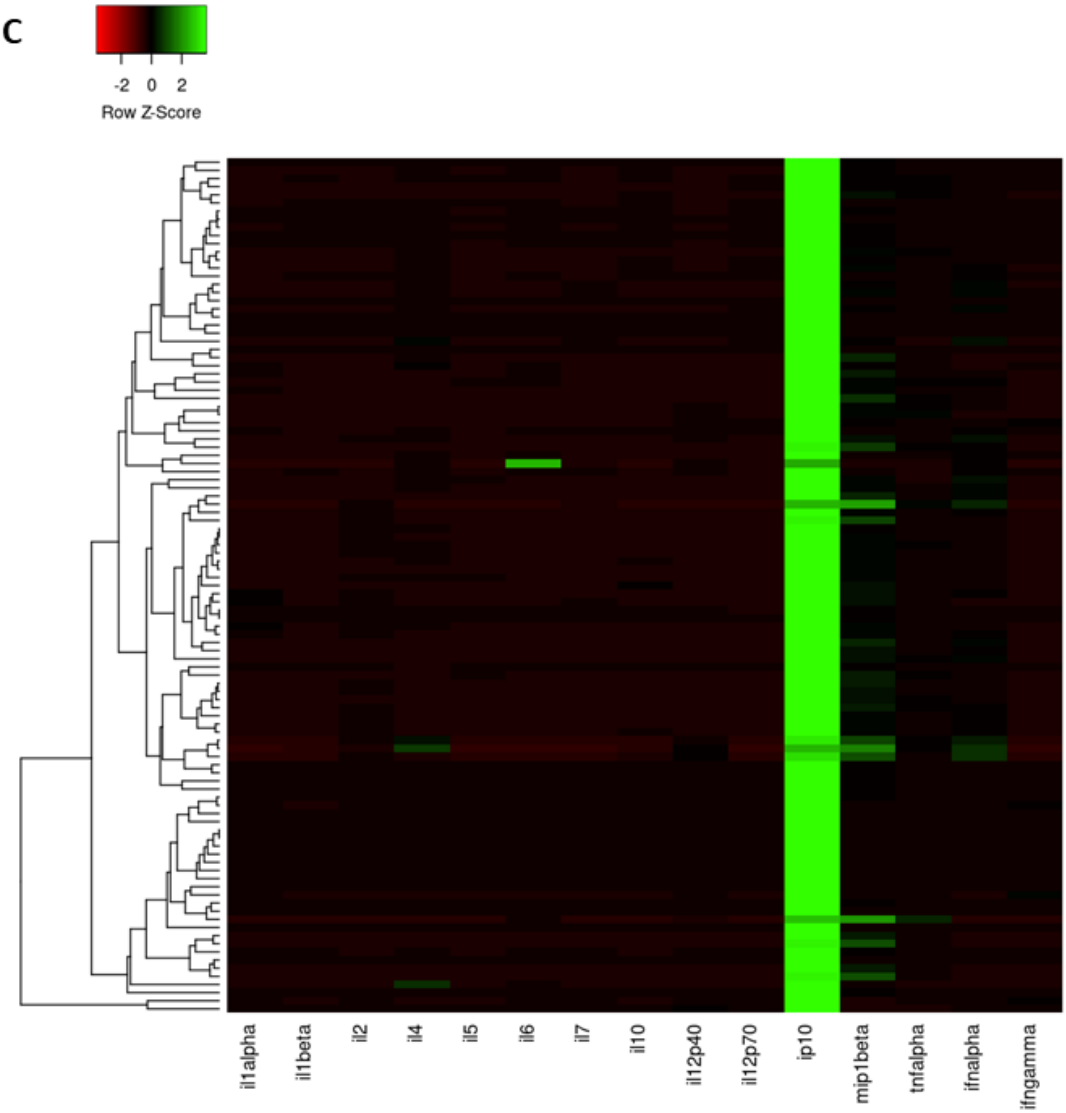
C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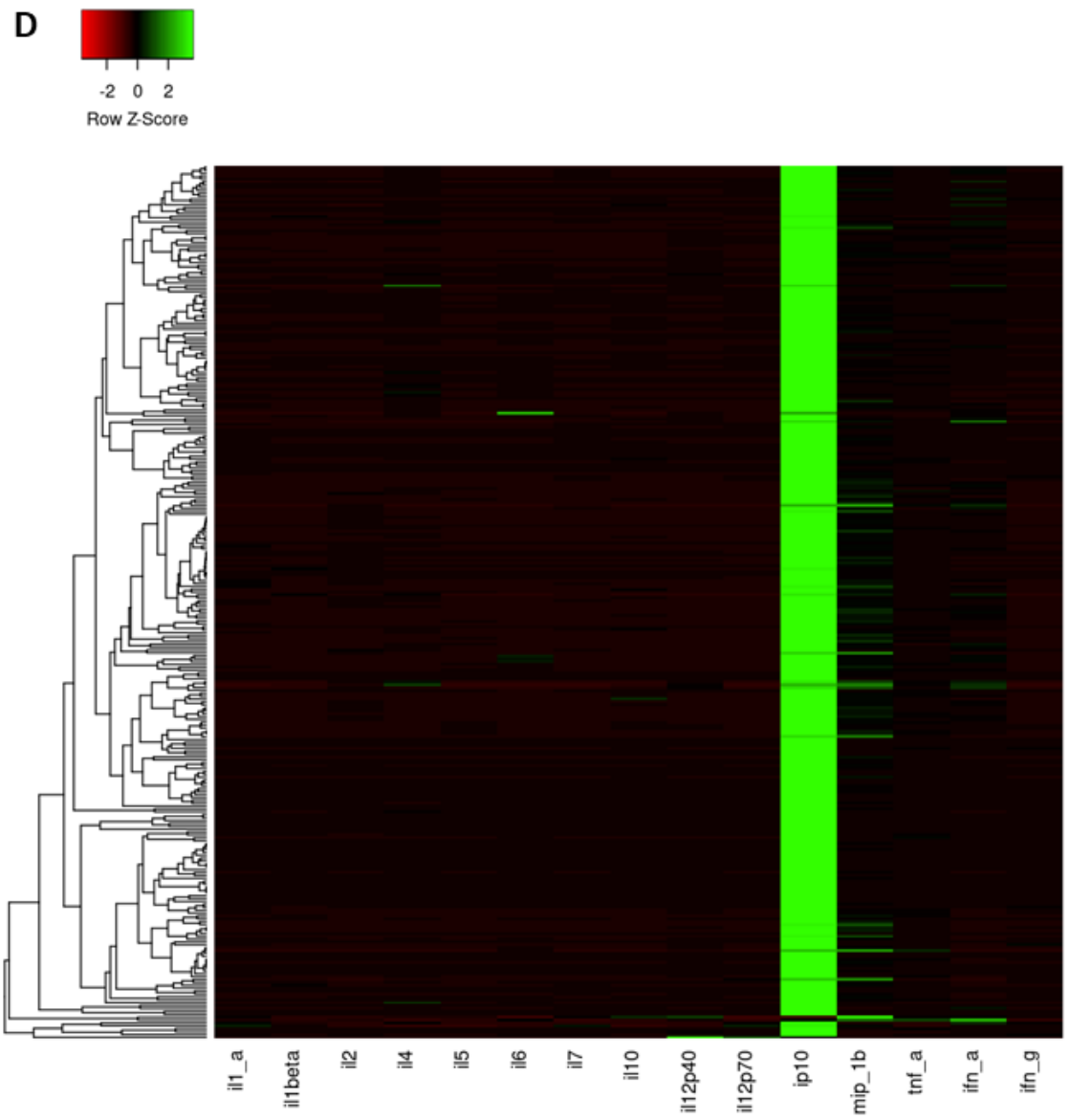

Figure 6.17: Cytokine profile over time. 15 cytokines were measured from HIV infected women (n=90) over time. A1 (baseline) A1.5 (two weeks post ART initiation) A2 (28 weeks) and A3 (birth) using the luminex platform. The method used for hierarchical clustering is based on average linkage using spearman rank correlation. A) This is a representation of cumulative cytokine responses for all participants, regardless of outcome. B) This is a representation of cumulative cytokine responses for all participants for the AGA outcome. C) This is a representation of cumulative cytokine responses for all participants for the SGA outcome. D) This is a representation of cumulative cytokine responses for all participants for the PTD outcome

Although we found high levels of IP-10, these did not differ over time, irrespective of the treatment status (Figure 6.18A). There were also no differences seen in patients when they were delineated by treatment and outcome for both initiators (red circles) and those stable on ART (blue circles). When we looked at MIP-1β expression, we did not find any significant difference over time in patients initiating and stable on ART (Figure 6.18B). There were no differences in outcomes over time in patients initiating ART. However, we noted a higher expression of MIP-1β in SGA compared to AGA at the second time point in patients stable on ART. There were no significant differences in TNF-α expression over time in patients initiating and stable on ART (Figure 6.18C) in line with previous studies (Osuji et al., 2018). There were no differences in the expression of IL-6 over time when comparing patients initiating and those stable on ART (Figure 6.18D) and when stratified by outcome.

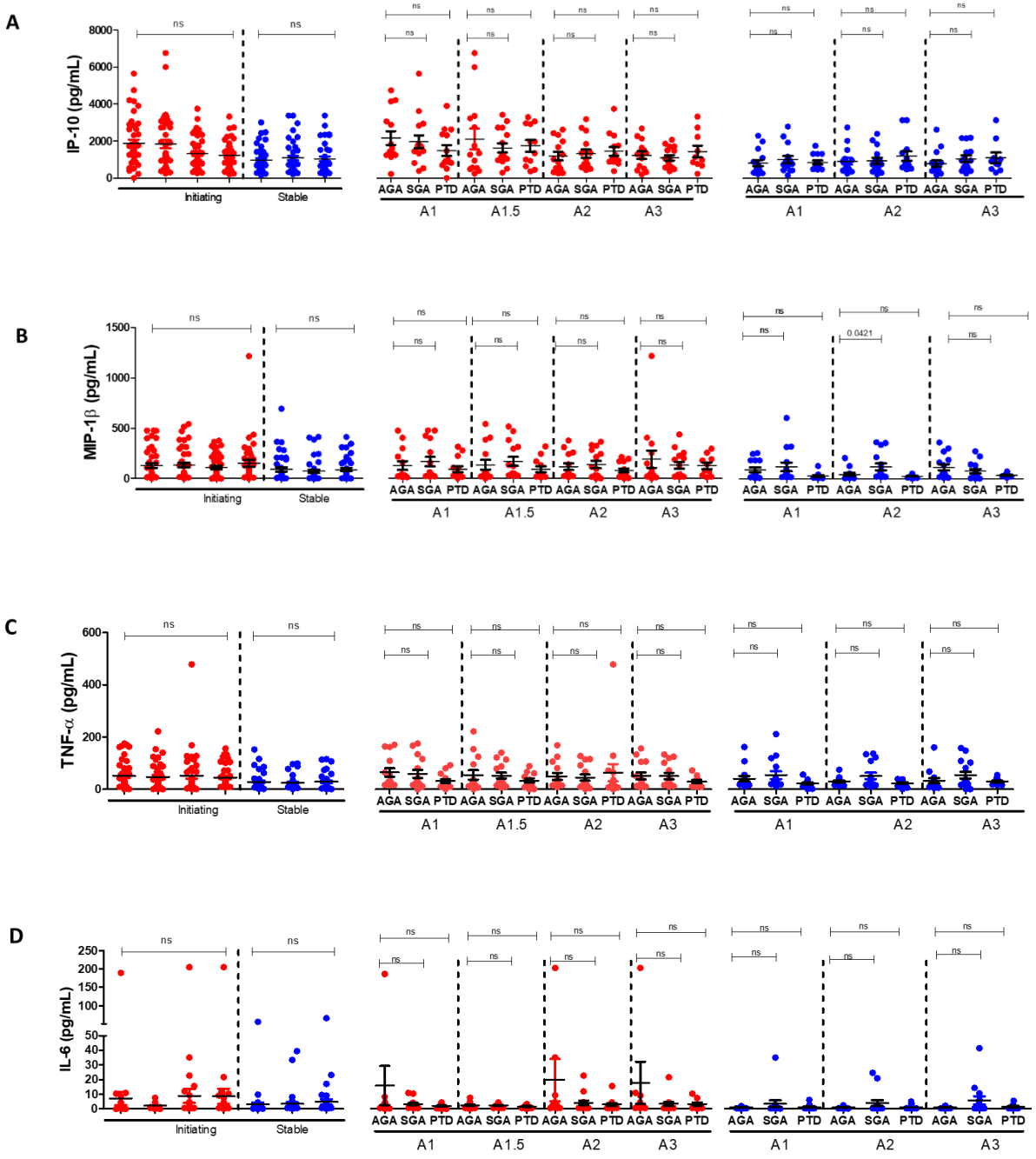

Figure 6.18: Expression of TH1 cytokines. A) IP-10 expression levels for women initiating (red circles) and stable on ART (blue circles) over time. Outcomes for each time point for women initiating ART (in red) and women stable on ART (blue). B) MIP-1β expression levels for women initiating (red circles) and stable on ART (blue circles) over time. Outcomes for each time point for women initiating ART (in red) and women stable on ART (blue). C) TNF-α expression levels for women initiating (red circles) and stable on ART (blue circles) over time. Outcomes for each time point for women initiating ART (in red) and women stable on ART (blue). D) IL-6 expression levels for women initiating (red circles) and stable on ART (blue circles) over time. Outcomes for each time point for women initiating ART (in red) and women stable on ART (blue).

6.6 Correlation of cytokine concentrations with treatment status

Levels of biomarkers were then assessed for correlations between one another (mutual correlation was determined using the cut-off of +0.5 and -0.5 for a significant positive and negative correlation respectively). At baseline (Figure 6.19), we observed a positive correlation between the following cytokines; IL-1α was positively associated with IL-1β and IL-7; IL-1β with IL-2, IL-7 and IFN-α. IL-2 positively correlated with MIP-1β and IFN-α. Other positive correlations were between IL-5 with Mip-1β; IL-12(p40) with IL-12(p70); IL-10 with IP-10; IP-10 with TNF-α and Mip-1β with TNF-α as well IFN-α. At this time point, there were no distinct Th1 or Th2 cytokine clusters seen.

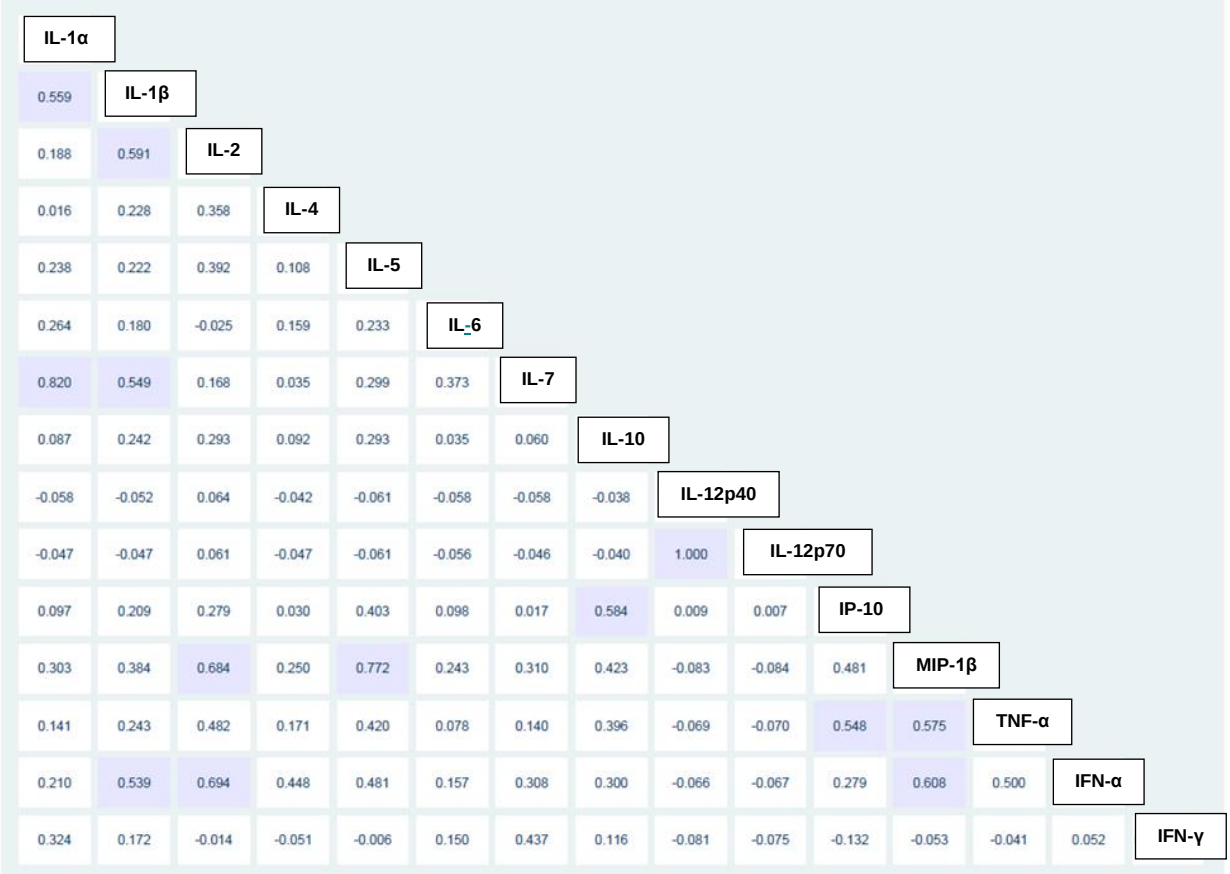

Figure 6.19: The correlation matrix of peripheral blood cytokine levels at baseline for patients initiating and stable on ART. Biomarkers present in HIV infected pregnant women were assessed (n =90) and analyzed by Spearman's correlation. The correlogram shown was constructed using unsupervised hierarchical clustering in STATA. A cut-off of +0.5 and -0.5 for a significant positive and negative correlation respectively. Additionally, positive associations are indicated by the shaded squares.

Using the same cut offs, we next looked at the correlations over time, not distinguishing treatment status (Figure 6.20), here we found positive correlations for IL-1β with IFN-α, IL-2 and IL-5 both positively correlated with MIP-1β and IFN-α respectively. IL-12 (p40) with IL-12 (p70) an association that was seen at baseline. IP-10 with MIP-1β and TNF-α, with MIP-1β associating with TNF-α and IFN-α. Lastly we found a positive correlation between TNF-α, and IFN-α.

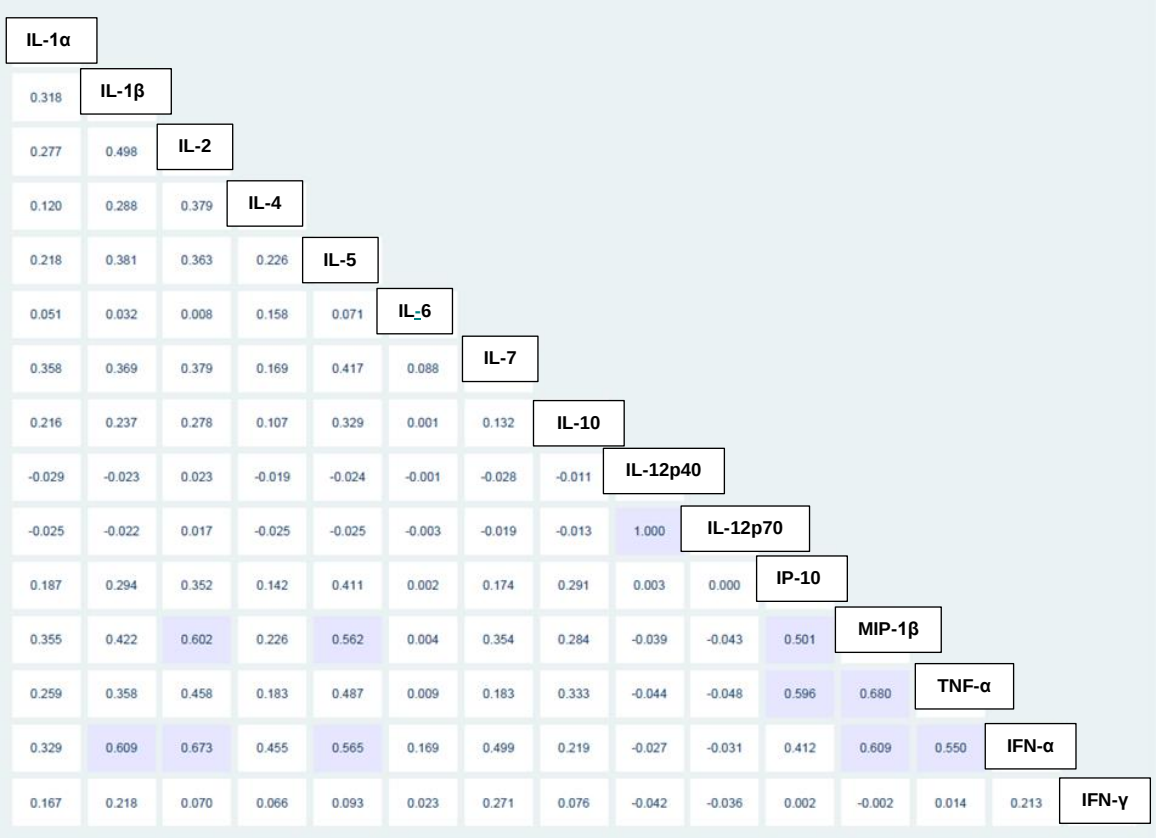

Figure 6.20: The correlation matrix of peripheral blood cytokine levels over time for patients initiating and stable on ART. Biomarkers present in HIV infected pregnant women were assessed (n =90) and analysed by Spearman's correlation. The correlogram shown was constructed using unsupervised hierarchical clustering in STATA. A cut-off of +0.5 and -0.5 for a significant positive and negative correlation respectively. Additionally, positive associations are indicated by the shaded squares.

For those initiating ART (Figure 6.21), the correlation was positive for IL-1β with IL-2 and IFN-α. IL-2 positively associated with MIP-1β, TNF-α and IFN-α. IL-5 positively associated with MIP-1β and TNF-α. IL-12 (p40) positively associated with IL-12 (p70); IP-10 with MIP-1β and TNF-α; also, MIP-1β associating with TNF-α, and IFN-α. Lastly we found a positive correlation between TNF-α, and IFN-α.

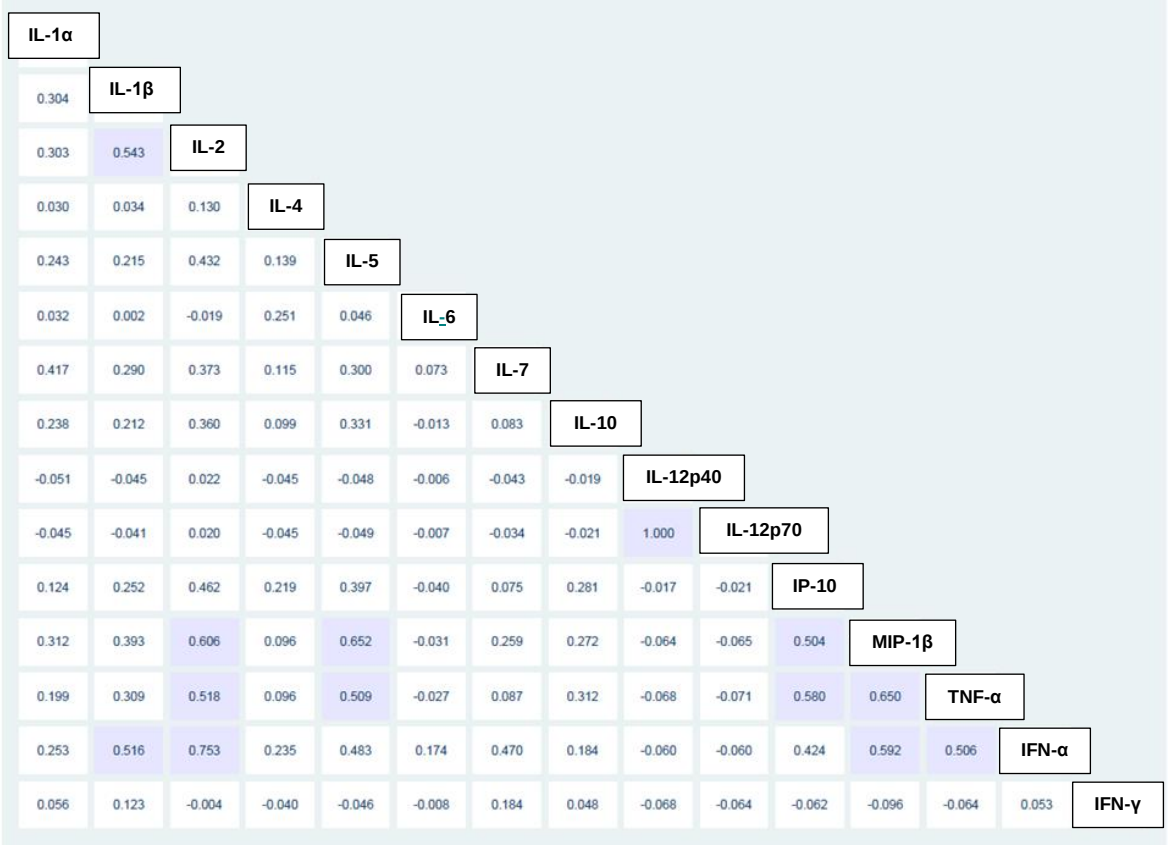

Figure 6.21: The correlation matrix of peripheral blood cytokine levels over time for patients initiating ART. Biomarkers present in HIV infected pregnant women were assessed (n =90) and analysed by Spearman's correlation. The correlogram shown was constructed using unsupervised hierarchical clustering in STATA. A cut-off of +0.5 and -0.5 for a significant positive and negative correlation, respectively. Additionally, positive associations are indicated by the shaded squares.

For those stable ART (Figure 6.22), IL-1β positively correlated with cytokines IL-4, IL-5, IL-7, IL-10, 1L-12(p40), 1L-12(p70) and IFN-α. IL-2 positively correlated with IL-4, IL-10, 1L-12(p40), MIP-1β, TNF-α as well as IFN-α. IL-4 had positive correlations with IL-10, IL-12(p40) and IFN-α. IL-5 positively correlated with IL-7, IL-10 and IFN-α. IL-6 positively correlated with IFN-α. IL-7 correlated with IL-10, 1L-12(p70), MIP-1β as well as IFN-α. IL-10 had positive correlations with MIP-1β, TNF-α as well as IFN-α. IL-12(p40) positively correlated with IFN-α. IL-12(p70) positively correlated with IFN-α and IFN-γ; IP-10 with TNF-α; MIP-1β with TNF-α; and TNF-α with IFN-α. This group also demonstrated the most TH1 associations.

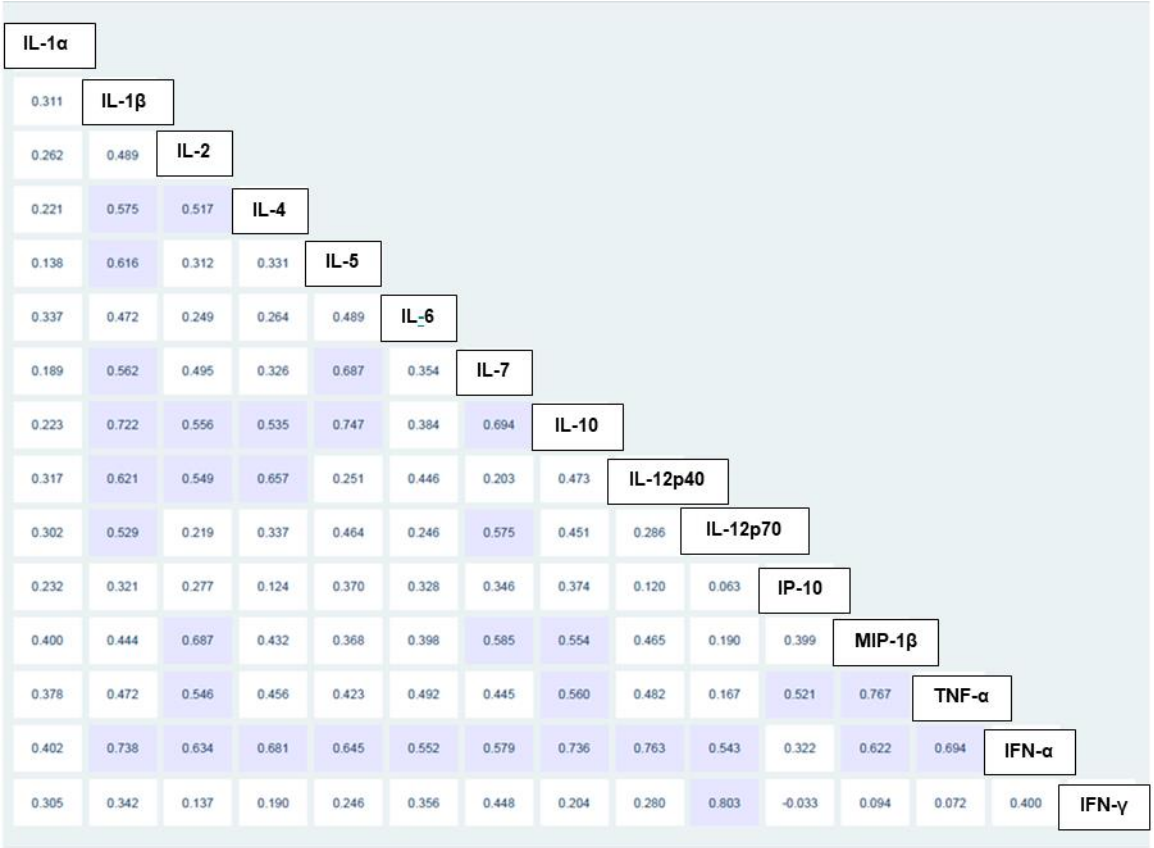

Figure 6.22: The correlation matrix of peripheral blood cytokine levels over time for patients stable ART. Biomarkers present in HIV infected pregnant women were assessed (n =90) and analysed by Spearman's correlation. The correlogram shown was constructed using unsupervised hierarchical clustering in STATA. A cut-off of +0.5 and -0.5 for a significant positive and negative correlation, respectively. Additionally, positive associations are indicated by the shaded squares.

6.7 Summary

The aim of this work was to carry out the quantification of plasma cytokine profiles associated with the initiation of ART during pregnancy and their impact on preterm birth. This was done using the Luminex platform measuring TH1/TH2 profiles in a cohort of pregnant women who either conceived on ART or initiated treatment during pregnancy and followed longitudinally. We hypothesised that an ART mediated cytokine shift in HIV infected women who initiate ART could be responsible for an increase in preterm deliveries.

The mechanism regulating the Th1: Th2 ratio is yet to be fully elucidated, but the importance of maternal immune tolerance during pregnancy is unquestionable (Sykes et al., 2012, Fiore et al., 2006). Research has also shown that pregnancy induces changes in maternal cytokine levels in order to prevent foetus rejection (Morelli et al., 2015, Reinhard et al., 1998). Previous studies demonstrated that higher peripheral concentrations of certain pro-inflammatory cytokines, e.g., IL-2, IL-6, IL-12, and IFN-γ, are associated with outcome such as preterm delivery (Kaartinen et al., 2019).

TH1 cytokines are involved in cell mediated immunity, and typically comprise of cytokines Interleukin (IL)-1, IL-2, IL-6, IL-12, IFN-γ, TNF-α, interferon (IFN) -γ, and tumour necrosis factor (TNF)-α, MIP-1β, and IP-10. Activation of the Th1 cytokines occurs as a specific response to infection caused by intracellular bacteria, parasites, and viruses (Yazdani et al, 2012). The interleukin-1 family members which form most of the cytokines in this family; are closely linked to damaging inflammation; however, the same members also function to increase nonspecific resistance to infection and development of the immune response to foreign antigens

(Sutton et al., 2004, Maharaj et al., 2017, Robert et al., 2013). Previous research has found that pro-inflammatory cytokines like IL-1 are increased during pregnancy (Osuji et al, 2017). IL-2 is generally decreased during HIV infection (Osuji et al, 2017). Maternal production of IL-2 has been directly associated with unsuccessful pregnancy as it activates cytotoxic activity (Fiore et al, 2006). There was no significant difference in the expression IL-2 over time and by outcomes. An increase in the IL-2/IL-4 and IFN-γ /IL-4 ratios, as well as elevated circulating levels of IL-6 and TNF-α have been reported in previous studies, suggesting a pro-inflammatory systemic increase when compared to normal pregnancy (Maharaj et al., 2017, Osuji et al., 2018, Raphael et al., 2015, Smith et al., 2007). IL-12 plays a key role in the regulation of cell-mediated immunity and that there is more secretion of IL-12p40 (a heterodimer) than IL-12p70 (subunit), a process tightly regulated mainly by antigen presenting cells (Byrnes et al., 2008). We did not see notable differences in the expression levels of IL-12 were seen. These data are an indication that the result is not favouring a Th1 response as these are key cytokines that define the pro-inflammatory response (Bhurani and Dalai, 2018).

Type I interferons (IFNs) are known to play a critical role in the control of HIV-1 transmission and replication and when stimulated in chronic viral infections, they have been shown to possibly lead to increased levels of immune activation, which has been shown to possibly be higher in HIV-1-infected women than in men (Ziegler and Altfeld, 2017). We saw no significant difference in the expression of IFN-α, a key player in the control of viral infections (Yazdani Brojeni et al., 2012, Ziegler and Altfeld, 2017). There was no difference in the expression of the cytokine over time in those initiating ART during pregnancy or those who were stable on ART. There was also no difference in expression by outcome; we did however see a higher expression in women initiating ART. IFN-γ which is known to play a role in immune responses against viral infections where large amounts of pro-inflammatory are

produced by activated T lymphocytes and natural killer (NK) cells (Robert et al., 2013) was also not significantly different over time for the cohort. Previous work has shown that the use of ART markedly increased plasma IFN-γ levels, we were however not able to see this as the expression of the cytokine seemed to be more increased in patients initiating ART (Osuji et al., 2018). Also, women with recurrent miscarriage have been shown to have increased IFN-γ compared with women that go on to have successful pregnancies (Sykes et al., 2012). We found a higher expression of IFN-γ in patients initiating ART in this cohort of women; our previous work has shown that women initiating ART have a likelihood of worsened outcome however a larger sample size of our cohort would be required to clearly elucidate the mechanisms (Mdletshe et al., 2021). Interferon (IFN)-γ inducible protein, CXCL10/IP-10, has pro-inflammatory and anti-angiogenic properties and has been proposed to be a key link between inflammation and angiogenesis (Gotsh et al, 2007b). A study found that patients with normal pregnancies had a significantly higher media concentration of IP10 than non-pregnant women (Gotsch et al., 2007b). We found high levels of IP-10 production when all women were included in the cumulative cytokine analysis but there was no statistical significance in the differences in expression when we looked at treatment status as well as outcomes. We also could not make direct comparisons as our cohort did not include non-pregnant women.

The TH2 cells secrete anti-inflammatory cytokines such as IL-4, IL-10, and IL-13. Based on previous research, we expected to find a prominent TH2 profile as that is associated with a desirable pregnancy outcome (Saito et al., 2010, Sykes et al., 2012). Interleukin-4 is a definer of the Th-2 subset known to exert anti proliferative effects; it is also identified among the critical cytokines promoting HIV immunopathology. Studies have found that with HAART commencement, levels of IL-4 have been found to gradually decline to those comparable with healthy controls and this may be linked to reduction in HIV viral load with subsequent

suppression of inflammatory episodes resulting from combined antiretroviral therapy (Osuji et al, 2018). We however did not find significant differences in its expression.

IL-10 inhibits synthesis of TH1 cytokines such as IFN-γ and IL-2 and functions to inhibit antigen-presenting cells, it is one of the cytokines that are increased during HIV infection (Osuji et al., 2018). In a study aimed at characterizing the Th1 and Th2 cytokine profiles among malaria-exposed and HIV-infected pregnant women, they found elevated levels of IL-10 which was indication of the essential anti-inflammatory role this cytokine plays in the context of maintenance of the pregnancy when exposed to inflammatory insults (Ibitokou et al., 2015). The use of ART has been seen to lower IL-10 systemic levels during HIV infection and during successful pregnancy, this cytokine has been associated with an increased expression (Sykes et al., 2012, Osuji et al., 2018, Fiore et al., 2006). We however found low levels of IL-10 and could not make an association of its expression with outcome. When we looked at expression profiles of individual cytokines, our data does not show any differences over time in patient's initiating or stable on ART. We also did not find differences in outcomes regardless of treatment.

When we looked at a pooled expression analysis over time, the data shows that there is a Th1 bias in expression of cytokines in general, regardless of outcome and ART timing. Th1 cells are the model cell type involved in cell-mediated inflammation, delayed-type hypersensitivity reactions and are important for immunity to intracellular pathogens (Raphael et al., 2015). TNF-α is characteristic of Th1 type immunity and mediates several cell mediated cytotoxic and inflammatory reactions (Maharaj et al., 2017). Its expression is increased during HIV infection and its levels significantly decline during HAART (Osuji et al., 2018). Women with recurrent

miscarriage have been shown to have increased IFN-γ and TNF-α levels compared with women that go on to have successful pregnancies (Sykes et al., 2012). In this subset of women studied, we were unable to associate the expression of TNF-α with outcome. Another study had indicated that expression of TNF-α is associated with IL-10 expression; we found both these cytokines to not be significantly (Stylianou et al., 1999).

IL-6, although possessing some anti-inflammatory properties, is present in many chronic inflammatory and autoimmune disorders and serves as a marker for the systemic activation of pro-inflammatory cytokines (Maharaj et al., 2017). It is one of the cytokines that are increased during HIV infection (Osuji et al., 2018). Studies aimed at understanding PTD have found that mice genetically deficient in IL-6 deliver one day later than wild-type mice, but this delay was normalized with exogenous IL-6 administration (Gotsh et al, 2007). We were unable to make this association, as we did not see significant differences in the expression of IL-6 over time in our cohort. Although highly expressed in the cohort, we were not able to associate its expression with outcome.

Our results do not distinctly show the effect of ART on pregnancy outcomes is mediated through its effect on the cytokine environment, a result contrary to previous research that was able to associate the two (Fiore et al., 2006). We saw differences in cytokine production when we looked at treatment initiation for some cytokines, where there was a higher expression for those initiating and lower in those stable on ART. We did not find any link between birth outcomes, treatment initiation and cytokine production. The measurement of proteins along with cytokines would have enlightened our findings further as these have been seen to promote a TH2 bias (Sykes et al., 2012). From these data, we would not be able to speculate any

immunological pathways or biomarkers that play an active role in predicting birth outcomes by treatment initiation. In future it might be easier to perform this analysis using freshly isolated plasma to increase sensitivity along with using a larger sample size in order to ascertain a more robust conclusion (Cardigan and Green, 2015).

Chapter 7 Discussion

The recommendations put forth for antiretroviral therapy (ART) initiation to all HIV positive mothers for the prevention of mother to child transmission (PMTCT) of the virus has resulted in the reduction of transmission rates. South Africa, as one of the countries with a high HIV prevalence, adheres to the WHO recommendations with all HIV positive pregnant women offered lifelong ART treatment. This intervention has resulted in a growing number of pregnant mothers and infants exposed to antiretroviral drugs, with the consequences of these exposures not well studied. Previous studies have focused on HIV infected pregnant women that had been exposed to ART for a short period (11.7 weeks) at most or not at all (Ekouevi et al., 2008b). These studies did not investigate the impact of ART exposure on immunological parameters over the course of the pregnancies, and did not explicitly distinguish between the impact of ART and HIV on birth outcomes. These studies have suggested that HIV infection is associated with both preterm delivery and low birth weight (Ekouevi et al., 2011). Studies have also reported an association between ART use in pregnancy and preterm delivery and low birth weight (Ekouevi et al., 2008b, Townsend et al., 2010a, Thorne, 2004a, Machado et al., 2009). Moreover, there are only a few studies that have been conducted in Africa and are associated with either ART use or the use of short-course PMTCT regimens. Only a handful of these studies have been associated the use of ART during pregnancy with an increased risk of adverse pregnancy outcomes, including stillbirth, premature delivery, small-for-gestational age infants and early neonatal death.

The success or failure of a pregnancy has been attributed to a cytokine shift from Th1 to Th2. Pregnancy can be defined as a process that is both pro-inflammatory and anti-inflammatory depending on the stage of gestation (Mor and Cardenas, 2010). It has been described that ART causes a shift from Th2 to Th1 cytokine balance (Lopez et al., 2012). This change may be able to prevent or modulate HIV disease progression, but can be associated with unsuccessful

pregnancy outcome, pre-term delivery (PTD) and SGA, although some studies have not found these correlations (Briand et al., 2009b).

Knowledge of the impact of maternal HIV infection on birth outcomes such as preterm delivery and SGA infants is needed in order to understand the potential impact of antiretroviral treatment. Particularly, there is a need to identify immunological risk factors that may predispose mothers to differential birth outcomes. To improve understanding of factors associated with premature delivery of either appropriate- or small-for-gestational age infants and inform understanding of possible causes; the hypotheses underlying this work were that (i) ART initiated in early pregnancy would be associated with a significantly increased risk of preterm delivery, and (ii) the reconstitution of cellular immune responses under ART in pregnant women would result in increases in preterm delivery when compared to appropriate-for-gestational age infants or small-for-gestational-age births.

Our study also hypothesised was that the immune activation status modulated by ART introduced before or during pregnancy would be associated with either PTD or SGA. Therefore, in a case control study, adaptive and innate immune cell subsets and phenotypes were analysed at baseline and over time to understand the effects. Firstly, upon baseline analysis at a median of 15 weeks gestation, we were able to ascertain that $CD8^+$ T cell activation was lower in women initiating ART (at the time when they were ART-naïve and at 2 weeks following ART commencement) with a subsequent PTD outcome, but not those stable on ART, which was contrary to our hypothesis. SGA cases presented a similar $CD8^+$ T cell immune activation profile to women in the control group (AGA). Further, frequencies of classical monocytes were significantly increased in PTD cases both in initiators and those stable on ART. PTD was also associated with significantly higher intermediate monocyte frequencies,

but only at the early time points (ART-naïve and two weeks post initiation) in women initiating ART in pregnancy.

Our findings at this time point suggested some differences in inflammatory monocytes frequencies that differed by birth outcomes, with lower frequencies among PTD cases initiating ART. Monocyte subset levels were associated with outcome, with strong associations noted between higher frequencies of classical and intermediate monocytes and PTD, and possible association with lower inflammatory monocyte frequencies. Lower levels of monocyte, mDC and pDC activation were also all strongly associated with both PTD and SGA in unadjusted analyses, especially among women initiating ART during pregnancy. For most of the immune cells' profiles studied, SGA tended to have similar profiles to control (AGA) women. Adjusted analysis further confirmed that lower monocyte activation and higher levels of classical/intermediate monocytes (or lower levels of inflammatory monocytes) were the most significant immune markers associated with PTD among initiators, with low monocyte activation below a threshold of 20% identified as a potential biomarker to predict women at risk for PTD.

When we examined the parameters longitudinally, there was a lowered expression of activation as defined by the expression of CD86 and CD69 in APCs, monocytes, mDCs and pDCs. All of which were strongly associated with a subsequent PTD outcome specifically for women initiating ART during pregnancy and in some cases those who started ART before pregnancy; after allowing for baseline viral load. The association of lower immune activation with PTD in ART initiators was observed before initiation of ART, at two weeks post-ART initiation up until the last time point, at birth.

Monocyte cell subsets were also analyzed over time. We noted an increase in frequencies of classical monocytes irrespective of treatment status. Previous work done on monocytes during viral infections like HIV has documented that the $CD16^{+}$ subset is expanded (Han et al., 2009). This previous result is consistent with our findings that women initiating therapy had an increase in the frequency of classical monocytes compared to the other monocyte populations. Intermediate monocyte frequencies in this cohort increased significantly immediately post ART initiation, with the significance subsequently lost in the later time points. When stratified by birth outcome and ART timing, intermediate monocyte frequencies were higher in PTD than in SGA cases and AGA controls for initiators over time, whereas frequencies in patients stable on ART were significantly higher at all time points after baseline no significant differences were seen when delinieated by outcome. Intermediate monocyte numbers are also expanded in the blood of patients with systemic infections, implying that they must play an important role in the rapid defence against pathogens (Wong et al., 2011). This subset is known to express CCR5 and has been shown to be involved in functions such as antigen presentation, cytokine secretion, apoptosis regulation, and differentiation (Kapellos et al., 2019). These cells also produce TNF-α, IL-1β, IL-6, and CCL3 upon TLR stimulation (Wong et al., 2012, Wong et al., 2011), however we did not observe an increase in these cytokines in this cohort when monocytes were stimulated.

The last monocyte subset investigated was that of the inflammatory monocytes. Here frequencies significantly decreased over time for patients initiating ART with no difference for those stable on ART over time. Notably, among initiators, inflammatory monocytes frequencies were lower in PTD than SGA cases and AGA controls at the time point when patients were ART naïve and 2 weeks post initiation only, with no significant differences in subsequent time points. These differences were not seen in the patients stable on ART. and

non-classical monocytes are involved in complement and Fc gamma-mediated phagocytosis and adhesion (Kapellos et al., 2019).

When monocyte activation was measured we were not able to differentiate outcomes based on ART timing as we saw the same responses in both those initiating ART and those stable on ART. Here the expression of CD86 in bulk monocytes was not significantly different over time for both treatment groups. The expression of CD86 on monocytes was significantly lower in PTD cases in both ART groups over time whereas the levels of CD69 expression in bulk monocytes were not different over time in all treatment groups. The expression CD69 on monocytes was significantly lower in PTD cases in both ART groups over time.

Unlike reports elsewhere (Townsend et al., 2010a, Townsend et al., 2010b, Ekouevi et al., 2008a), higher $CD4^+$ T cell counts were not associated with PTD in this study. Instead, our findings suggest that a lower level of immune cell activation, particularly of monocytes, accompanied by increased classical and intermediate monocyte levels, are an immunological factor associated with PTD particularly in women initiating ART in pregnancy (who were ART naïve at this time). These findings are consistent with observations that PTD risk is higher in women initiating ART during pregnancy, which is associated with reduction in overall immune activation- reversing the physiologic increase in immune activation in normal pregnancy (Robinson and Klein, 2012, Mikyas et al., 1997).

On the other hand, some studies have suggested that increased inflammation may potentiate detrimental pregnancy outcomes including PTD (Romero et al., 2006, Cappelletti et al., 2015). Although increased inflammation as an underlying factor for PTD is hard to reconcile with the findings of lower immune activation being associated with PTD in the present study, inflammation may be indicative of underlying infection as a causative factor for PTD, with the

reduced immune activation observed in our study a possible surrogate of reduced potential of immune cells to become activated and respond to infection or antigen encounter.

A recent transcriptomic study identified adaptive and innate immune genes as risk factors for PTD, although results contrasted when maternal peripheral blood and cord blood samples were analyzed, with data from the latter samples (downregulation of innate immune genes) more consistent with our results (Vora et al., 2018). Our findings are consistent with previous reports that PTD infants display a reduced ability to respond to pathogens ex vivo (Goedicke-Fritz et al., 2017, Lavoie et al., 2010), which would be expected if they express lower levels of the costimulatory molecule CD86 on monocytes as observed in the current study, although the previous studies did not address that question specifically. Overall, our findings suggest that reduced immune activation, which may be linked to reduced immune responsiveness to pathogen insult, could precede PTD and indicate an underlying mechanism, particularly in women initiating ART during pregnancy. (Chang et al., 2012).

It is well characterised that the innate immune system is the first line of defence against pathogens, thus the timeous response to pathogens by the immune system is the key element to fighting infections. It is made up of innate immune cells that are able to respond to infections in a timeous manner. These cells function through the recognition of PAMPs by receptors such as TLRs (Chang et al., 2012, Stacey et al., 2009) . We also know that the immune system is dysregulated during HIV-1 infection and those infected are also prone to suffering from hyper activation of the immune system. The constant immune challenge with high levels of pro inflammatory cytokines along with an increase in immune cell activation all contribute to viral pathogenesis.

Antigen presenting cells, namely monocytes, mDCs and pDCs pDCs were analysed following stimulation with TLR agonists LPS, CpG and CL097. These cell types reacted differently to stimulus and the data shows a reduced expression in inflammatory cytokines IFN-α, TNF-α and MIP-1β mostly in patients initiating ART and to a less extent. s in patients stable on ART. The PTD outcome tended to have a lower expression in cytokines when compared to AGA or SGA. Specifically, TLR-4 induced expression of IFN-α, and TLR4/TLR-7/8 induced expression of MIP-1β in monocytes was decreased in PTD cases in initiators, suggesting reduced immune activation. Traditionally, monocytes produce high amounts of TNF-α in response to LPS stimulation (Piguet et al., 2014). The same was seen in mDCs, TLR-9 induced expression of TNF-α, MIP-1β and IFN-α expression was nited. There was also TLR-7/8 and TLR 4 induced IFN-α expression in these cells for the PTD outcome. There was minimal TLR induced TNF-α and MIP-1β expression for pDCs withTLR-4 and TLR-7/8 induced expression of IFN-α in women initiating ART andin women stable on ART.

We saw differences in cytokine production when we looked at treatment initiation for some cytokines, where there was a higher expression for those initiating and lower in those stable on ART. We did not find any link between birth outcomes, treatment initiation and cytokine production. From these data, we would not be able to speculate any immunological pathways or biomarkers that play an active role in predicting birth outcomes by treatment initiation.

Interestingly, TLR-4 induced expression of IFN-α, and TLR4/TLR-7/8 induced expression of MIP-1β in monocytes was decreased in PTD in initiators, suggesting reduced immune activation may be indicative of reduced responsiveness to antigen stimulation (immune senescence) as an underlying factor for PTD. Overall, our findings strongly implicate reduced immune activation, particularly lower monocyte activation and higher frequencies of classical and intermediate monocytes (with concomitant increase in inflammatory monocytes), as an underlying factor for PTD but not SGA (Mdletshe et al., 2021). Protease inhibitors have been

associated with adverse outcomes (Watts and Mofenson, 2012). We however did not find any association with protease inhibitors as the majority of our patients were on the TDF regiment (Table 2.1).Overall, our findings suggest that reduced immune activation, which may be linked to reduced immune responsiveness to pathogen insult, could precede PTD and indicate an underlying mechanism, particularly in women initiating ART during pregnancy.

We know that pregnancy causes changes in the maternal cytokine levels in order to regulate and prevent foetus rejection. The aberrant production of proinflammatory cytokines such as IL-1β, TNF-α, and IFN-γ at the maternal-fetal interface has been found to be harmful to pregnancy (Peltier, 2003). Our results do not distinctly show the effect of ART on pregnancy outcomes is mediated through its effect on the cytokine environment, a result contrary to previous research that found an association between the two (Fiore et al., 2006). We saw differences in cytokine production when we looked at treatment initiation for some cytokines, where there was a higher expression for those initiating and lower in those stable on ART. We did not find any link between birth outcomes, treatment initiation and cytokine production. The measurement of proteins along with cytokines would have enlightened our findings further as these have been seen to promote a TH2 bias (Sykes et al., 2012). We found levels of IP-10 to be high in all patients in the cohort. Studies looking IP-10 levels in other infections like lupus and pyelonephritis found high levels of the cytokine and associated its expression with an inflammatory state (Gotsch et al., 2007a, Björkander et al., 2012). It would have been interesting to determine cytokine levels for IL-10 as it has been shown to suppresses the production of pro-inflammatory cytokines by other cells and numerous studies have documented its production at the maternal-fetal interface (Peltier, 2003). Understanding the endocrinology profile in this cohort might reveal more as these are know to work hand in hand. From these data, we were not be able to establish with certainty any immunological pathways

or biomarkers that play an active role in predicting birth outcomes following by treatment initiation. If it safe to speculate that if any exist, these could be beyond what was measured in this study.

Chapter 5 of this thesis assessed Tregs at baseline and follow up; looking at the relationship between Tregs and birth outcomes (AGA, SGA and PTD), or to illustrate the effect of ART initiation on the Treg balance. Treg frequencies in this cohort were expanded in patients before the initiation of ART which was not seen at follow-up time points. There was differential expression of co-inhibitory molecules on Tregs, however these were not detected when assessed by birth outcome. Lastly, co-expression data showed that Tregs mostly expressed Tigit with either CTLA4 or CD39 respectively.

The data here showed that SGA outcome in patients stable on ART expressed higher levels on CD39 at both time points while no correlations could be made in patients initiating ART during pregnancy. Previous research has shown that the expansion of the $CD39^{+}$ Tregs subset correlates positively with the level of immune activation and negatively with CD4 T-cell counts in HIV-infected subjects (Nikolova et al., 2011). The data further suggests that ART initiation may disrupt the function of these Tregs, as co-expression of these molecules is indicative of Treg effector function (Anderson et al., 2016), this is at least true for CTLA-4 and CD39 whereas Tigit may be minimally affected.

There was a negative correlation between Tigit vs CD39 at baseline before the commencement of treatment which was lost upon follow-up. This result could suggest that the expression of these receptors on Tregs might be co-dependant and that ART may influence this expression.

Co-expression analysis showed that the Tregs produced are suppressive because they express either one of the surface molecules that aid the cells in their function (Schulze zur Wiesch et al., 2011).

The SGA outcome of patients stable on ART had significantly higher levels of CD39. The expression of CD39 could indicate an immune checkpoint mediator which can be used to identify naïve Tregs that are prone to apoptosis. It was also interesting that most Tregs expressed Tigit indicating that the cells form part of the distinct Treg cell subset that specifically suppresses pro-inflammatory T helper 1 (Th1) and Th17 cells, but not Th2 cell responses (Joller et al., 2014).

We had hypothesised that the reconstitution of immune responses under ART in pregnant women would result in enhanced immunity against the fetus resulting in increased rates of preterm birth. Our data suggest that that the Tregs produced are high in patients initiating ART and subsequently altered by ART, however this alteration did not predict birth outcome. It is highly plausible that Tregs measured here are naïve and immune-suppressive $Foxp3^{+}$ regulatory T (Treg) cells owing to their expression of CD39 (Borsellino et al., 2007, Zhao et al., 2017), however further research is required as the direct functionality of Tregs was not measured in this study. Nonetheless, the modulation of co-inhibitory molecules and Tregs function is undoubtedly important as a therapeutic potential for treatment of a wide variety of inflammatory disorders, including cancer, chronic infection, autoimmune disease, and pregnancy complications.

7.1 Conclusion

In conclusion, it is important to highlight that pregnancy is a complex process and its regulation or dysregulation are multifaceted. We attempted to understand this by testing the hypothesis that immune dysregulation occurring during immune reconstitution under ART leads to preterm delivery.

Our study highlights the role of the immune system as a mechanistic factor underlying pregnancy outcome, particularly low immune activation and increases in classical and intermediate monocyte subsets with concomitant reduction of inflammatory monocyte subsets during pregnancy as a risk factor for PTD but not SGA in women who initiate ART during pregnancy. This interpretation is further supported by the reduced expression of inflammatory cytokines TNF-α and MIP-1β, particularly in monocytes, following TLR stimulation. The study further highlights a relationship between ART initiation and a diminished inhibitory ability in Treg cells in patients with a PTD outcome. This study was not able to associate outcome TH1/TH2 related cytokines with outcomes and ART initiation.

7.2 Limitations of the study and future directions.

Our study was limited by a number of factors. First, due to the small sample size we could not stratify women by treatment regimen; however, 88% of women were on a TDF-3TC-EFV regimen, which is the first line regimen in South Africa. This was indeed a small sample size, this was however a case-control study, with the focus being to understand the immunological changes over the course of pregnancy in the cases versus controls.

We were also unable to allow for other factors known to be associated with risk of PTD or SGA; however, baseline comparison suggest few differences between the two groups, except for age of the women, with women stable on ART significantly older than those initiating.

The use of frozen rather than freshly obtained immune cells is also a potential limitation as markers and phenotypic profiles of cells may be altered; however, cases and controls had samples managed to the same protocol, and their samples were taken at approximately the same time, and would have been frozen for the same duration.

Further work will be needed to confirm these immunological parameters as potential biomarkers for PTB among women initiating treatment in pregnancy and to explore the exact underlying mechanisms to facilitate better diagnosis and clinical interventions.

Our Treg analysis in chapter 5 did not show major differences in the frequencies of circulating Treg cells. It would be worthwhile to further reliably distinguish Tregs from activated effector $CD4^+$ T cells. To do this additional quantification of the overall frequency of functional Tregs will need to be performed based on the methylation status of the Treg-specific demethylated

region (TSDR) of the FOXP3 gene using qPCR. This will need to be determined using established flow-based CFSE proliferation assays and cytokine secretion profiles with special focus on cytokines associated with Tregs such as IL-10, IL-27 and TGF-ß as recently published (Angin et al., 2012). Given the small percentage of $CD4^+$ Tregs, some of the functional assays will require flow-sorting of $CD3^+CD4^+CD127^{dim}$ regulatory T cells followed by short term Treg expansion to obtain sufficient cell numbers for the functional assays. The suppressive capacity of Tregs can be quantified using a co-culture of Tregs and autologous effector T cells stimulated polyclonally with anti-CD2/anti-CD3/anti-CD28 microbeads or HIV-1-clade C peptide pools as previously described (Angin et al., 2012). This work could not factor this due to lack of samples.

Treg differentiation is associated with the acquisition of corresponding chemokine receptors and adhesion molecules responsible for directed homing. They are subdivided into naive cells (nTregs), central memory cells (cmTregs), effector memory cells (emTregs), and effector Treg (eTreg) lymphocytes, (Shevyrev and Tereshchenko, 2020). One of the study limitations is the lack of this phenotypic data for this cohort as the data in these cells as this would have complemented the co-stimulatory receptor data and confirmed the type of Tregs that are expressed in this cohort.

Several pregnancy-related proteins are known to promote Th2 bias such as leukemia inhibitory factor, progesterone, progesterone-induced blocking factor, and estradiol. Prostaglandin D2 (PGD2) promotes IL-4, IL-13, IL-5, and IL-10 production in T helper 2 cells in vitro via the second PGD2 receptor; chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). PGD2 is produced by the placenta and may play a role in the chemoattraction of

Th2 cells via the CRTH2 receptor to the maternal fetal interface to produce a localised Th2 bias (2). A successful human pregnancy has a number of elements which are needed, inclusive of which are the endocrine and metabolic changes that result from physiological alterations at the boundary between mother and fetus (Kumar and Magon, 2012). Progesterone is known to prevent preterm birth. Its implications have not been determined fully. Therefore, its measurement in this cohort would give insight as to how it impacts the different outcomes and how it is affected by treatment initiation.

Chapter 8 References

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