Characteristics of decidual macrophages and Hofbauer Cells in placentas of HIV positive women on antiretroviral therapy

by

MICHAEL ZAKHELE ZULU

ZLXMIC001

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Supervisor: Professor Clive M. Gray

DECLARATION

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

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LIST OF PRESENTATIONS & PUBLICATIONS

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2. KEYSTONE SYMPOSIA: MATERNAL-FOETAL CROSSTALK: HARMONY VS. CONFLICT; 2017. The Westin Hotel, Washington DC, USA. 4 - 8 October 2017. **Differential distribution of M1 and M2 Macrophages in the decidua and chorionic villi of placentas from HIV-1 infected mothers on combination antiretroviral therapy.** Michael Z. Zulu, Nadia Chanzu, Nozwakazi Bangani, Alexander Perez, Thoko Malaba, Hlengiwe Madlala, Landon Meyer, Gianguido Cianci, Tamara Tilburgs, Marie-Louise Newell and Clive Gray. **Poster presentation.**

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5. Michael Z. Zulu, Fernando O. Martinez, Siamon Gordon, Clive M. Gray (2019). **The Elusive role of Placental Macrophages: The Hofbauer Cell**. *Journal of Innate immunity,* 2019; 1-10. (Appendix D, in this thesis).

ABBREVIATIONS

AIDS:	Acquired Immunodeficiency Syndrome
ART:	Antiretroviral Therapy
ARV:	Antiretroviral
CA:	Chorioamnionitis
cART:	Combination Antiretroviral therapy
CCR5:	C-C chemokine receptor 5
CXCR4:	C-X-C chemokine receptor 4
DAB:	3,3'-diaminobenzidine tetrahydrocloride
DAPI:	4',6-diamidino-2-phenylindole
DB:	Decidua Basalis
DC-SIGN:	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DCs:	Dendritic Cells
dNK cells:	Decidual natural killer cells
DP:	Decidua Parietalis
EDTA:	Ethylenediaminetetraacetic acid
EVTs:	Extra-villous trophoblasts
FCS:	Foetal-Calf Serum
FcγR:	Fc receptors for IgG
FIs:	Fusion Inhibitors
FXIIIa1	Human coagulation factor 13 A1
GDM:	Gestational diabetes mellitus
HCs:	Hofbauer Cells
HIV-1:	Human Immunodeficiency Virus, type 1
HIV-2:	Human Immunodeficiency Virus, type 2
HLA:	Human Leukocyte Antigen
HPA:	The Human Protein Atlas
IDO:	Indoleamine 2,3-dioxyenase
IF:	Immunofluorescence
IFN-γ	Interferon-gamma
IGF-2:	Insulin-like growth factor 2

IHC:	Immunohistochemistry
IL:	Interleukin
INSTIs:	Integrase strand transfer inhibitors
IRF-5	Interferon regulatory factor 5
IUGR	Intrauterine growth retardation
M-CSF:	Macrophage-colony stimulating factor
M1:	Classically-activated Macrophages
M2:	Alternatively-activated Macrophages
MDMs:	Monocyte-derived Macrophages
MHC:	Major Histocompatibility Complex
MMP9:	Matrix Metallopeptidase 9
MR:	Mannose receptor
NK cell:	Natural Killer cell
NNRTIs:	Non-nucleoside reverse inhibitors
NO:	Nitric Oxide
NRTs:	Nucleotide reverse transcriptase inhibitors
OIP5:	Opa-Interacting protein 5
PBS:	Phosphate-buffered saline
PBST:	Phosphate-buffered saline & Tween 20
PIs:	Protease Inhibitors
RNA:	Ribonucleic Acid
SGA:	Small-for-gestational age
SIVs:	Simian Immunodeficiency Viruses
SR:	Scavenger receptor
TNF:	Tumor necrosis factor
TNIP1:	TNFAIP3-interacting protein 1
Tregs:	Regulatory T cells
Tris:	Triaminomethane
UNAIDS:	The Joint United Nations Programme on HIV/AIDS
VT:	Villous Tissue
WHO:	World Health Organization
ZIKV:	Zika Virus

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ABSTRACT

Macrophages are the most abundant immune cells at the maternal-foetal interface of term placentas. They play a role in regulating pregnancy, promoting immunological tolerance, and maintaining a homeostatic environment crucial for foetal development. Numerous studies have reported the dysregulation of maternal uterine-derived macrophages, the decidual macrophages and the foetal placental-derived macrophages, the Hofbauer Cells (HCs) in pregnancies complicated by maternal viral infections. The immune mechanisms associated with the high prevalence of adverse birth outcomes amongst HIV-1 infected women on combination antiretroviral therapy (ART) are not well characterized. In this Ph.D. dissertation, the impact of HIV-1 infection and the duration of ART exposure on the phenotype and function of decidual macrophages and HCs was studied. Birth outcomes and placenta pathologies of HIV-infected women categorized into two groups, 1). HIV-1 infected pregnant women who initiated ART during pregnancy (*Initiating ART n=16*); and 2). HIV-1 infected women who initiated ART before pregnancy (*Stable on ART n=14*) were compared. Immunohistochemical and immunofluorescence staining of formalin-fixed placental tissues from the two groups was performed to compare the expression of macrophage markers associated with inflammation (M1) and immune regulation (M2). Lastly, the Human Protein Atlas database was used to identify novel markers of decidual macrophages and HCs and their potential biomarkers of HIV-1 infection.

The expression of various M1 and M2-specific macrophage markers significantly varied between membranes of the maternal-foetal interface within the initiating and stable group. The duration of ART exposure had no effect on the expression of typical M1 and M2 macrophage markers on decidual macrophages and HCs of HIV-1 infected women who initiated a similar ART regimen before pregnancy and during pregnancy. The macrophage populations in the maternal-foetal interface co-expressed markers associated with M1 and M2 macrophage polarisation, suggesting functional plasticity of these cells. Human Coagulation Factor XIIIa1 (FXIIIa1) and Insulin-like growth factor 2 (IGF2) were identified as potential novel markers of HCs and decidual macrophages respectively, however, further validation is required. There is a paucity of data on the characteristics of decidual macrophages and Hofbauer cells in both normal and complicated pregnancies. Data presented in this dissertation suggests the

possibility that HIV-infection and / the duration of ART exposure may not play an active role in dysregulating immune mechanisms associated with macrophages at the maternal-foetal interface. However, further characterization of these cells in placentas exposed to different ART regimens is required using the novel markers discovered in this Ph.D.

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1. Review of the literature

1.1. Global prevalence of HIV infection

Human Immunodeficiency Virus-1 (HIV-1) is one of the greatest burdens to human health globally. It has been implicated in more than 35 million deaths worldwide over the past 30 years (Hemelaar, 2013). According to the UNAIDS/WHO-2018 fact sheet, in the year 2017, 36.9 million (31.1 million – 43.9 million) people were living with HIV globally and approximately 59% of these people were on antiretroviral therapy (ART) (UNAIDS, 2017b). They also reported that 80% (61 - 95%) of pregnant women living with HIV had access to antiretroviral therapy. Despite this, women of child-bearing age are most affected by the HIV pandemic, especially in developing countries. In the African continent, an estimated 1 in 25 adults is living with HIV, that makes up approximately two-thirds of the world's population of people living with HIV (UNAIDS, 2017b). Global trends suggest that HIV infections are still on the rise, however, AIDS-related deaths have decreased significantly and this is mainly attributed to widespread use of antiretroviral treatment. Figure 1.1 below shows recent global trends in HIV-1 prevalence for the year 2017 (World Health Organization (WHO), 2017).

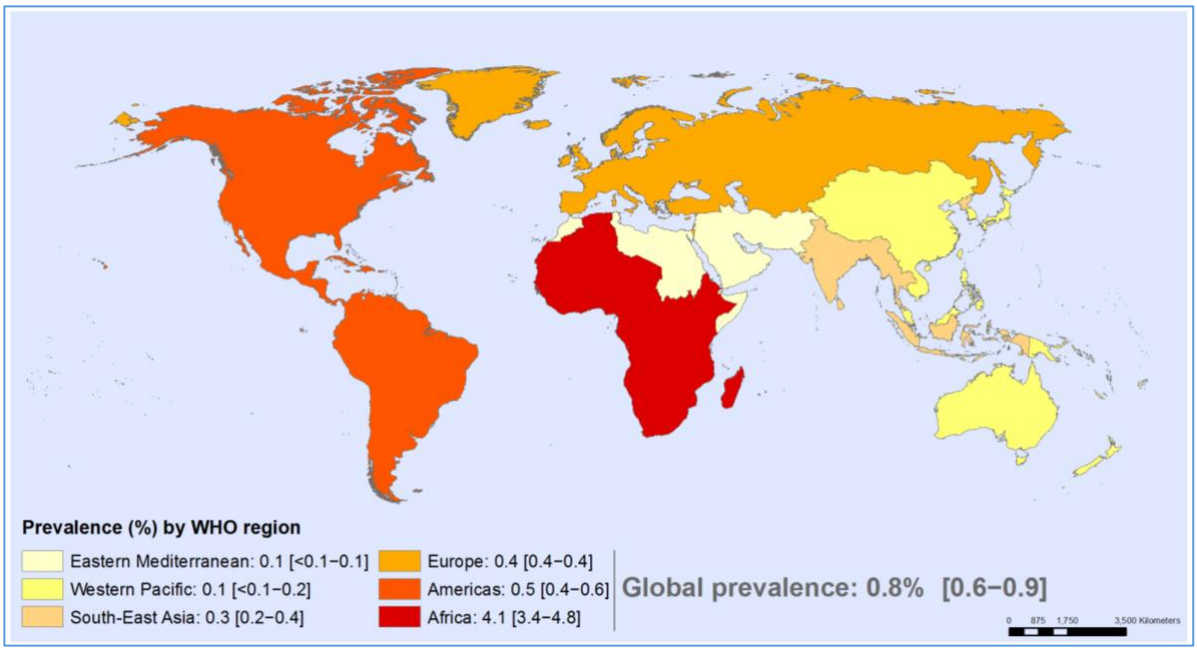

Figure 1.1: Global prevalence of HIV among adults, aged 15-49 in the year 2017 (WHO, 2017).

1.2. HIV infections in South Africa

An estimated 7.1 million South Africans were living with HIV in 2016. HIV prevalence is at 18.9% among the general population, making it the highest in the world (UNAIDS, 2017b). Despite having the world's largest ART programme, the HIV epidemic continues to have devastating social, economic and health effects on South Africans. Women and children are the most affected population. The prevalence of HIV infections among pregnant women increased continuously from the year 1990 to 2005, but slowly decreased when the country increased its ART roll-out programmes between 2005 and 2010 (Figure 1.2). The HIV pandemic is more than just a health problem. From 2015, the South African government invested more than $1.34 billion in its HIV prevention programmes (UNAIDS, 2017a). However, the HIV pandemic continues to have negative effects on the country's social and economic development (Dixon et al., 2002).

Increased mortality and morbidity due to HIV infection has not only affected the country's economy, it has also weakened the family system leading to an increased number of child-headed households and homelessness (Taraphdar et al., 2011). It is estimated that more than 2 million South African children are orphaned due to the HIV

pandemic (UNICEF, 2016). Orphaned children are at higher risk of contracting HIV because of economic and social insecurities. They often become sexually active at a younger age than other children and are at a risk of being forced into sex in exchange for financial support ((SANAC), 2017). Primitive cultural and sexual practices make women of childbearing age much more vulnerable to HIV infection than men. Although the number of Acquired Immunodeficiency syndrome (AIDS)-related deaths has decreased by 29% since the year 2010, it was reported that more than 100 000 people died from AIDS-related deaths in the year 2016 alone (UNAIDS, 2017a). The country's economy has plummeted due to loss of skilled labour and lack of investor confidence. The country's weak economy has led to high levels of youth unemployment and subsequent social problems such as drug abuse and crime. A weakened family system resulting from AIDS-related deaths and lack of financial support can also be associated with high levels of prostitution which further increases the risk of HIV-infection amongst women and the youth.

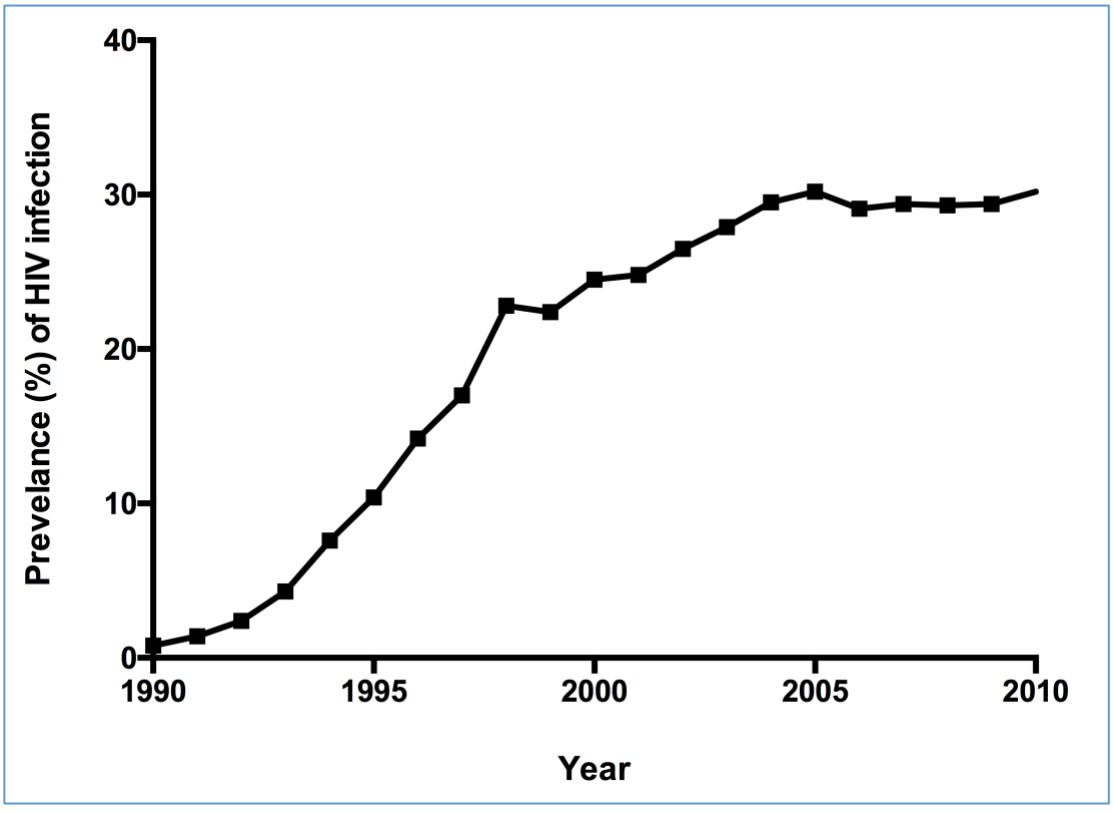

Figure 1.2: HIV prevalence among pregnant women in South Africa (Adapted from **www.avert.org** , accessed November 2018).

1.3. HIV/AIDS Treatment: Antiretroviral therapy

Management of HIV infections has improved in recent years; this is attributed to increased access to combination antiretroviral therapy (cART). Life-time dependency on ART due to persistent HIV viral reservoirs presents major health and economic challenges. In the past two decades, the use of Antiretroviral therapy (ART) by pregnant women living with HIV has had remarkable results on the epidemic of HIV in infants. There was a drastic 80% decrease in new infant infections, a decrease from 590 000 in 1998 to 120 000 in 2016 (UNAIDS, 1998, UNAIDS, 2017b). Despite this, women of reproductive age (15-24 years) are highly susceptible to HIV infections. It was reported that 51% of people living with HIV are female (UNAIDS, 2017b). Antiretroviral drug regimen used to for the prevention of mother-to-child transmission of HIV has evolved from zidovudine single-drug prophylaxis used in 1994 to the current triple-drug regimens (Connor et al., 1994, Fowler et al., 2017).

The hypothesis is that ART controls HIV viral replication, resulting in low or undetectable viraemia that drastically decreases the risk of HIV transmission. However, the mechanisms of how these drugs prevent HIV vertical transmission is not fully understood. Since 2009, the number of people accessing ART in South Africa has increased exponentially (Figure 1.3). However, there is very little information on the effect of prolonged ART exposure on the immune system of people living with HIV, especially women of childbearing age. The effect of different ART regimens administered during pregnancy on birth outcomes is not well understood. A recent study reported that ART drug, dolutegravir, causes neural tube birth defects in infants born to HIV-infected mothers that received treatment during pregnancy (Zash et al., 2018). There is a need for pharmacovigilance to assess immune dysregulation caused by ART exposure during pregnancy and in infants exposed to ART while *in utero*.

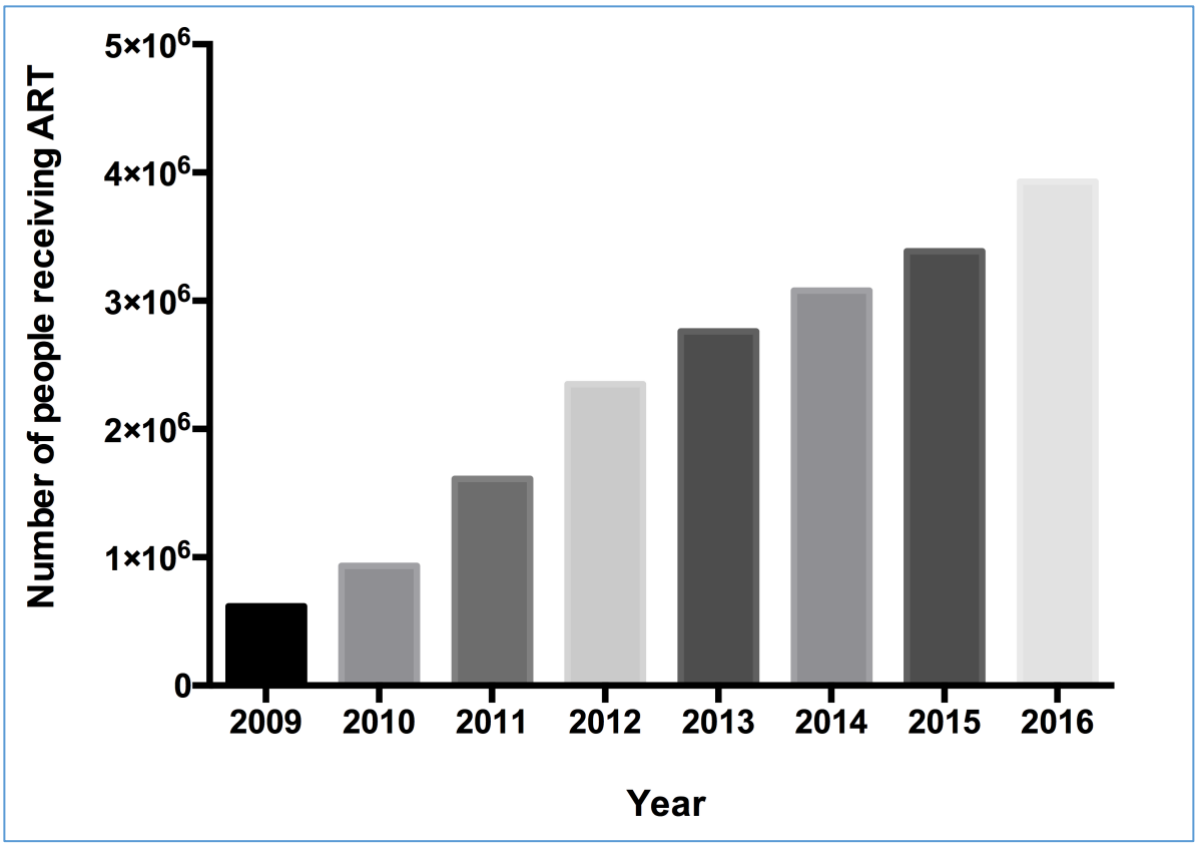

Figure 1.3: Exponential increase in the number of people receiving antiretroviral therapy in South Africa. (Adapted from **www.avert.org**, accessed in November 2018).

1.4. Classification and geographical distribution of HIV

The virus responsible for causing AIDS was first isolated from severe immunocompromised patients and classified to the lentivirus family of retroviruses (Barresinoussi et al., 1983, Levy et al., 1984, Popovic et al., 1984) and termed Human Immunodeficiency Virus (HIV). The virus originated from Simian Immunodeficiency Viruses (SIVs) of primates and it was classified into HIV-1 and HIV-2 (Galea and Chermann, 1998, Gallo and Montagnier, 2003, Hahn et al., 2000, Sharp and Hahn, 2010, Sharp and Hahn, 2011). HIV-1 was derived from the SIV of chimpanzees (SIV_{cpz}) while HIV-2 came from the SIV of sooty mangabey monkeys (SIV_{mm}) (BarreSinoussi, 1996, Gallo and Montagnier, 2003). The SIVs did not cause immunodeficiency in their native host. However, both HIV-1 and HIV-2 are tropic for CD4+ lymphocytes and cells of the mononuclear phagocyte system, they also have similar genetic structures and open reading frames but differ slightly as their nucleic

acid sequences are 40% homologous (Dorman and Lever, 2000, L'Hernault et al., 2012).

There are numerous clades of HIV-1. The subtypes of HIV-1 were phylogenetically classified based on the 20-50% difference in their envelope (*env*) nucleotide sequences (Spira et al., 2003, Starcich et al., 1987). HIV-1 is grouped into major (M), new (N) and outlier (O) groups; these may also represent three episodes of zoonotic transmission from chimpanzees. Groups M and O *Env* proteins differ by 30-50% while Group N is phylogenetically equidistant from both groups M and O (Hemelaar, 2013). Group M HIV subtypes are mainly responsible for the pandemic and they are divided into subtypes A-K. The HIV-1 subtypes of Group M have an inter-subtype variation of 20-30% while intra-subtype variation is between 10-15% (Gao et al., 1998). Due to high diversity in HIV-1, diagnostic testing, ART regimen and pathogenesis needs to be carefully monitored as these may be subtype-specific. Geographical distribution (Figure 1.4) of patients infected with different subtypes of HIV-1 is heterogeneous (Takebe et al., 2004). The remarkable genetic diversity of viral subtypes in different regions is due to high mismatch error rate of the HIV reverse transcriptase (RT) enzyme coupled with the absence of exonuclease proof-reading activity (Sierra et al., 2005).

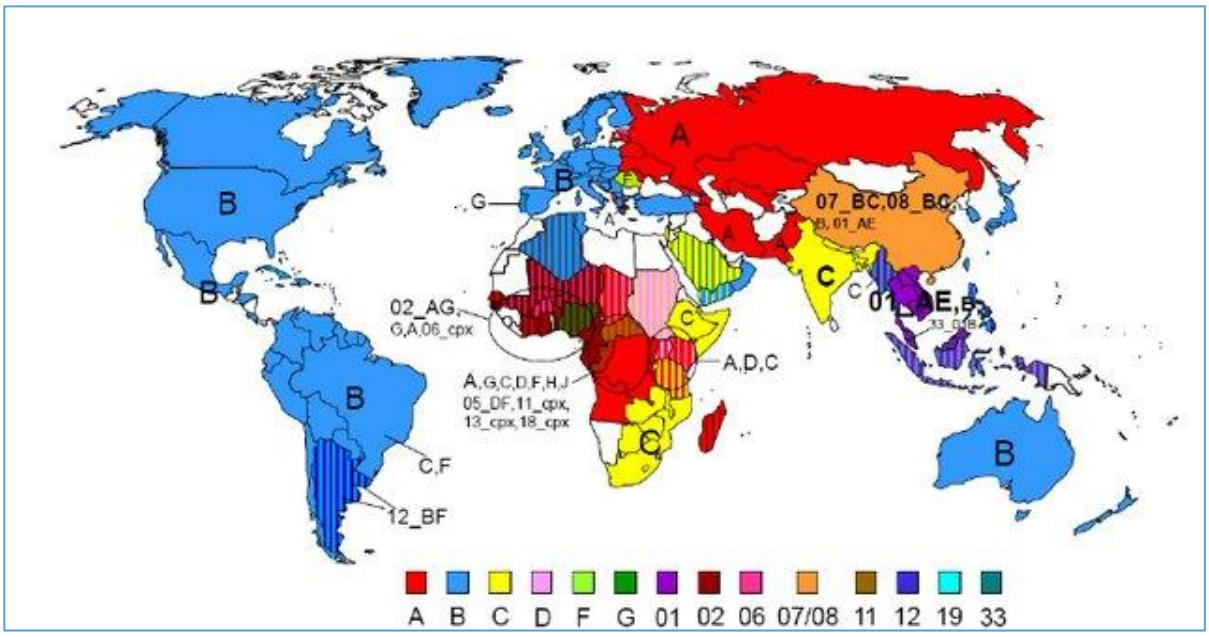

Figure 1.4: Geographical distribution of HIV-1, Group M subtypes (2016). (Adapted from WHO-UNAIDS HIV Vaccine Initiative).

The most predominant subtype in Southern and South-Eastern African countries such as South Africa, Lesotho, Swaziland, Namibia, Botswana, Zimbabwe, Zambia, and Mozambique is subtype C (Janssens et al., 1997, Papathanasopoulos et al., 2003). This is also the main subtype found in South-East Asian countries like Nepal and India. Subtype B is found mostly in Australia, Europe and the Americas. Increased migration and globalization has introduced new recombinant subtypes. Reports suggests that 40% of new HIV infections in Europe were non B-African and Asian variants (Spira et al., 2003, Cohen et al., 2008). Subtypes A and A/G recombinants are found mainly in West and central Africa (Papathanasopoulos et al., 2003) while Subtype D is the most predominant subtype in East and central Africa (Janssens et al., 1997). Subtype E has been detected in countries like Thailand, the Philippines, China and central Africa as an A/E mosaic (Paladin et al., 1998). Subtype F was detected in HIV-infected people in Central Africa (Triques et al., 1999), South America and Eastern Europe. Subtype G and A/G recombinant viruses were detected in patients from Western and Eastern Africa (Papathanasopoulos et al., 2003). Subtypes H, J and K were detected in patients from central Africa and Central America (Triques et al., 1999). However, it is important to note that due to vast changes in global migration in recent years, geographical distribution of HIV-1, group M subtypes may differ significantly in 2019 and this may increase the prevalence of recombinant subtypes.

1.5. HIV-1 Pathogenesis

1.5.1. The structure and replication cycle of HIV-1

Retroviruses have an RNA genome which consists of two identical 9.2 kb single-stranded RNA molecules (Sierra et al., 2005). The HIV virion is spherical in shape with a diameter of about 100 – 120nm (Sierra et al., 2005). Each virion consists of a lipid bilayer membrane that surrounds the cone-shaped nucleocapsid that houses the genomic RNA molecules, Vpu, Vif, Vpr, Nef; and the viral enzymes: protease, reverse transcriptase and integrase and cellular factors (Figure 1.5). It has about 72 spikes of the viral Env glycoproteins and the principal structural proteins forming the core are in the virion matrix (MA or p17), capsid and nucleocapsid (Gelderblom et al., 1987).

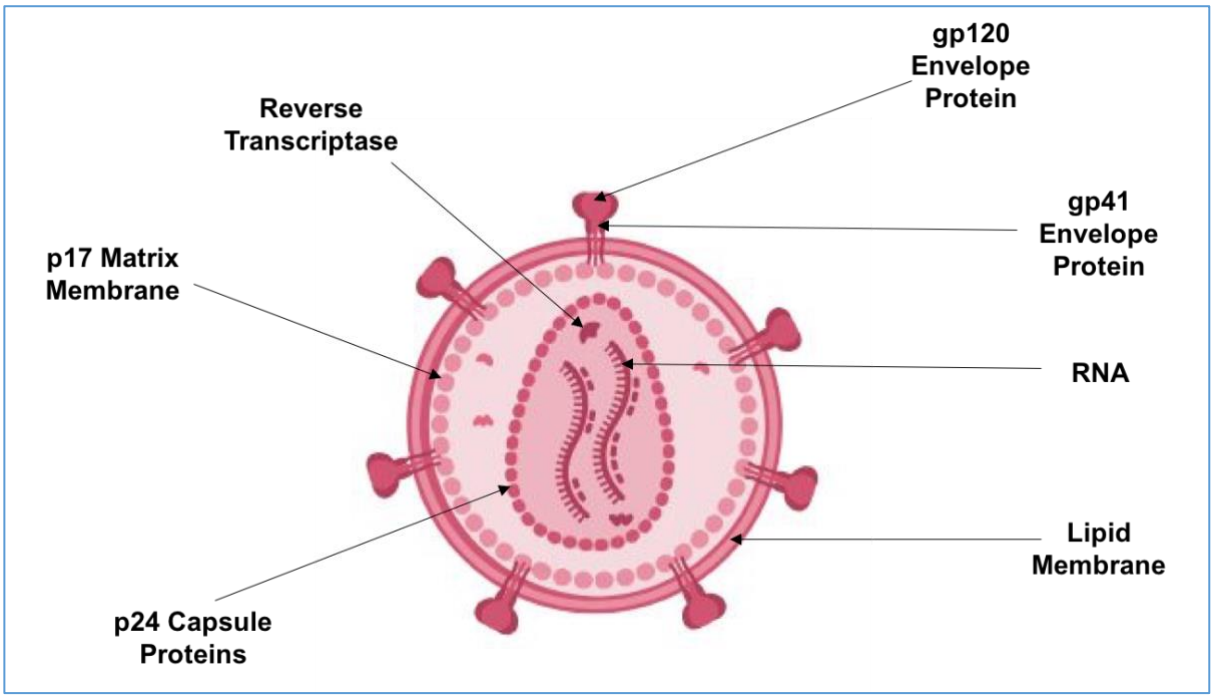

Figure 1.5: A schematic diagram of the structure of HIV-1. (Created with Bio-render)

The global HIV-1 pandemic over the years has indicated that the virus is able to evade innate, adaptive and intrinsic immunity to establish an infection (Emerman and Malim, 1998, Bieniasz, 2004). However, there are various viral and host factors that are major determinants of the outcome of HIV-1 infection and the rate of disease progression,

these factors vary between individuals (Naif, 2013). The virus gains entry into CD4+ T lymphocytes by attaching its gp120 envelope proteins to the CD4 receptor, this marks the first step in the replicative cycle of HIV. The virus binds further to co-receptor, C-C chemokine receptor 5 (CCR5) or C-X-C chemokine receptor 4 (CXCR4) resulting in the fusion of the viral envelope and the cellular membrane. This is then followed by the release of viral nucleocapsid into the cytoplasm (Dragic et al., 1996). The enzyme reverse transcriptase facilitates the reverse transcription of viral RNA into double-stranded proviral DNA that migrates to the nucleus where integrase allows viral DNA to become integrated into the host chromosome as pro-viral DNA. RNA polymerase II transcribes the proviral DNA into mRNAs which are translated by cellular polysomes into viral proteins and genomic DNA; these are then transported to the cellular membrane and assembled. A fully-formed virion is then reproduced after processing of the polypeptide precursors (Dragic et al., 1996). Figure 1.6 shows the HIV replication cycle, starting with viral fusion at the surface of the host cell to the formation of a newly synthesized polyprotein creating a mature infectious virus.

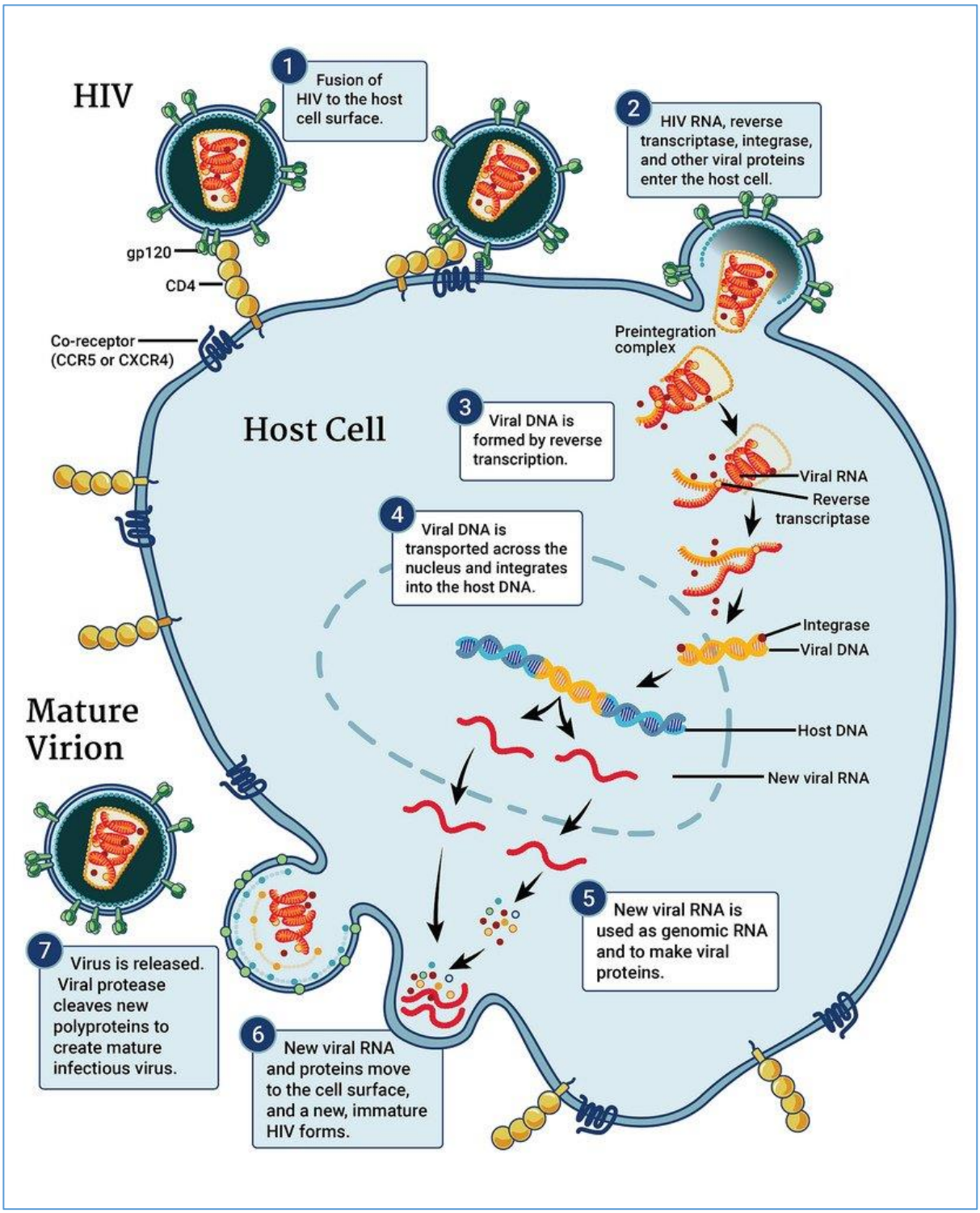

Figure 1.6: HIV replication cycle. (https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle , accessed November 2018).

1.5.2. The role of macrophages in the acquisition of HIV-1

HIV is transmitted from one infected person to another through direct contact of body fluids such as blood, semen, vaginal fluids and breast milk. Sexual intercourse is a predominant mode of HIV-1 transmission in humans (Royce, 1997). During this mode of transmission, HIV-1 crosses the genital mucosal barrier through small mucosal lesions and epithelial abrasion that may occur during sexual intercourse or because of other sexually transmitted infections. The first immune cell that HIV encounters in the epithelium are macrophages, dendritic cells and CD4+ memory T cells monitoring the mucosal surface (Shen et al., 2011). HIV targets immune cells that express the CD4 receptor at these sites (Wilen et al., 2012). It gains entry into these immune cells by attaching itself unto the CD4+ receptor on the cell surface, upon entry, viral-cell fusion occurs. Viral fusion triggers a number of intracellular mechanisms that regulate expression of viral regulatory and accessory genes, that subsequently leads to a productive or latent infection (Levy, 1993). Using the macaque-simian immunodeficiency virus as a model for vaginal acquisition of HIV, Stieh and colleagues reported that the Th17 lineage CCR6 CD4+ T cells are preferentially infected during vaginal infection (Stieh et al., 2016). A number of studies have demonstrated how mucosal infection with a variety of sexually-transmitted infections plays a role in the transmission and pathogenesis of HIV-1 (Dutta et al., 2017, Kolodkin-Gal et al., 2013, Schust et al., 2012, Tugizov, 2016).

HIV is also able to gain entry into cells of the myeloid cell lineage such as monocytes, macrophages and dendritic cell (DCs) by utilizing the expression of CCR5 or CXCR4. These receptors are widely expressed by DCs, monocytes, macrophages and microglia of the central nervous system (Jazin et al., 1997, Moepps et al., 1997, Torres et al., 2001, Zhu et al., 2012, Wei et al., 2018, Creery et al., 2006). HIV is able to solely utilize CCR5 or CXCR4 during cell infection (Wilen et al., 2012, Venuti et al., 2017, Zhou et al., 2001). *In vivo* and *in vitro* studies demonstrated that both monocytes and macrophages can be infected with HIV-1 (Zhu et al., 2002, Crowe et al., 1987, Kalter et al., 1991), with macrophages being more susceptible (Iordanskiy et al., 2013). Monocytes are less susceptible to HIV-1 infection, but become more susceptible during their differentiation into macrophages (Rich et al., 1992, Sonza et al., 1996).

Although they express low levels of the CD4 receptor, monocytes and macrophages have been shown to express markers suitable for productive HIV-1 infection *in vivo* (Koenig et al., 1986). Although few macrophages are infected by HIV compared to CD4+ T cells, their phenotype, tissue localization and immune functions makes them an ideal immune cell type for infection and dissemination of the virus (Vijayan et al., 2017, Sattentau and Stevenson, 2016). Therefore, macrophages play a critical role in the establishment of infection and pathogenesis of HIV.

Gartner and colleagues were the first to report that tissue-resident macrophages are permissive to HIV infection and are capable of replicating the virus (Gartner et al., 1986). Their study not only reported that HIV does not induce a sudden decrease in macrophage cell numbers but also showed that there is prolonged viral replication compared to the infection of CD4+ T cells. HIV infection of tissue macrophages which persists during combination antiretroviral therapy (cART) has been confirmed at all stages of the disease (Cory et al., 2013). Macrophage infection by HIV is not only dependent on CCR5 or CXCR4 surface expression; it also requires initial absorption of the virus by the cell surface. This process is facilitated by lectin-like receptors, integrin, and heparin sulfate proteoglycans (DiFronzo et al., 1997, Duncan and Sattentau, 2011). It was suggested that entry occurs after virion internalization in macropinosomes (Marechal et al., 2001) and endosomes where fusion between the viral envelope and the host is believed to occur (van Wilgenburg et al., 2014). In support of these findings, a recent study demonstrated that internalization of fluorescent quantum dots encapsulated by infectious HIV-1 particles in primary macrophages (Li et al., 2017).

Upon productive HIV-infection of tissue-resident macrophages, they migrate from the mucosal sites of infection to regional lymph nodes where the virus disseminates systemically in the host (Rodrigues et al., 2017). In humans, this process is not very well understood and is impractical to investigate. However, in nonhuman primates such as Simian Immunodeficiency Virus (SIV)-infected rhesus macaques, the virus was detected as early as day 1 post intravaginal inoculation in the gastrointestinal tract and the spleen, and systemic distribution was observed at day 7 (Barouch et al., 2016). This viral dissemination is accompanied by an acute phase of HIV infection with rapid viral replication in many tissues (Li et al., 2005, Mattapallil et al., 2005). These findings

support the involvement of HIV-1 infected macrophages in systemic distribution of the virus.

1.5.3. Macrophages contribute to the HIV-1 cellular reservoir

The myeloid lineage cells play an essential role in HIV-1 infection and; continuously aid in its pathogenesis throughout disease progression (Kedzierska and Crowe, 2002). Immune functions of macrophages and their susceptibility to infection prompted numerous studies to investigate their role in the development of viral reservoirs and chronic inflammation observed in HIV-infected individuals on ART (Sereti et al., 2017, Hunt, 2017). ART drastically reduces HIV replication in the body. However, the virus has been shown to persist in cellular and anatomical reservoirs (Chun et al., 1997, Finzi et al., 1997, Wong et al., 1997, Sennepin et al., 2018, Su et al., 2018). When ART is interrupted, these reservoirs become a source of viral rebound. Viral reservoirs represent a major obstacle to the eradication of HIV (Rose et al., 2018). Ubiquitous distribution of macrophages in human tissues and their ability to infiltrate virtually all organs of the human body makes them an integral HIV target cell for spreading HIV-1 in an infected individual (Gras and Kaul, 2010) and for establishing the development of the cellular viral reservoirs (Meltzer et al., 1990, Poles et al., 2006).

The macrophage viral reservoirs cannot be controlled by current ART regimens because macrophages have a longer lifespan and are resistant to the cytopathic effects of viral replication (Barton et al., 2016). Although latently infected CD4+ T cell populations such as naïve T cells and memory T cells have been reported to be a source of HIV reservoir (Blankson et al., 2002, Chun and Fauci, 1999, Finzi and Siliciano, 1998), various tissue-specific macrophage populations have also been implicated (Wong and Yukl, 2016). In macrophages, viral replication is much slower compared to CD4+ T cells; therefore, they express less viral proteins which enables them to evade the immune response (Alexaki et al., 2008).

1.6. Polarisation of Macrophages

1.6.1. Polarisation of Macrophages

Macrophages play a role in virtually every aspect of an organism's biology, from development and homeostasis to tissue repair and immune responses to pathogens. They are a heterogeneous population of immune cells that constantly change their functional state in response to changes in tissue physiology or environmental challenges (Martinez and Gordon, 2014a). Their plasticity allows them to respond to various environmental signals and change their phenotype and physiology in response to cytokines and microbial signals (Mosser and Edwards, 2008). Macrophages are tissue-resident immune cells that perform crucial immunological functions such as antigen presentation, phagocytosis, cytokine secretion, and coordination of innate and adaptive immune responses (Fujiwara et al., 2011). For the longest time, tissue-resident macrophages were believed to be continuously replenished by blood-circulating monocytes that originate from progenitors in adult bone marrow. This concept was central in defining the "Mononuclear Phagocyte System" (MPS) that grouped together precursors of monocytes in the bone marrow, monocytes in the peripheral blood, and macrophages in the tissues (van Furth et al., 1972, Yona and Gordon, 2015). Advanced techniques for studying cellular ontogeny showed that the homeostatic contribution of peripheral blood monocytes to tissue-resident macrophage populations may be confined to specific tissues such the gut, the dermis, and the heart with tissue-specific turnover rate. Alternatively, most tissue-resident macrophages arise from embryonic precursors prior to birth and maintain themselves locally throughout adulthood, independent of circulating monocytes (Hoeffel and Ginhoux, 2015, Ginhoux and Guilliams, 2016).

The functional maturation of macrophages has recently been described in a manner similar to the well-characterized concept of T helper type 1 (T_H1) and T helper type 2 (T_H2) polarisation of CD4+ T cells (Romagnani et al., 2000, Mills et al., 2000). They are classified as M1 (classically activated) and M2 (alternatively activated) macrophages (Biswas and Mantovani, 2010, Gordon, 2003), M1-like macrophages secrete pro-inflammatory cytokines, mediate resistance to pathogens and contribute

to tissue destruction, while M2 macrophages secrete anti-inflammatory cytokines and promote tissue repair and remodeling (Gordon, 2003, Martinez et al., 2008). However, this paradigm of macrophage activation has not yet been fully characterized, especially in humans (Martinez and Gordon, 2014a). This definition only considered the effects of stimuli on macrophage polarisation neglecting complex mechanisms that lead to macrophage activation in different tissues. A great variety of intermediates have been described based on their plasticity and adaptability (Mosser and Edwards, 2008) so that this simplistic concept has to be revised. One M1-like phenotype has been described compared with several of the M2 phenotypes, such as M2a, M2b, M2c and M2d (Mantovani et al., 2004, Ferrante et al., 2013). The M2b macrophages share similar characteristics with M1-like macrophages (Sironi et al., 2006). Each of the M2 macrophages subtypes differs in a number of aspects such as expression of certain surface molecules, cytokine secretion, and function (Mosser and Edwards, 2008).

1.6.2. Classical activation of Macrophages

Classically-activated (M1) macrophage stimuli can be grouped according to their ability to induce prototypic inflammatory responses and markers. However, their source, role, receptor, and signaling pathways differ dramatically (Martinez et al., 2008). The M1 phenotype results from stimulation of resting macrophages by microbial products or pro-inflammatory cytokines such as interferon–gamma (IFN-γ), tumor necrosis factor (TNF) or Toll-like receptor (TLR), characteristically, they have been shown to produce high levels of IL-12, IL-13, and nitric oxide (NO) (Verreck et al., 2004). Interferon regulatory factor-5 (IRF-5) is a transcriptional regulator of the M1 macrophage phenotype and has been shown to play a critical role in the induction of pro-inflammatory cytokines such as TNF, IL-6, IL-12 and IL-33 (Krausgruber et al., 2010b). There is a high expression of IRF-5 in human M1 macrophages which adds to the plasticity and polarisation of macrophages to the M1 phenotype and initiation of potent T_H1-T_H17 responses (Krausgruber et al., 2011b). Interestingly, IRF5 has the ability to promote differential gene expression suggesting a mechanism by which IRF5 can form either repressor or activator complexes at selected target genes (Eames et al., 2012). In human macrophages, prostaglandin E_2 (PGE_2) and cyclooxygenase (Cox) enzymes contribute to the production of pro-inflammatory cytokines (Arias-

Negrete et al., 1995). Cox-1 and Cox-2 initiate PGE_2 synthesis by catalyzing the first two steps of eicosanoid metabolism using arachidonic acid as a predominant substrate (Williams and Shacter, 1997). Cox enzymes are indistinguishable in their biosynthetic catalytic activities, however, they have been shown to have different physiological functions. Cox-1 (prostaglandin synthase-1) protein is constitutively expressed in most cell types and is thought to be responsible for regulating normal physiological functions and its cellular activity is primarily regulated by substrate availability while Cox-2 is an inducible enzyme expressed by activated macrophages and fibroblasts (Giroux and Descoteaux, 2000).

1.6.3. Alternative activation of Macrophages

There are four subtypes of M2 macrophages that have been described so far, these are interrelated and have been termed M2a, M2b, M2c and M2d. They have various functions including regulation of immunity, maintenance of tolerance, inhibition of inflammation and tissue repair or wound healing (Martinez and Gordon, 2014b). Exposure to glucocorticoids and cytokines such as interleukin (IL)-4, IL-10 and IL-13 has been shown to polarize macrophages towards the M2 subset (Gordon, 2003). M2 polarized macrophages are characterized by enhanced expression of innate immunity receptors, which includes scavenger receptors such as CD163 and the macrophage mannose receptor CD206 and also by an up-regulation in arginase activity which counteracts nitric oxide (NO) synthesis (Munder et al., 1998). CD68 and CD163 are markers used to identify macrophages in tissue sections (Barros et al., 2013). Several *in vitro* studies suggested CD163 as a possible marker for M2 macrophages (Sica and Mantovani, 2012, Buechler et al., 2000, Sulahian et al., 2000) and other studies of immune tissues considered $CD163^+$ cells identified by immunohistochemistry as M2 macrophages (Zaki et al., 2011, Ino et al., 2013). CD206, a macrophage mannose receptor has been shown to be upregulated by IL-4 supporting the concept of alternative activation of macrophages (Stein et al., 1992). A schematic diagram depicting the polarisation of macrophages based on the M1/M2 paradigm is shown in Figure 1.7.

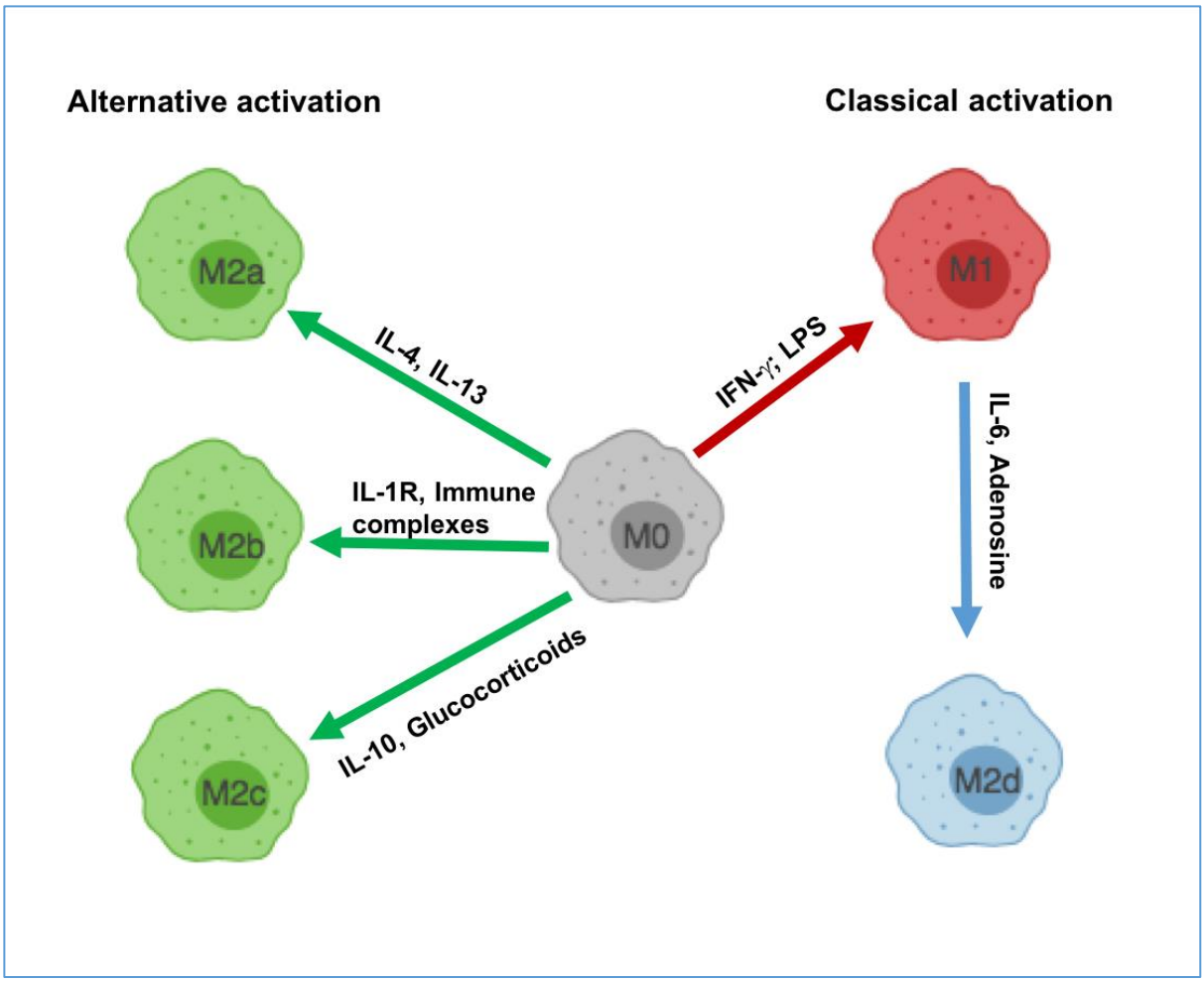

Figure 1.7: An overview of macrophages polarisation into M1 and M2. Resting macrophages (M0) can be classically activated by IFN-γ or lipopolysaccharide (LPS) stimulation into M1 macrophages. M1 macrophages are responsible for pro-inflammatory cytokine secretion, phagocytosis and initiation of immune response. Treatment of M1 macrophages with IL-6 or adenosine switches their polarisations towards an M2-like phenotype termed, M2d (Ferrante et al., 2013). IL-4, IL-1R and IL-10 stimulation polarizes M0 macrophages towards M2a, M2b, M2c respectively. M2 macrophages secrete high levels of the immune regulatory cytokine, IL-10 while expressing markers such as CD163, CD206 and CD209. (Created with Bio-render)

1.6.4. HIV-1 infection of polarized macrophages

Viral replication upon macrophage infection is regulated by cytokines that activate and/or polarize macrophages (Jimenez et al., 2012). Depending on the state of the cell at the time of infection, macrophage polarizing cytokines could either enhance or

inhibit HIV-1 replication (Cassol et al., 2010). Numerous studies have shown that HIV-1 infection of alternatively activated macrophages resulted in significantly lower levels of reverse transcription and p24 production (Montaner et al., 1997, Schuitemaker et al., 1992, Wang et al., 1998). However, treatment of these macrophages with IL-4, increased viral replication (Kazazi et al., 1992, Naif et al., 1994). Cassol *et al*., and others have also shown that cytokine-induced polarisation of human monocyte-derived macrophages (MDMs) into either classical (M1) or alternatively (M2a) MDM was associated with decreased capacity to support productive CCR-5-dependent HIV-1 infection (Cassol et al., 2009). They also reported an increased inhibition of viral replication in MDMs that were stimulated with INF-γ, IL-4, IL10 and IL-33 (Cassol et al., 2009, Cassol et al., 2010, Rasool et al., 2008). Classically-activated macrophages secrete high levels of pro-inflammatory cytokines which aid in HIV-infection of these cells and disease pathogenesis (Herbein and Varin, 2010). Alternative activation of macrophages induced by IL-4 and IL-13 decreases their susceptibility to HIV-1 infection through down-regulation of CCR5 expression (Wang et al., 1998). HIV-1 infection of MDMs has been associated with increased secretion of M1-associated chemokines such as CCL3, CCL4, and CCL5; and decreased expression of M2-associated markers, such as CD163, and CD206 (Brown et al., 2008, Porcheray et al., 2006). These findings further reinforce the hypothesis that HIV-1 infection of macrophages polarizes them towards an inflammatory (M1) phenotype which enhances disease progression. Whether pre-existing polarized M1 macrophages (due to inflammation) are more susceptible to HIV-1 infection is not yet clear.

1.7. The immunology of pregnancy

1.7.1. The Maternal Foetal Interface

Pregnancy presents a great challenge to the maternal immune system. It is a unique and complex immunological phenomenon in that a foetus consisting of both maternal and paternal alleles, develops within an active maternal immune system without succumbing to immunological rejection (Gomez-Lopez et al., 2014, Erlebacher, 2013a). This occurs simultaneously with defense against pathogenic microorganisms (PrabhuDas et al., 2015). It represents a complex immunological paradox that has

been the focus of numerous research for over half a century. The maternal-foetal interface is a unique anatomical site between the uterine mucosa and the extra-embryonic tissue of the developing conceptus (Erlebacher, 2013a). It is made-up of three well-defined compartments, namely the placenta (foetal origin), the decidua and myometrium (both of maternal origin) which are both infiltrated by extra-villous trophoblasts (EVT) during implantation. During gestation, these compartments undergo drastic changes in architecture and leukocyte composition (Brown et al., 2014, Gomez-Lopez et al., 2014).

Flow cytometric analysis studies have shown that 40% of the cell populations in the decidua are leukocytes (Vince et al., 1990). At conception, there is a general increase in immune cell populations, such as Natural Killer (NK) cells and macrophages at the maternal-foetal interface (Heikkinen et al., 2003). In normal conditions, most decidual macrophages are characterized by an immunosuppressive phenotype with M2 polarisation (Svensson et al., 2011) whereas, in complicated pregnancy, there is an increase of pro-inflammatory M1 macrophages (Kacerovsky et al., 2014). We recently highlighted the lack of consensus in literature on the impact of complications of pregnancy on the activation status of macrophage populations in the maternal-foetal interface (Zulu *et al*., 2019, *Journal of Innate immunity*, *in press*). Macrophages are the most abundant antigen presenting cells (APCs) in the human decidua during pregnancy (Nagamatsu and Schust, 2010). They have been shown to play a role in regulating pregnancy (Abrahams et al., 2004), promoting tolerance of the semi-allogeneic foetus (Svensson-Arvelund et al., 2014), and in the maintenance of a homeostatic environment necessary for normal foetal development (Erlebacher, 2013b). There is limited knowledge about the quantities and characteristics of the potent type of APCs, the dendritic cells (DCs) at the maternal-foetal interface of humans throughout pregnancy. In mice models, uterine DCs were phenotypically characterized as cells with high expression of CD11c and MHCII surface molecules with lymph node-homing and T-cell-stimulating capacity (Collins et al., 2009, Keenihan and Robertson, 2004). Whereas in humans, decidual DCs isolated from first trimester decidua were described as cells expressing CD83 with potent antigen-presenting capacity *ex vivo* (Kammerer et al., 2000). The presence of phenotypically and functionally distinct DCs at the maternal-foetal interface of both humans and rodents has been proposed to contribute to tolerance and immunity (Liang and Horuzsko,

2003). However, more sophisticated procedures are required to characterize populations of DCs of the maternal-foetal interface and how they play a role in tolerance.

1.7.2. The Placenta

The placenta is a transient organ made-up of foetal-derived tissues and the maternal decidua which develops from the uterine mucosa (Ferreira et al., 2017). Placenta formation occurs through a complex but well-coordinated effort between the foetus's extra embryonic tissues and the gravid endometrial tissues. In humans, it is a disc-shaped organ with a diameter of 15-20cm, thickness of 2-3cm and weighs approximately 500g at term (Griffiths and Campbell, 2015). It plays a crucial role in orchestrating the local circulatory system, provides a large surface area for maternal-foetal exchange, and also acts as an immune regulatory tissue during pregnancy (Wetzka et al., 1997). Therefore, the placenta is a major interface between the mother and the developing foetus, where its functions include gaseous exchange, nutrients transfer, excretion of waste products, and foetal protection through hormone secretion and transfer of immunity from the mother to the foetus (Knipp et al., 1999, Malek, 2013). The placenta acts as a semi-permeable membrane that allows for transfer of certain drugs across the placental membranes depending on the physical properties the drugs (Pacifici and Nottoli, 1995, van der Aa et al., 1998).

During embryo implantation, the uterine mucosa undergoes a specialized tissue reaction known as decidualization to support the development and function of the placenta. The decidua is therefore the specialized endometrial stromal tissue surrounding the conceptus (Erlebacher, 2014). Trophoblasts are the extraembryonic epithelial cells that compose the bulk of the placenta and replace maternal endothelial cells in remodeled spiral arterioles. There are two types of villi in the placenta, the villi that float in the maternal blood and the villi that attach the foetus to the mother's endometrial wall. Each villus has a connective tissue core which contains foetal blood vessels and many macrophages, called Hofbauer cells found under a thick basement

membrane (Pereira et al., 2005). A schematic diagram of a transverse section through a full-term placenta is shown in Figure 1.8.

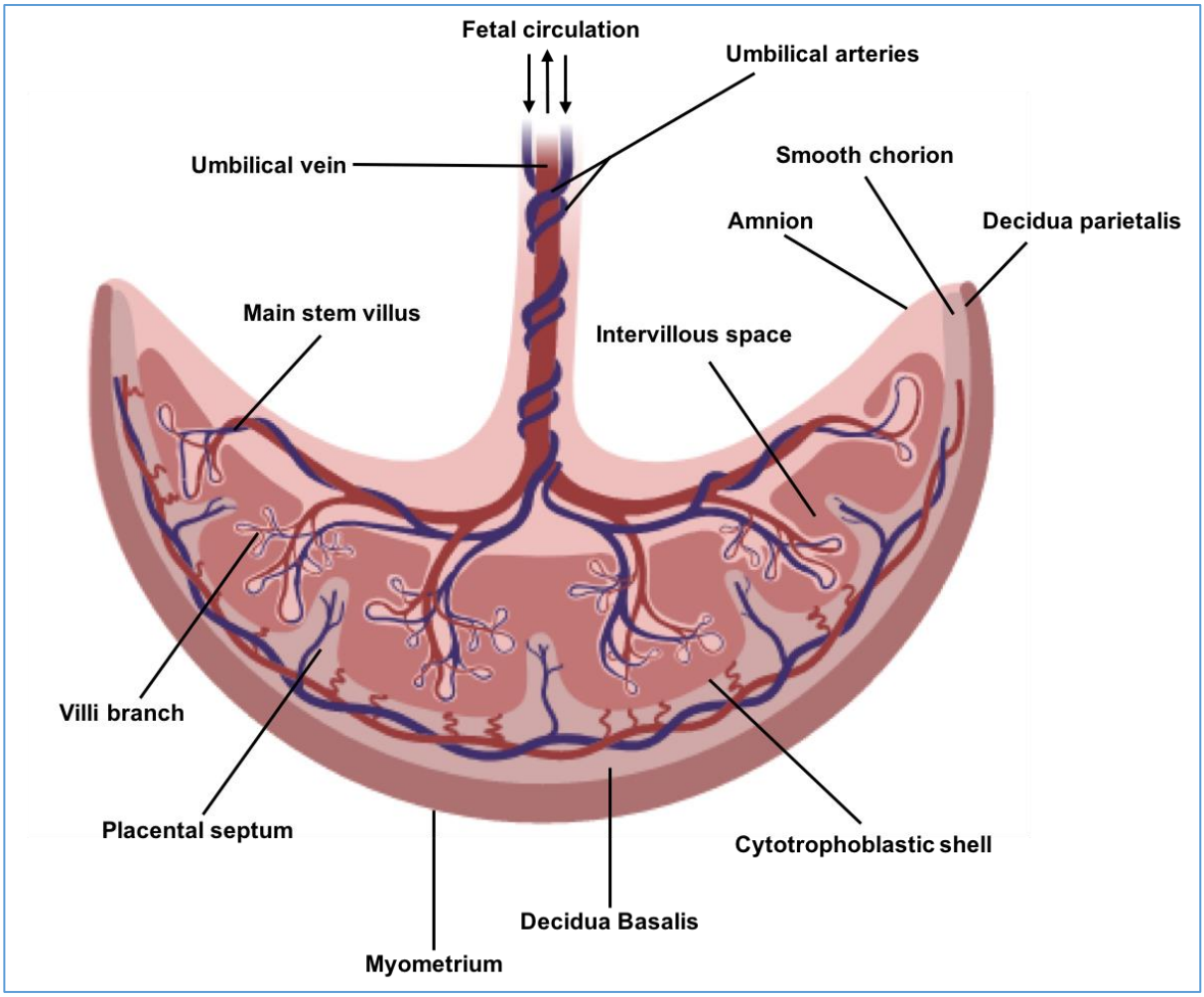

Figure 1.8: A schematic diagram of a transverse section through a full-term placenta. (Created using Bio-render).

1.7.3. Maternal-Foetal Tolerance

The mechanism by which the maternal immune system tolerates the foetus is not yet fully understood. Successful pregnancy is facilitated and is dependent on continual development of maternal-foetal tolerance (Finn et al., 1977). It has been 66 years since Medawar (Medawar, 1953) proposed the presence of immunological tolerance towards the semi-allogeneic foetus. Initially, it was proposed that immune tolerance is brought about by the failure of maternal immune cells to respond to foetal cells expressing foreign molecules (Medawar, 1953). However, It was later shown that,

pregnancy induces the development of foetal antigen-specific cytotoxic T cells and antibody-mediated immune responses against the paternal-derived HLA class I antigens of the foetus (van Kampen et al., 2002). These immune responses are suppressed through the modulation of effector T cells and NK cells functions at the maternal-foetal interface by the induction of regulatory T cells (Tregs)-specific for foetal antigens (Tilburgs et al., 2008, Tilburgs et al., 2009, Samstein et al., 2012).

Numerous other mechanisms on how the foetus is protected from maternal immunity have been proposed. These include catabolism of tryptophan, an amino acid involved in T lymphocytes activation by the enzyme indole-amine 2,3-dioxygenase (IDO) secreted by trophoblasts and macrophages (Munn et al., 1998); trophoblast cell induced tolerance through expression of certain MHC molecules (King et al., 2000, Ishitani et al., 2003); and various mechanisms induced by regulatory T lymphocytes (Tregs) have been proposed (Aluvihare et al., 2004, Sasaki et al., 2005, Somerset et al., 2004). The EVTs that infiltrate the maternal decidua during implantation lack the expression of classical MHC class I molecules, HLA-A and HLA-B like most cells, but express HLA-C and the non-classical MHC class I molecules HLA-E and HLA-G (Apps et al., 2009). HLA-G is specifically expressed by EVT and has low levels of polymorphism suggesting that it may play a role in the induction of immune tolerance at the maternal-foetal interface (Kovats et al., 1990).

Several studies have reported the critical role of decidual macrophages in the induction and maintenance of immune tolerance. Svensson-Arvelund and colleagues reported that placental trophoblast cells secrete IL-10 and M-CSF which polarizes the decidual macrophages (maternal-derived) towards the homeostatic, immune regulatory M2 phenotype; so leading to an expansion of regulatory T cells (Tregs) and inhibition of T helper cells activation. Collectively, these cells contribute to foetal tolerance and maintenance of a homeostatic microenvironment suitable for foetal development (Svensson-Arvelund et al., 2014). Figure 1.9. is an illustration of the mechanism that decidual macrophages interact with decidual NK cells and T cells to bring about maternal-foetal immune tolerance.

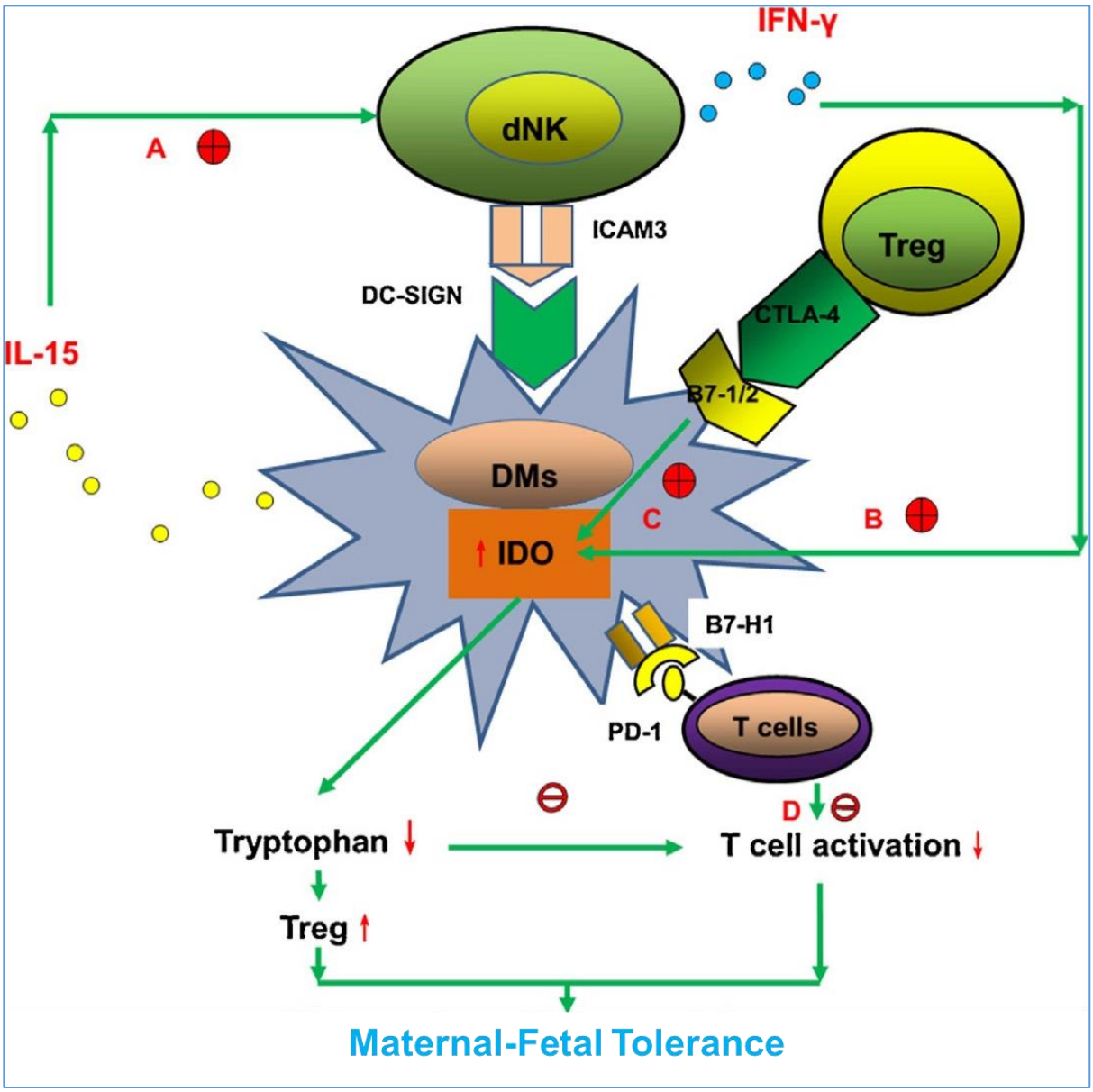

Figure 1.9: The interaction of decidual macrophage (DMs) with decidual NK cells and T cells to bring about maternal-foetal tolerance (Tang et al., 2015). DMs stimulate the differentiation of endometrial NK cells into activated decidual NK (dNK) cells by secreting IL-15. DMs interact with dNK cells via CD209 (DC-SIGN), leading to the secretion of IFN-γ that then stimulates the up-regulation of IDO in DMs. IDO catabolizes tryptophan leading to impaired T cell activation and expansion of Tregs. Tregs interact with DMs via CTLA-4/B7-1/2, further upregulating the production of IDO. DMs suppress excessive T cell activation via B7/H1/PD-1 interactions.

1.8. The role of macrophages in pregnancy

1.8.1. Decidual Macrophages

Following embryo implantation, the decidua divides into two distinct regions: the decidua basalis, which is the portion of the uterus attached to the placenta, and decidua parietalis, which is the layer closest to the endometrium, found between the amnion and the chorion (Brown et al., 2014). Macrophages derived from both the mother and foetus play a very important role in all stages of pregnancy. Labour is an inflammatory process (Bollopragada et al., 2009) and presence of inflammatory macrophages has been shown in the decidua, cervix and foetal membranes during labour (Osman et al., 2003). Macrophages are among the primary innate immune cells that play a role in the processes of term and preterm labour (Houser, 2012). Decidual macrophages are the most abundant antigen presenting cells (APCs) throughout gestation (Bartmann et al., 2014), and they constitute more than 15% of $CD45^+$ cells or at least 10% of all decidual cells (Vince et al., 1990, Singh et al., 2005). They are critical in establishing the balance between tolerance and pro-inflammatory responses. According to Heikkinen *et al* they are phenotypically characterized by co-expression of CD14 and CD68 (Heikkinen et al., 2003). They have been shown to have characteristics associated with homeostatic M2 macrophages, including expression of the homeostatic scavenger receptor CD163 and the pattern recognition receptors CD206 and CD209 while preferentially secreting cytokines and chemokines such as IL-10, CCL-2 and CCL-18 (Svensson et al., 2011, Houser et al., 2011). Human decidual macrophages were recently characterized as $CD163^+CD206^+CD209^+IL\text{-}10^+CCL18^+$ cells (Svensson-Arvelund et al., 2014, Sayama et al., 2013).

1.8.2. Hofbauer Cells

Hofbauer Cells (HCs) are foetal-derived macrophages found within the chorionic villi of the placenta (Goldstein et al., 1988, Reyes et al., 2017, Kim et al., 2008). These cells express all three subtypes of the IgG Fc receptors (FcγR) found on human leukocytes and classical monocyte/macrophage markers, such as CD68 (Vinnars et al., 2010, Bright et al., 1994). They were identified more than 100 years ago as large

(10-30µm), pleomorphic cells, highly vacuolated with granular cytoplasm that is associated with phagocytic activity (Castellucci et al., 1980, Castellucci et al., 2000, Enders and King, 1970). Histological analyses of normal placental beds showed a large number of macrophages, localized and within the vicinity of apoptotic cells (De and Wood, 1990).

Although their ontogeny of HCs is poorly characterized, they are found within the foetal villi of the placenta from the first trimester of pregnancy until birth (Wetzka et al., 1997). During the first trimester of pregnancy, they are believed to originate from mesenchymal progenitor cells (Abumaree et al., 2013) while in the second and third trimester, they differentiate from circulating monocytes of the foetus (Selkov et al., 2013, Moskalewski et al., 1975). Their role in placental physiology is also not well understood; however, there is some evidence that they may play a role in transport within the villous stroma and in immunological reactions through their Fc receptors (Jensen and Matre, 1995, Saji et al., 1994). Histological analyses showed their location to be in the stroma of the placental villus, close to foetal vessels and trophoblasts making them likely candidates for involvement in regulatory functions during placental development and homeostasis (Katabuchi, 2014).

HCs have a regulatory phenotype consistent with that of M2 anti-inflammatory macrophages. Several studies have shown that HCs are stimulated by glucocorticoids (Tang et al., 2013) and IL-10 (Svensson et al., 2011) to express CD163, CD206 and CD209 (Svensson et al., 2011) while secreting IL-10 and TGF-β (Johnson and Chakraborty, 2012). HCs have also been reported to constitute a mixture of M2a, M2b and M2c macrophages that differ in surface expression of certain molecules, in cytokine secretion and functions (Loegl et al., 2016). Functionally, HCs have been shown to play a critical role in maternal immunological tolerance against the foetus (Svensson-Arvelund et al., 2015). This further reinforce the regulatory rather than the inflammatory role of HCs. Hofbauer cell dysfunction is associated with numerous pregnancy complications such as chorioamnionitis, miscarriage, and preterm delivery (Tang et al., 2011), highlighting that these cells play an important role in maintaining a healthy pregnancy. Numerous publications reporting on the polarity and function of maternal decidual macrophages have been thoroughly reviewed by Brown *et al*. and

others (Brown et al., 2014). However, there is very little information on the effect of complications of pregnancy and maternal infections on foetal HCs polarity, function and gene expression profile.

HCs have been shown to limit HIV-1 replication by the induction of immuno-regulatory cytokines (Johnson and Chakraborty, 2012) and they also possess intrinsic adaptations which facilitate the sequestration of HIV-1 that may serve as a protective viral reservoir to allow for antiretroviral drug entry *in utero* and potentially the neutralization of the virus (Johnson et al., 2015). Therefore, HCs are pivotal mediators in HIV-1 transmission *in utero*. Although *in vitro* permissiveness of HCs to HIV-1 infection has been reported (Al-Husaini, 2009), there is a paucity of data on the effect of HIV-1 and/or antiretroviral drugs exposure on the quantity, phenotype and function of both decidual macrophages and HCs in placentas from pregnancies complicated by maternal HIV-1 infection.

1.9. HIV-1 infection and Pregnancy

1.9.1. Impact of HIV infection on birth outcomes

During pregnancy, the mechanism of trans-placental transmission of HIV-1 from the mother to her foetus is still not yet clear. However, in a few cases, vertical transmission of HIV was reported to be due to trans-placental spread by an unknown mechanism (Newell, 1998). Apart from transmission of the virus from the mother to her foetus, a number of studies have reported a significant association between maternal HIV-1 infection and adverse birth outcomes (Newell et al., 1994, Coley et al., 2001, Miotti et al., 1990, Braddick et al., 1990, Johnstone, 1992). Small-for-gestational age (SGA) infants, stillbirth, preterm birth and intrauterine growth retardation (UIGR) which leads to perinatal and neonatal mortality and morbidity have all been reported in pregnancies complicated by HIV-1 infection regardless of transmission (Dos Reis et al., 2015, Ndirangu et al., 2012, Chetty et al., 2018, Jao et al., 2012).

Numerous studies have reported the transfer of maternal immune cells and humoral immunity across the placenta into the foetal circulation (Mold et al., 2008, Nijagal et

al., 2011, Zhou et al., 2000). During maternal HIV-1 infection, antibody- and cell-associated HIV-1 virions, cell-free virions and maternal broadly neutralizing antibodies cross the placental barrier to interact with Hofbauer cells (HCs) prior to entering the foetal circulation (Palmeira et al., 2012). The phenotype of HCs favors productive infection with HIV-1 (Simister, 1998). Despite this, vertical transmission of HIV-1 is very low and that is mainly attributed to the widespread use of ART. Combination antiretroviral therapy has significantly improved mortality and slowed disease progression amongst HIV infected individuals. It has also been associated with life-threatening, non-AIDS health complications such as cardiovascular diseases, malignancies, and neurological diseases (Hasse et al., 2011, Guaraldi et al., 2011) and adverse birth outcomes, as discussed above.

1.9.2. Classes of antiretroviral drugs

There are 6 classes of antiretroviral drugs that are currently prescribed to HIV-1 infected individuals (Meintjes et al., 2017). These drugs target different stages of HIV replication cycle. They are as follows:

1) Nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs).
2) Non-nucleoside reverse transcriptase inhibitors (NNRTIs).
3) Protease inhibitors (PIs).
4) Integrase strand transfer inhibitors (INSTIs).
5) Fusion inhibitors (FIs).
6) Chemokine receptor antagonists (CCR5 Antagonist).

In South Africa, an ART regimen usually consists of two NRTIs [tenofovir (TDF) and emtricitabine (FTC)] and a third drug, either from the NNRTIs [efavirenz (EFV)] or the PIs class of antiretroviral (ARV) drugs. It is not yet known whether metabolites of these drugs are able to cross the placental barrier, also the long term effects of these drugs on foetal and neonatal immunity are poorly understood. The ART regimens currently available in southern Africa are made-up of the ARV drugs summarized in Table 1 (Meintjes et al., 2017).

Table 1.1: Characteristics of ARV drugs currently prescribed in southern Africa.

Class of drug	Generic name	Recommended dosage
NRTI	Tenofovir (TDF)	300 mg, daily
NRTI	Lamivudine (3TC)	300 mg, daily
NRTI	Emtricitabine	200 mg, daily
NRTI	Abacavir (ABC)	300 mg, 12-hourly
NRTI	Zidovudine (AZT)	300 mg, 12-hourly
NRTI	Stavudine (d4T)	300 mg, 12-hourly
NRTI	Didanosine (ddI)	400 mg, daily
NNRTI	Efavirenz (EFV)	600 mg at night
NNRTI	Nevirapine (NVP)	200 mg, 12-hourly
NNRTI	Rilpivirine (RPV)	25 mg, 12-hourly
NNRTI	Etravirine (ETR)	200 mg, 12-hourly
PI	Atazanavir (ATV)	400 mg, daily
Boosted PI	Lopinavir/ritonavir (LPV/r)	400/100 mg, 12-hourly
PI	Darunavir	600 mg, 12-hourly
PI	Saquinavir (SQV)	1000mg, 12-hourly
InSTI	Raltegravir	400 mg, 12-hourly
InSTI	Dolutegravir (DTG)	50 mg, daily
CCR5 blocker	Miraviroc (MVC)	150 mg, daily

1.9.3. Antiretroviral therapy and birth outcomes

Among pregnant women, studies have reported an increased rate of adverse birth outcomes with maternal highly active antiretroviral therapy (HAART) (Thorne et al., 2004, Papp et al., 2014, Mofenson, 2016). There is a high prevalence of preeclampsia and foetal death amongst HIV-1 infected women on combination antiretroviral therapy (cART), and ART has been shown to aggravate pre-eclampsia (Tooke et al., 2016). A South African based, cohort study by Chetty and colleagues showed no association between preconception and post-conception use of non-nucleoside reverse

transcriptase-based regimens tenofovir disoproxil fumarate (TDF)-lamivudine (3TC)/emtricitabine (FTC)-efavirenz (EFV) on preterm birth and small-for-gestational (SGA) infant births compared to other regimens (Chetty et al., 2018). However, another study comparing birth outcomes among women initiating dolutegravir-based ART with women initiating efavirenz-based ART, reported that initiation of dolutegravir-based therapy during pregnancy may be associated with a major congenital abnormality called skeletal dysplasia, (Zash et al., 2018). The aetiology of adverse birth outcomes among HIV-infected women on ART and how different ART regimens interact with cells of the maternal-foetal interface is yet to be fully understood.

In this PhD, It is hypothesized that HIV-1 and/ ART exposure, given either before or during pregnancy, dysregulates the balance of M1 and M2 macrophages at the maternal-foetal interface leading to adverse birth outcomes. The following questions were asked:

1). How does the duration of ART exposure affect the phenotype and polarisation of decidual macrophages and Hofbauer cells in HIV-1 infected, South African women?

2). What markers can be used to characterize and differentiate between decidual macrophages and Hofbauer cells irrespective of their tissue localization?

3). What are the decidual macrophage and Hofbauer cell-specific biomarkers of HIV-1 infection?

2. Study Aim & Objectives

Study Aim: To investigate the impact of HIV-1 and antiretroviral drug exposure on the phenotype and function of maternal uterine-derived, decidual macrophages and foetal placental-derived macrophages, the Hofbauer cells.

Objective 1: To phenotypically characterize decidual macrophages and Hofbauer cells from placentas of HIV-1 infected women who initiated antiretroviral therapy during pregnancy (*Initiating ART*) and before pregnancy (*Stable ART*) using immunohistochemistry and immunofluorescence staining.

Objective 2: To identify novel cell markers for decidual macrophages and Hofbauer cells based on the Human Protein Atlas database.

Objective 3: To determine placental macrophage-specific biomarkers of maternal HIV-infection and activation status based on microarray datasets generated from monocyte-derived macrophages (MDM) that were stimulated with various stimuli and infected with CCR5-using (R5) HIV-1.

3. Materials and Methods

3.1. Human Placentas

Placentas (*n=30*) used in this study came from the Prematurity Immunology in HIV-infected Mothers and their infants Study (PIMS) cohort based in Cape Town, South Africa. The PIMS cohort consisted of HIV-1 infected pregnant women who were already on combination antiretroviral therapy (cART) before pregnancy and HIV-1 infected women who initiated cART at week 20 during gestation. Placentas used in this study were collected under a protocol approved by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee (HREC REF: 739/2014). Placentas were collected at birth from consenting mothers by midwives and transported to the University of Cape Town, Division of Immunology Laboratory in a sealed container submerged in 500ml of RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal-calf serum (FCS) and 1% penicillin/streptomycin antibiotic (Life Technologies, Grand Island, NY, USA) (complete medium). This study subset received ethical approval (HREC REF: 665/2015).

3.2. Isolation of decidual macrophages and Hofbauer cells

Decidual leukocytes and Hofbauer cells were isolated from the decidual membrane and placental foetal-chorionic villi respectively. The Decidua Parietalis (DP) was obtained by firstly removing the amnion before scraping the DP off from chorion while Decidua Basalis (DB) was obtained from the maternal side of the basal plate. The Villous Tissue (VT) was obtained from the basal plate of the placenta. The dissection of membranes of the maternal-foetal interface from a term human placenta is illustrated in Figure 3.1. After dissection, the DP, DB and VT were rinsed in phosphate buffered saline (1X PBS) (Sigma-Aldrich, St. Louis, MO) and minced using scissors. Finely minced tissues were then incubated in 1% Collagenase IV (1 mg/ml) (Sigma-

Aldrich, St. Louis, MO, USA) and 0.1% DNase I (0.1 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) in a shaking water bath at 37^0C for 75 minutes. After digestion, tissues were washed in complete medium and filtered through a 100μm, 70μm and 40μm pore cell strainers (Falcon; Corning Life Sciences, Durham, NC, USA) successively into a 50ml falcon tube. The resulting cell suspension was washed and re-suspended in 10ml of complete medium and then added into 10ml of 50% Percoll solution (GE Healthcare Biosciences, Uppsala, Sweden). The 20ml of the sample in Percoll solution was carefully layered on top of the 45% Percoll in PBS phase that was already layered on top of the 70% Percoll in RPMI medium. The resulting Percoll gradient was then topped up with 5ml of 1X PBS before gradient centrifugation (2000rpm for 25 minutes). Decidual macrophages and Hofbauer cells were collected from the interface higher than the lymphocytes layer of the density gradient (Figure 3.2) and washed twice in 1X PBS supplemented with 1% FCS. The following protocol was optimized for the isolation of Hofbauer cells and decidual macrophages from the maternal-foetal interface.

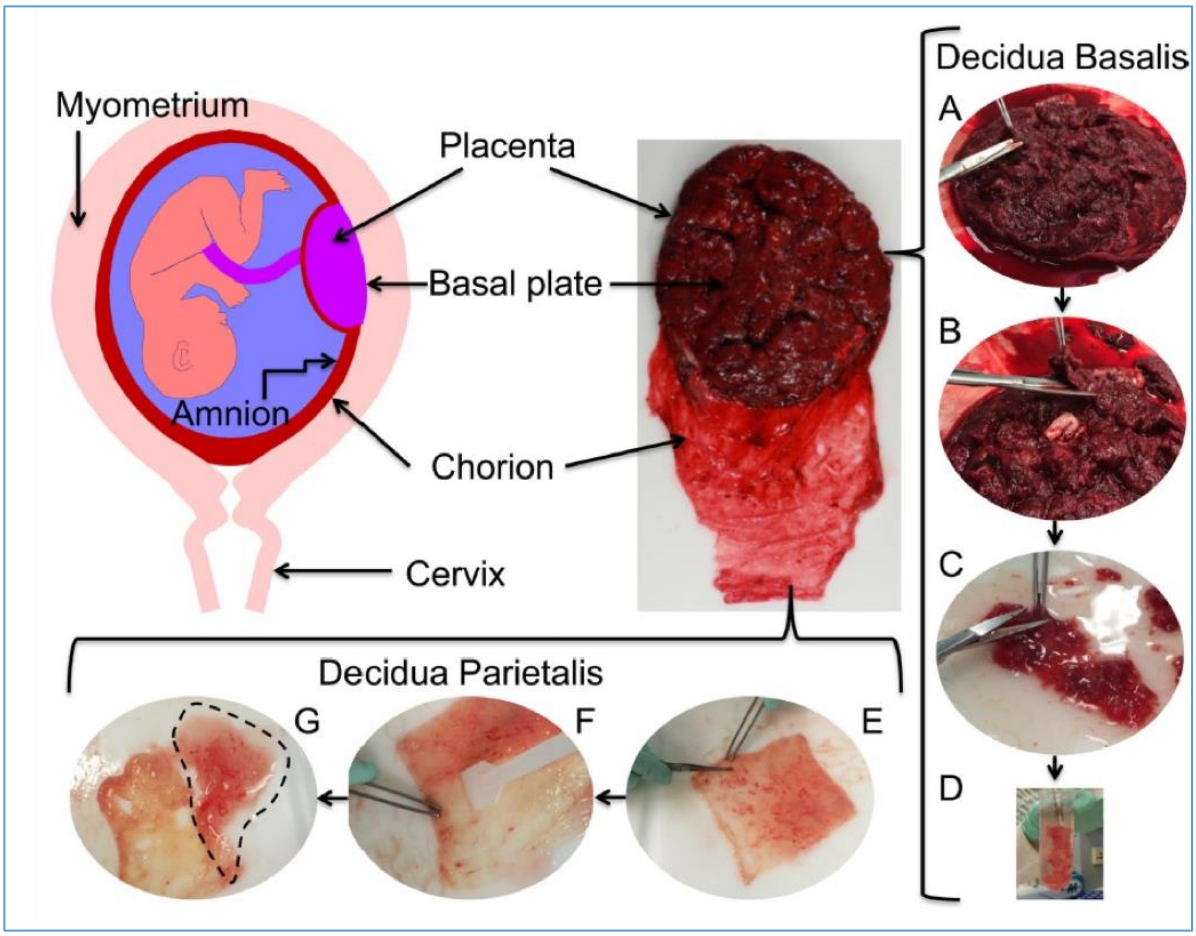

Figure 3.1: Dissection of placental membranes and isolation of leukocytes from the maternal-foetal interface of term human placenta (Xu et al., 2015). (A) Dissection of basal plate from the placenta; (B) Separation of the basal plate from the placental villi; (C) Trimming of the placental villi from the decidua basalis; (D) Rinsing of the decidual basalis in 1x PBS; (E) A piece of the chorionic membrane is dissected; (F) Decidua parietalis is gently scrapped-off to remove blood clots; and (G) Decidua parietalis (dotted line) is separated from the chorionic membrane.

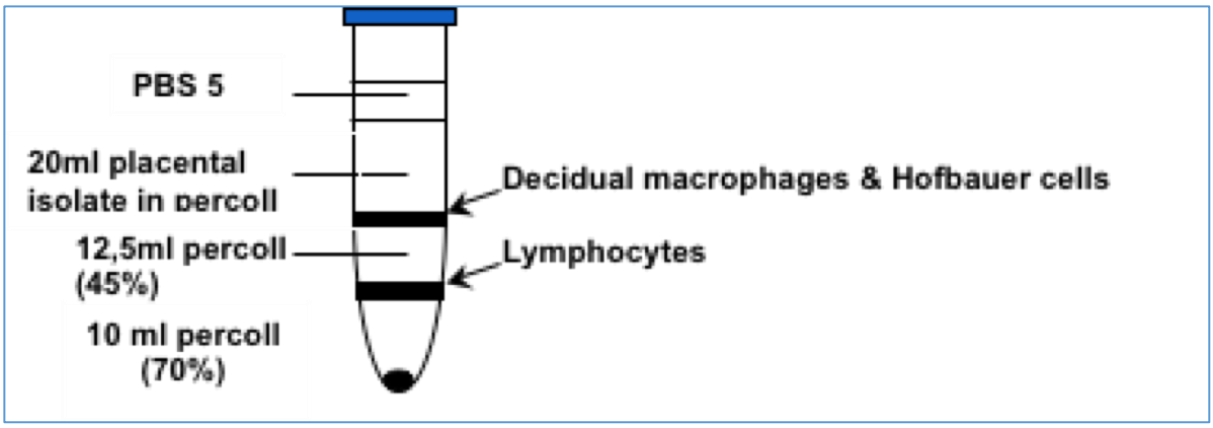

Figure 3.2: Percoll gradient layers after centrifugation for the isolation of decidual macrophages and Hofbauer cells.

3.3. Fixation of decidual macrophages and Hofbauer cells

Placenta-isolated decidual macrophages and Hofbauer Cells were stained with a Live/Dead cell marker, Zombie NIR Fixable Viability Kit (Biolegend, San Diego, CA) prior to fixing. The kit is composed of lyophilized Zombie NIR dye and anhydrous DMSO. A standard cell staining protocol was followed whereby cells were re-suspended at 1.0 x 10^6 cells per 100μl of 1x PBS before adding 1μl of zombie NIR dye and incubating at room temperature for 20 minutes. Cells were then washed twice in RPMI medium (2100rpm for 3 minutes). After removing supernatant, cells were then incubated in 10ml of 1x BD FACS™ lysing solution (BD Biosciences, San Jose, CA) which lyses red blood cells while fixing the remaining blood cells and incubated at room temperature for 10 minutes. Cells were then washed in 1x PBS containing 1% FCS and centrifuged 2100rpm for 3 minutes. After removing the supernatant, cells were frozen at 1 x 10^6 cells/ml in 10% DMSO and FCS. Cells were then stored in the -80^{o}C freezer.

3.4. Immunohistochemistry and Immunofluorescence

Immunohistochemistry (IHC) is an extensively used technique in pathology. It combines the fields of anatomy, physiology, immunology and biochemistry. It is useful for visualizing the distribution and localization of specific-antigens or cellular components on tissues sections (Ramos-Vara, 2017). Immunofluorescence (IF)

staining is an extension of IHC which allows for localization of proteins in cells by immunofluorescence (Donaldson, 2015). In this study, IHC and IF were used to phenotypically characterize decidual macrophages and Hofbauer cells from placentas from HIV-1 infected mothers on cART from the two groups. Human placental DP, DB and VT were dissected from placentas and fixed in 10% formalin (10% Formaldehyde and 10g of anhydrous sodium chloride in distilled water) (KIMIX chemicals and lab Suppliers, Cape Town, South Africa) for at least 24 hours. Tissues were then trimmed and placed onto labelled tissue processing/embedding cassettes (Sigma-Aldrich, St. Louis, MO, USA) for processing on the Leica TP1020 processor (Leica Biosystems, Wetzler, Germany). After processing, placental tissues were embedded in paraffin on the Leica EG1140H (Leica Biosystems, Wetzler, Germany). Formalin-fixed paraffin-embedded DP, DB and VT blocks were sectioned into 5μm thick sections onto labeled-Leica surgipath X-tra adhesive slides (Leica Biosystems, Wetzler, Germany) using the Leica Rotary Microtome (Leica Biosystems, Wetzler, Germany).

For subsequent immunohistochemistry experiments, wax was removed from tissue slides using xylene and 100% ethanol. Endogenous peroxidase activity was blocked with methanol containing 0.3% H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes. Tissue sections were then rehydrated with 70% and 50% ethanol before antigen retrieval. Two different antigen retrieval methods were performed depending on the primary antibody data sheet. Tissue sections were pre-treated by microwaving the sections for 10 minutes in boiling citrate buffer (10mmlo/l, pH 6) (Sigma-Aldrich, St. Louis, MO, USA) or in boiling Tris/EDTA buffer (1mmol/l, pH 9) (Sigma-Aldrich, St. Louis, MO, USA). After two washes in PBS, tissue sections were incubated with primary antibodies at primary antibody-specific concentrations in PBS with 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) at 4^0C overnight. Next day, tissue sections were washed three times in PBS and incubated for 30 minutes with the ultra-view universal DAB detection kit (DAB Substrate) (Agilent technologies, Santa Clara, CA, USA). Tissue sections were then counterstained with Mayer's hematoxylin (Agilent Technologies, Santa Clara, CA, USA) for 4 minutes, and then washed for 5 minutes with distilled water. The slide was then covered with a coverslip using Entellan mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Details of the primary antibodies used in IHC experiments are listed on Table 3.1.

Table 3.1: Details of primary antibodies used in IHC experiments.

Primary Antibody	Source	Species	Cat. Number	Isotype	Clone	Antigen retrieval
CD68	Abcam	Mouse	ab49777	IgG2a, κ	514H12	Tris/EDTA
CD163	Abcam	Rabbit	ab189915	IgG	EPR146 43-36	Citrate
CD206	Abcam	Mouse	ab117644	IgG1, κ	5C11	Citrate
CD209	Abcam	Rabbit	ab5715	IgG	9E9A8	Tris/EDTA

For Immunofluorescence experiments, wax was removed from tissue slides using xylene and 100% ethanol. Antigen retrieval was done using 10mM Citrate buffer (pH6) (Sigma-Aldrich, St. Louis, MO, USA) in a pre-heated pressure cooker for 2 minutes. After antigen retrieval, tissue slides were blocked in 5% normal goat serum (Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes in a humid chamber at room temperature. The blocking solution was then rinsed with PBS-Tween 20 (PBST) (Sigma-Aldrich, St. Louis, MO, USA). Tissue sections were incubated with the primary CD163 antibody at 1:200 dilutions in PBST and incubated in the dark at room temperature for 90 minutes. After incubation with the primary antibody, tissue slides were washed three times with PBST. Slides were then incubated with the Cy3-labelled Donkey-anti-rabbit secondary antibody in the dark at room temperature for 30 minutes. After washing with PBST, tissues sections were permeabilised by staining with 0.1% Triton-X100 (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes. Slides were washed three times with PBST and then stained with the second primary antibody, Alexa Fluor 488-conjugated IRF-5 at 1:100 dilutions. The slides were incubated at 4^{o}C overnight in a moist chamber. Next day, slides were washed three times with PBST, stained with DAPI and incubated at room temperature for 20 minutes. Slides were washed twice with PBST, tissue autofluorescence was quenched with a solution of 0.1% Sudan Black B (Sigma-Aldrich, St. Louis, MO, USA) in 70% ethanol, and incubated in the dark for 10 minutes. After washing with PBST, the slides were mounted using fluorescent mounting medium (Sigma-Aldrich, St. Louis, MO, USA), sealed, and stored at 4^{o}C until image acquisition. As negative controls, corresponding slides were concurrently stained with universal isotype control, Rabbit IgG for both markers.

Table 3.2: Details of primary antibodies used for IF experiments.

Primary Antibody	Source	Species	Cat. Number	Isotype	Clone	Fluorophore
IRF-5	Abcam	Rabbit	ab193245	IgG	EPR6094	Alexa Fluor 488
CD163	Abcam	Rabbit	ab189915	IgG	EPR14643-36	N/A

3.4.1. IHC image acquisition, quantification and statistics

Images were acquired using the NIS-elements software to capture 5 random images on the Nikon Eclipse 90i microscope (Nikon Inc. Minato, Tokyo, Japan) at x20 magnification with oil immersion. Images were analyzed using a validated ImageJ (National Institute of Health, Bethesda, Maryland) algorithm to quantify the percentage of DAB staining present in each image as previously described by Franklin and colleagues (Franklin et al., 2014). Briefly, the algorithm divides the acquired image in a red, green, and blue colour space into separate colour channels by a colour deconvolution method. For each antibody stain, DAB threshold levels were manually determined and kept constant for each antibody dataset. The percentage of DAB staining was quantified as the percentage of the total field area stained positive for DAB. Statistical analyses and graphs were done using GraphPad Prisms (GraphPad Software, La Jolla, CA, USA). Due to the small number of participants included in the study, medians and interquartile ranges were used, and tested for signficance using non-parametric testing.

3.4.2. IF image acquisition

Immunofluorescence images were acquired on a Zeiss LSM880 Airy Scan (Zeiss, Oberkochen, Germany) confocal microscope using a 40x Plan-Apochromatic oil immersion objective (numerical aperture, 0.95). The fluorophores for DAPI, Alexa Fluor 488 and Cy were excited using the 405, 488 and 633-nm laser lines, respectively. All channels were acquired sequentially to avoid bleed-through and the two-dimensional image was reconstructed using the Zeiss 2.1 software (Zeiss, Oberkochen, Germany). Composite images were created using ImageJ (National Institute of Health).

4. Expression of classical M1 and M2 macrophage markers on decidual macrophages and Hofbauer cells

4.1. Introduction

It is now standard of care for HIV-1 infected women to be on ART once their HIV status is known. However, some women are only diagnosed with HIV infection during pregnancy and the implications of the timing of ART initiation on adverse birth outcomes are not yet fully understood. Uthman and colleagues reported that women who initiated ART before conception were significantly more likely to deliver preterm and low-birthweight infants than those who initiated ART after conception (Uthman et al., 2017). Another study reported that a tenofovir disoprixil fumarate, emtricitabine, and efavirenz (TDF-FTC-EFV) regimen was associated with lower risk for adverse birth outcomes than other commonly prescribed ART regimens in women who initiated ART before conception (Zash et al., 2017). The impact of HIV-1 and/ ART exposure on the phenotype and function of immune cells of the maternal-foetal interface is not yet clear. In this chapter, placental samples were investigated for presence of macrophages in relation to the timing of ART initiation: before or during gestation. It is hypothesized that HIV and/ ART exposure dysregulates the balance of immunoregulatory and pro-inflammatory macrophages at the maternal-foetal interface.

4.2. Results

To investigate the effect of the duration of ART exposure on birth outcome and placenta pathology, we compared birth outcomes and placenta pathologies of HIV-infected women categorized into two groups, 1). HIV-1 infected pregnant women who initiated ART at during of pregnancy (at +/- 20 weeks of gestation) (*Initiating ART n=16*); and 2). HIV-1 infected women who initiated ART before pregnancy (*Stable on*

ART n=14). Table 4.1 shows a summary of the characteristics of women, their infants and placentas from the two study groups. At birth, the median age of women who were stable on ART before pregnancy was significantly higher than that of women who initiated ART at around 20 weeks of gestation (p=0.01; Mann-Whitney; Fig. 4.1A). Overall, there were no significant differences in the birth weight of infants born to women who initiated ART before pregnancy compared to those born to women who initiated ART during pregnant. There was no statistical significant difference in gestational age between these two groups, although, there were more women in the *initiating* group with gestational age of ≤ 40 weeks compared to women in the *stable on ART* before pregnancy group (p>0.05; Mann-Whitney; Fig. 4.1C). There were also no statistical significant differences in placental basal plate weight, umbilical cord length and umbilical cord insertion from the nearest margin of the basal plate between these two study groups (p>0.05; Mann-Whitney; Fig. 4.1D, E & F). These data suggest that the timing of ART initiation has no effect on these in HIV-1 infected mothers.

Table 4.1: Characteristics of placentas from women who were stable on ART before pregnancy (*Stable on ART*) and women who initiated ART at +/- 20 weeks of gestation (*Initiating ART*).

	INITIATING ART	STABLE ON ART
Sample Size (n)	16	14
Mean Age at Delivery	28.6	31.8
Mean Gestational Age (weeks)	38.3	38.7
Mean Birth weight (g)	3071	3088
Mean Umbilical Cord length (mm)	369	311
Mean Cord Insertion from the nearest margin (mm)	48.5	45.2
Basal Plate weight (g)	408.1	398.1
Chorioamnionitis*	3/11 (27%)	1/11 (9%)

* Presence or absence of Chorioamnionitis was reported in 11 pathology reports in both study groups

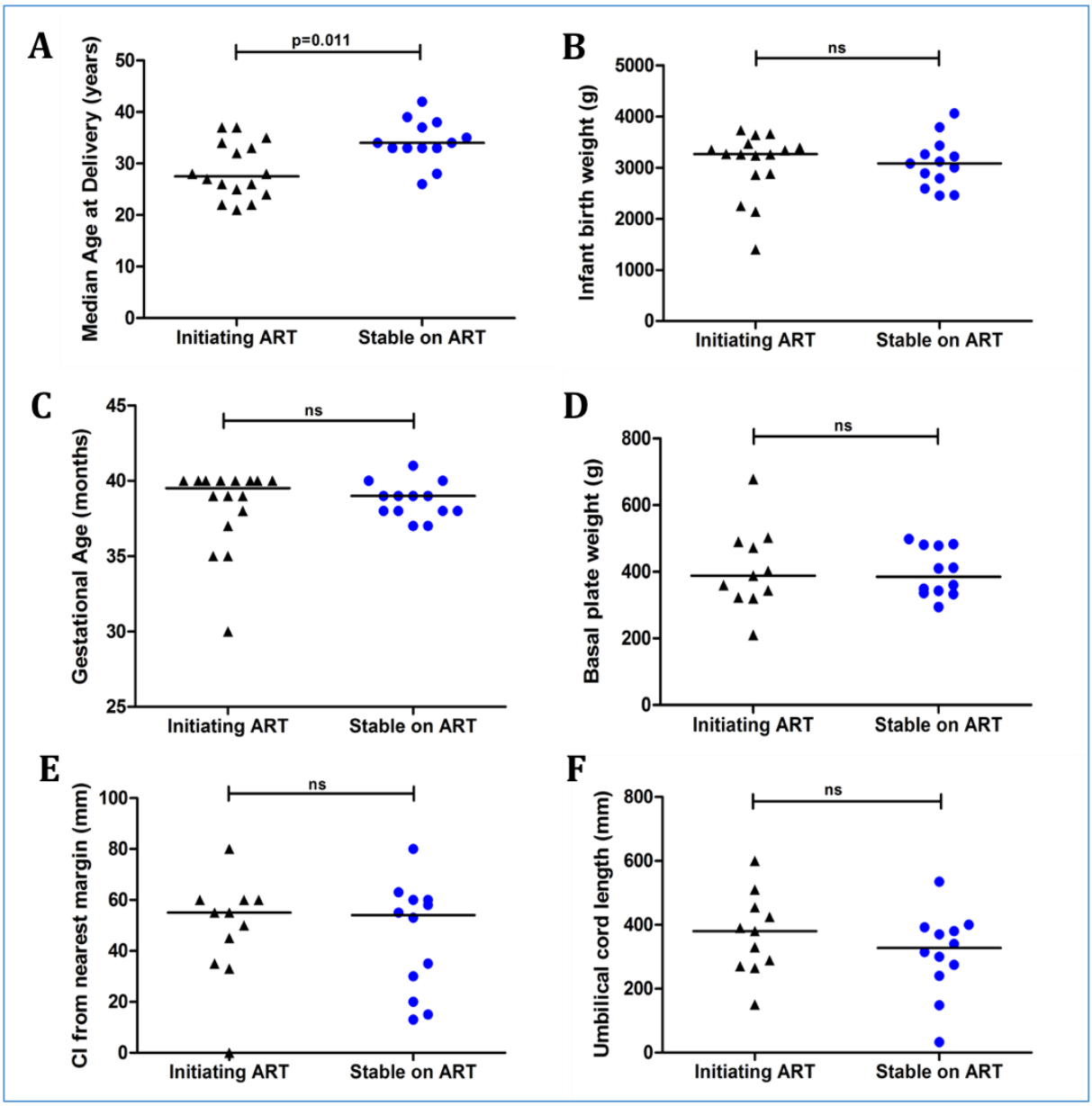

Figure 4.1: Differences in the characteristics of women, infants and their placentas between the two study groups. A comparison of (A) the median age of mothers at delivery (B) Infant's birth weight (C) gestational age in months (D) Placental basal plate weight (E) umbilical cord insertion from the nearest margin of the placenta basal plate, and (F) the umbilical cord length between the two study groups.

To investigate the effect of the duration of ART exposure on the phenotype of decidual macrophages and Hofbauer cells, placental tissue sections, DP, DB and VT were stained with Meyer's hematoxylin & Eosin (H&E), pan-macrophage marker (CD68), Scavenger receptor (CD163), Mannose receptor (CD206) and DC-SIGN (CD209). CD68 is a 110kD intracellular glycoprotein that is associated with cytoplasmic granules. Its expression localizes in the cytoplasm of macrophages and other mononuclear phagocytes (Gottfried et al., 2008). CD163 is a scavenger receptor for haptoglobin-hemoglobin complex that is highly expressed on the cell membrane of

monocytes and macrophages (Buechler et al., 2000). CD206 is a mannose receptor that is highly expressed by macrophages, dendritic cells and Langerhans cells (Martinez-Pomares, 2012). CD209 is the dendritic cell-specific ICAM-grabbing non-integrin that is highly expressed by macrophages and dendritic cells (Soilleux, 2003). In this study, M2 macrophages were regarded as CD68 positive cells that also express CD163, CD206 and CD209 while M1 macrophages are those that only express CD68. The staining controls for these markers are shown in Figure 4.2A-D.

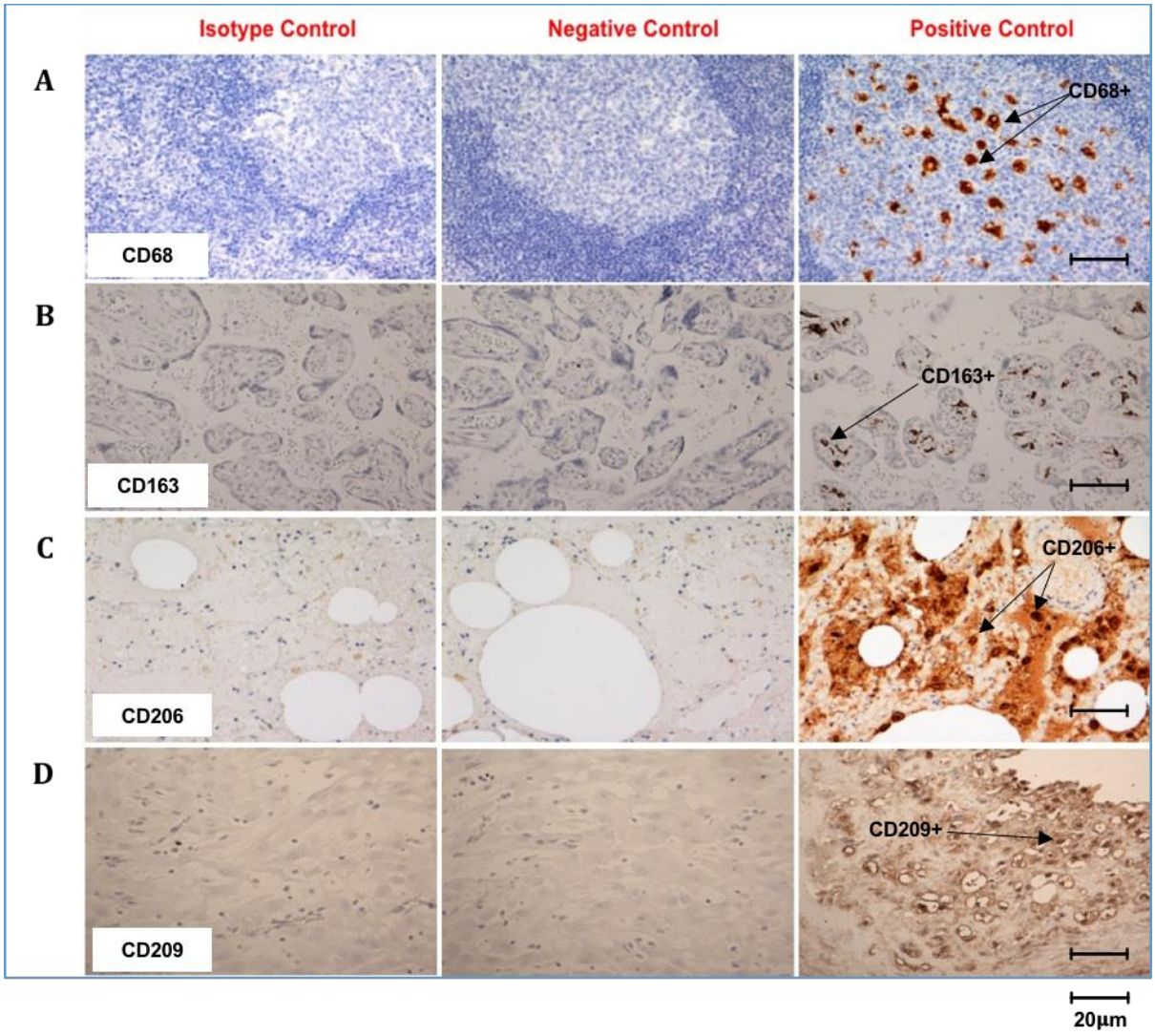

Figure 4.2: Isotype control, negative control and positive control for each marker. (A) CD68 control staining on human tonsil sections (B) CD163 control staining on human placental villi tissue (C) CD206 control staining on human lung tissue, and (D) CD209 control staining on human placental decidual basalis.

There were conspicuous differences in the staining pattern and specificities of the markers on control tissues used (Fig. 4.2), where these differences may be due to differences in antigen retrieval method, tissues and antigenicity of each marker. Figure

4.4 and Figure 4.5 are representative IHC images of placental membranes from a participant *initiating ART* during pregnancy and *stable on ART* before pregnancy respectively. H&E staining of the placental membranes making up the maternal-foetal interface revealed differences in tissue histology between the membranes. The villous tissue of participants *initiating ART* during pregnancy had larger and conspicuous intervilli spaces compared to the villous tissue of participants *stable on ART* before pregnancy (Figure 4.3 A& B). We also observed that most villi tissue of participants that were stable on ART before pregnancy had more maternal blood contamination compared to that of participants that initiated ART during pregnancy (Figure 4.3B). The significance of larger or smaller intervilli spaces is not yet known. Histologically,

there was no visible differences between the maternal decidual membranes, DP and DB within each group and between the two ART groups.

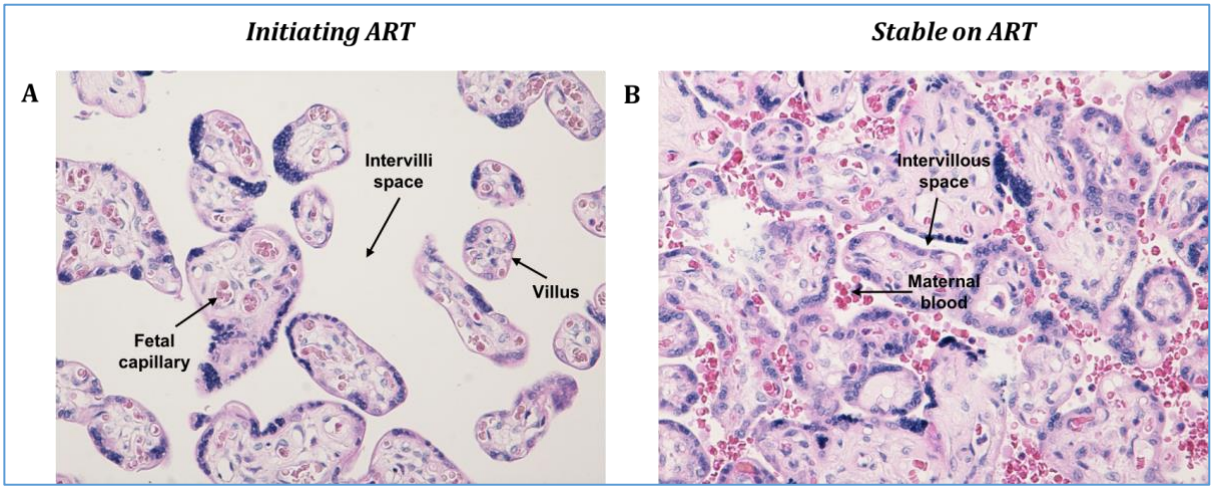

Figure 4.3: Histological differences in the villous tissue between a participant who initiated ART during pregnancy (A) and a participant who was stable on ART before pregnancy (B).

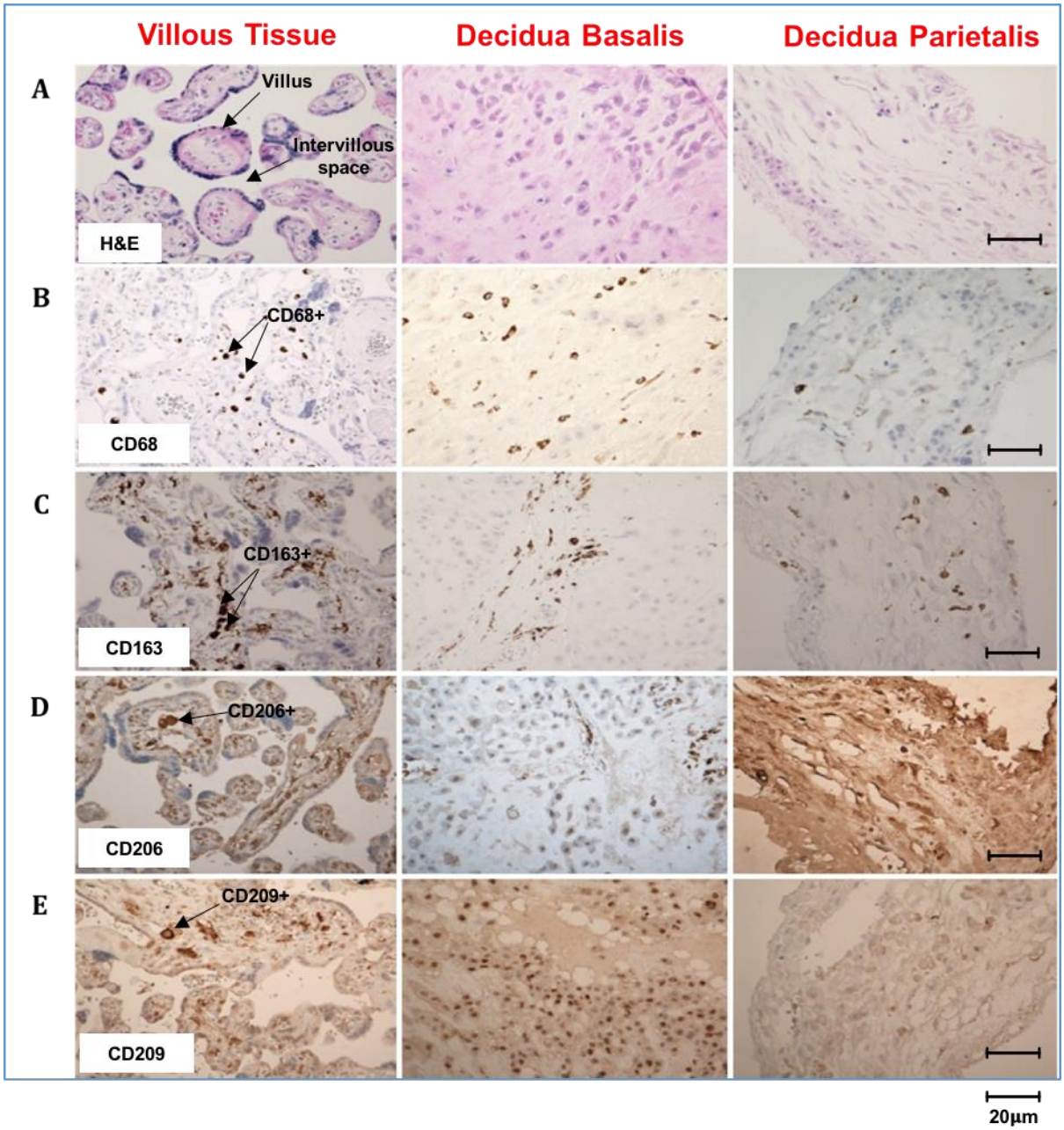

Figure 4.4: Representative IHC images of placental membranes from a participant *initiating ART* during pregnancy. (A) Hematoxylin and Eosin (H&E) staining (B) CD68, (C) CD163, (D) CD206, and (E) CD209 staining of the villous tissue, decidua basalis

and decidua parietalis of a placenta from a mother who initiated ART during pregnancy.

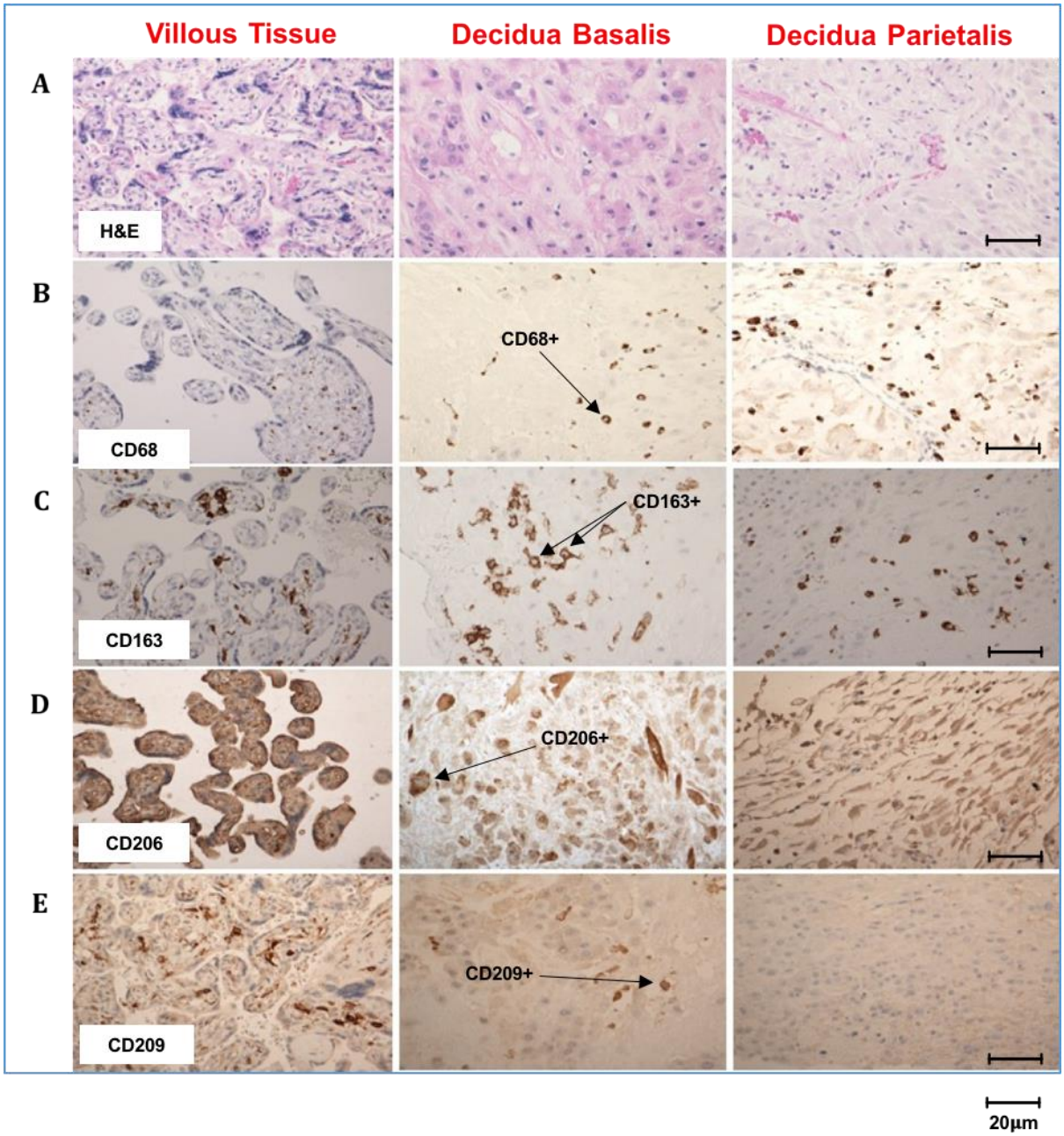

Figure 4.5: Representative IHC images of placental membranes from a participant who was *stable on ART* before pregnancy. (A) Hematoxylin and Eosin (H&E) staining (B) CD68, (C) CD163, (D) CD206, and (E) CD209 staining of the villous tissue, decidua basalis and decidua parietalis of a placenta from a mother who was stable on ART before pregnancy.

CD68+ (Figure 4.4B and Figure 4.5B); CD163+ (Figure 4.4C and Figure 4.5C); CD206+ (Figure 4.4D and Figure 4.5D); and CD209+ (Figure 4.4E and Figure 4.5E)

macrophages were differentially distributed between the membranes of the maternal-foetal interface. In all placental tissue sections stained irrespective of when ART was initiated (*n=30*); the number of CD68+ Hofbauer cells (foetal-derived macrophages in the villous tissue) was significantly higher than that of decidual macrophages of the decidua basalis ($p<0.0001$; Mann-Whitney; Fig. 4.6A) and decidua parietalis ($p<0.0001$; Mann-Whitney; Fig. 4.6A). The decidual macrophages of the decidua basalis had significantly higher CD68 expression than those of the decidua parietalis ($p=0.0101$; Mann-Whitney; Fig. 4.6A). There was no significant difference in CD163 expression between Hofbauer cells (HCs) and decidual macrophages of the decidua basalis (DB), and between decidual macrophages of the decidua basalis and decidua parietalis (Fig 4.6B). However, the number of CD163+ HCs was significantly higher than that of decidual macrophages of the decidua parietalis ($p<0.0001$; Mann-Whitney; Fig. 4.6B, ns refers to p-values greater than 0.05). Interestingly, there were more decidual macrophages of the DB expressing CD163 compared to the Hofbauer cells of the villous tissue and the other decidual macrophages of the DP. The expression of Mannose receptor (CD206) by HCs was significantly higher than that of the decidual macrophages of the DB ($p<0.0001$; Mann-Whitney; Fig. 4.6C) and DP ($p=0.0007$; Mann-Whitney; Fig. 4.6C). CD206+ decidual macrophages were higher in the DP compared to the DB ($p=0.0043$; Mann-Whitney; Fig. 4.6C). There was no difference in the number of CD209+ decidual macrophages between the DB and DP (Fig. 4.6D). The number of CD209+ HCs was significantly higher than both the CD209+ decidual macrophages of the DB ($p<0.0001$; Mann-Whitney; Fig. 4.6D) and DP ($p=0.0009$; Mann-Whitney; Fig. 4.6D).

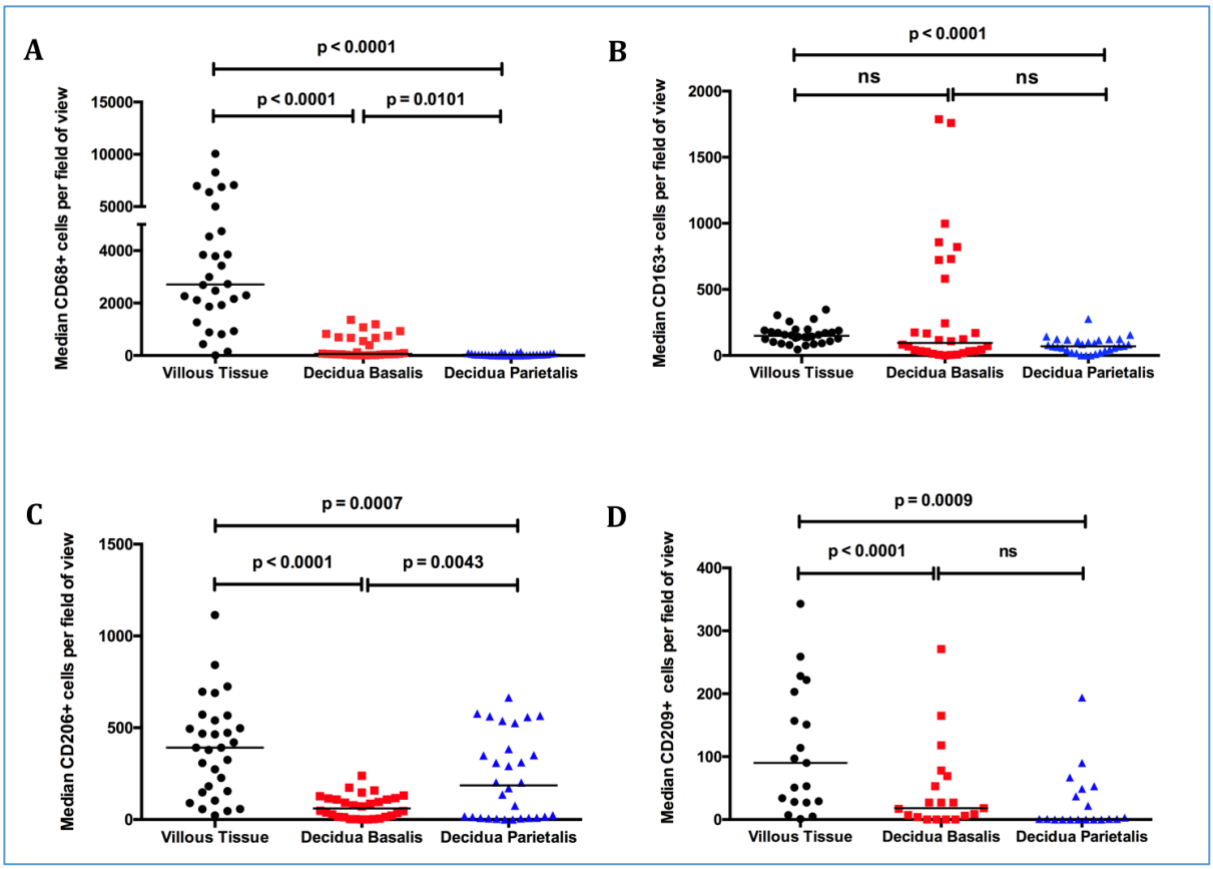

Figure 4.6: Differential distribution of classical M1 and M2 macrophage markers on the decidual macrophages and Hofbauer cells of the maternal-foetal interface of placentas from HIV-1 infected mothers. Distribution of (A) CD68+ cells, (B) CD163+ cells, (C) CD206+ cells and, (D) CD209+ cells in the villous tissue, decidua basalis and decidua parietalis of HIV-1 infected mothers.

Comparison of each of these macrophage marker expression in each membrane of the maternal-foetal interface between ART study groups was made. CD68 expression on Hofbauer cells was slightly higher in the villous tissue of placentas from women who initiated ART during pregnancy compared with those who were on ART before pregnancy. However, their median values were not statistically significant (Fig. 4.7A). There were also no significant differences for all our markers of interest between Hofbauer cells and decidual macrophages of women who initiated ART during pregnancy compared to those women on ART prior to pregnancy (Fig. 4.7B, C &D).

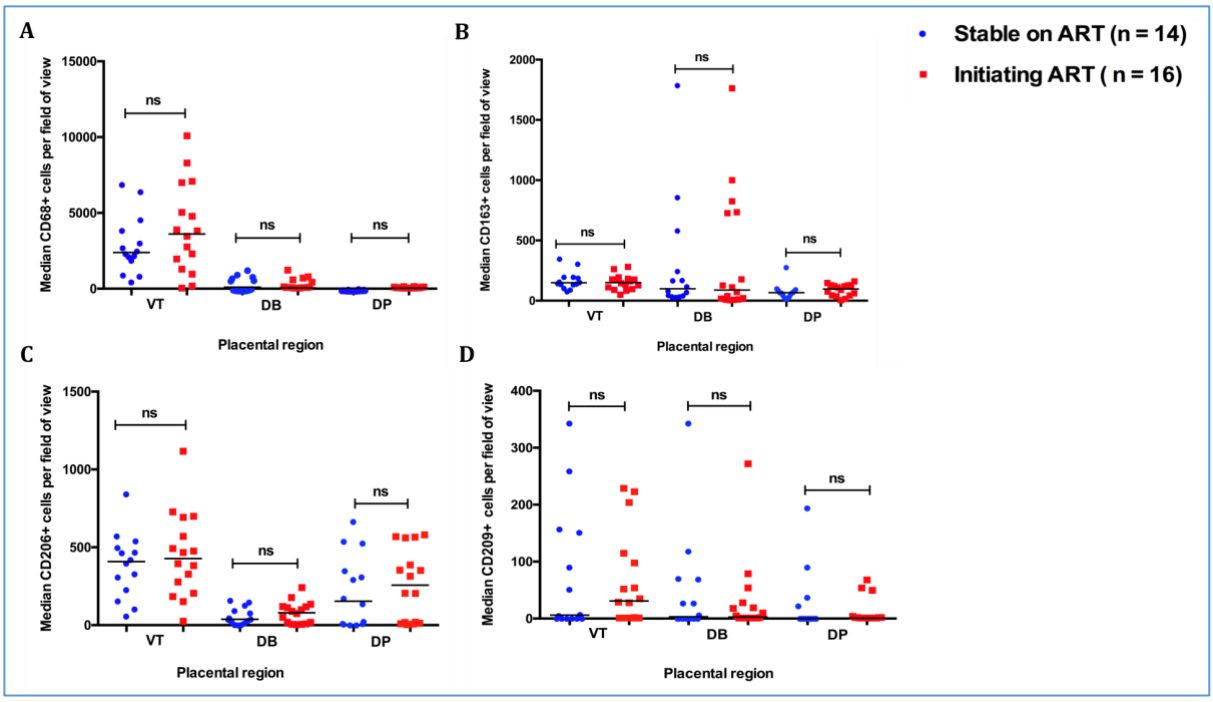

Figure 4.7: Effect of the duration of ART exposure on the distribution of classical M1 and M2 macrophage markers on decidual macrophages and Hofbauer cells of women who initiated ART before pregnancy compared to those whom initiated ART during pregnancy. Differences in the distribution of (A) CD68+ cells, (B) CD163+ cells, (C) CD206+ cells and, (D) CD209+ cells in the villous tissue (VT), decidua basalis (DB) and decidua parietalis (DP).

The activation status of decidual macrophages and Hofbauer cells in the two study groups was further investigated by immunofluorescence (IF) staining of placental tissues, DP, DB and VT with the M1-specific marker, interferon regulatory factor-5 (IRF-5) and the M2-specific marker, CD163 (as described above). Figure 4.8. shows IF staining controls. IRF-5 is a member of the interferon-regulatory factor (IRF) family is involved in the activation of genes encoding type I interferon and pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-6, IL-12 and IL-13 (Krausgruber et al., 2011a). It has been identified as a specific marker for inflammatory M1 macrophages (Krausgruber et al., 2010a, Weiss et al., 2013). As described previously, CD163 is a plasma membrane glycoprotein, a member of the scavenger receptor cysteine-rich super family class B (Fabriek et al., 2005) and a marker of regulatory M2 macrophages. The macrophage populations at the maternal-foetal interface (VT, DB and DP) of HIV-1 infected women, co-expressed markers associated with M1 (IRF-5)

and M2 (CD163) polarisation, irrespective of the time of ART initiation (merged images in Fig.4.9A-C, Fig. 4.10A-C and Fig. 4.11).

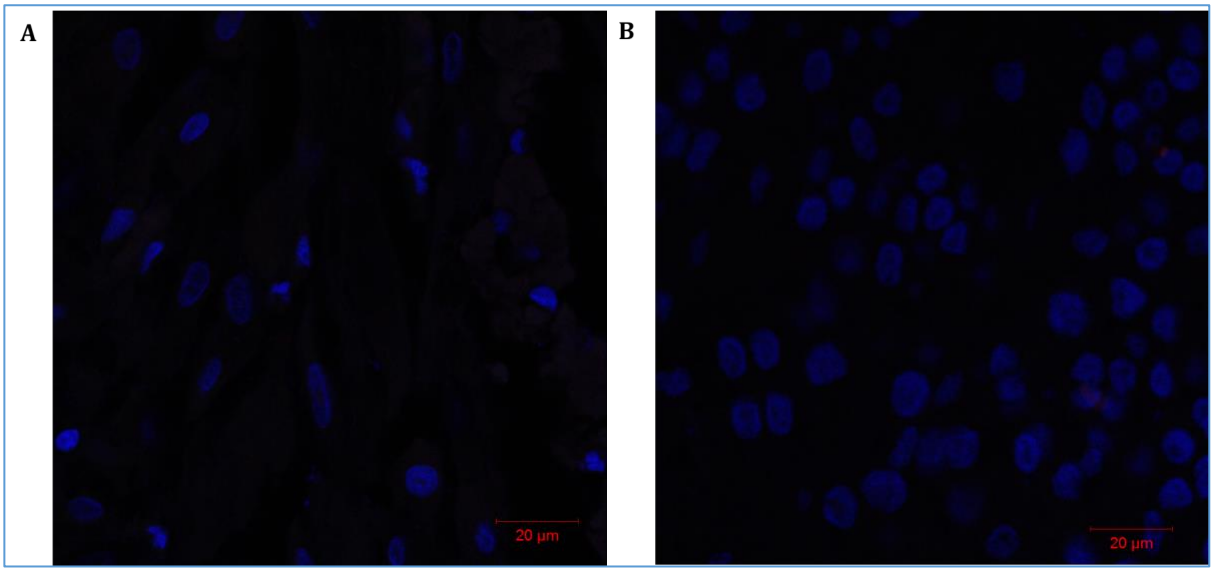

Figure 4.8: Immunofluorescence (IF) staining controls. (A) staining of placental villous tissue with DAPI and Cy3-labeled donkey anti-rabbit secondary antibody. (B) staining of placental villous tissue with DAPI and a non-specific isotype control, Rabbit IgG. Showing no non-specific staining of both the secondary antibody and the antibody isotype.

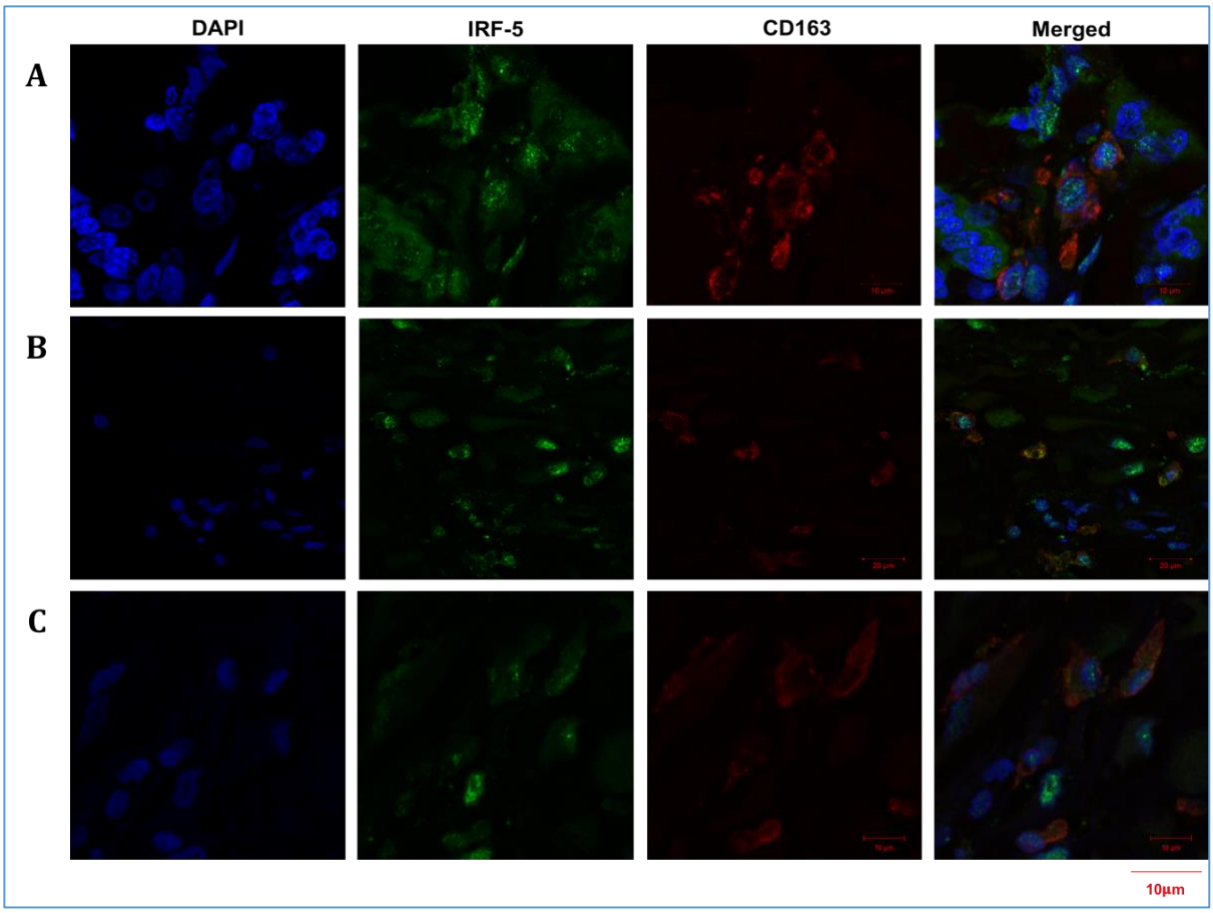

Figure 4.9: Representative IF images of placental membranes from a participant that *initiated ART* during pregnancy. (A) Villous Tissue staining of DAPI (Blue), IRF-5 (Green), CD163 (Red) and the composite image (B) Decidua Basalis staining of DAPI (Blue), IRF-5 (Green), CD163 (Red) and the composite image (C) Decidua Parietalis staining of DAPI (Blue), IRF-5 (Green), CD163 (Red) and the composite image.

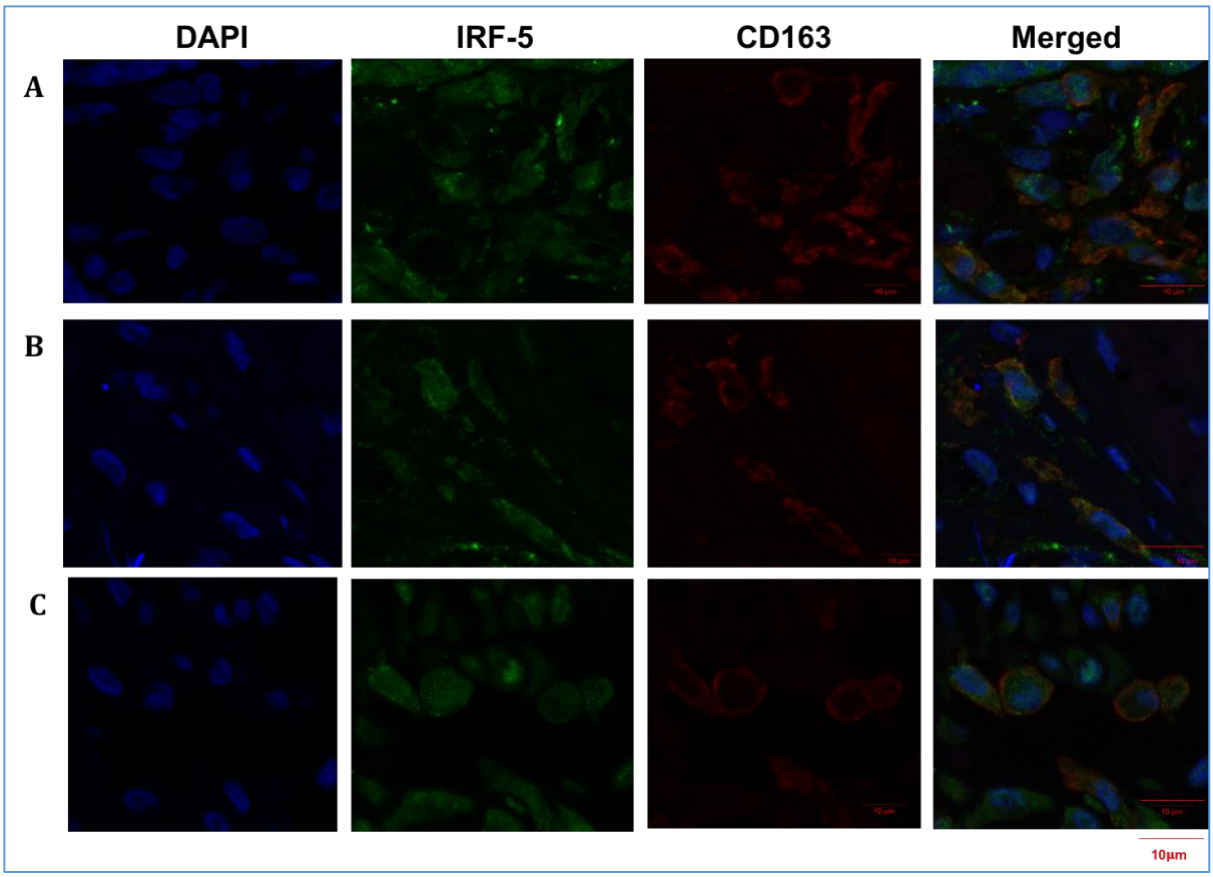

Figure 4.10: Representative IF images of placental membranes from a participant that was *stable on ART* before pregnancy. (A) Villous Tissue staining of DAPI (Blue), IRF-5 (Green), CD163 (Red) and the composite image (B) Decidua Basalis staining of DAPI (Blue), IRF-5 (Green), CD163 (Red) and the composite image (C) Decidua Parietalis staining of DAPI (Blue), IRF-5 (Green), CD163 (Red) and the composite image.

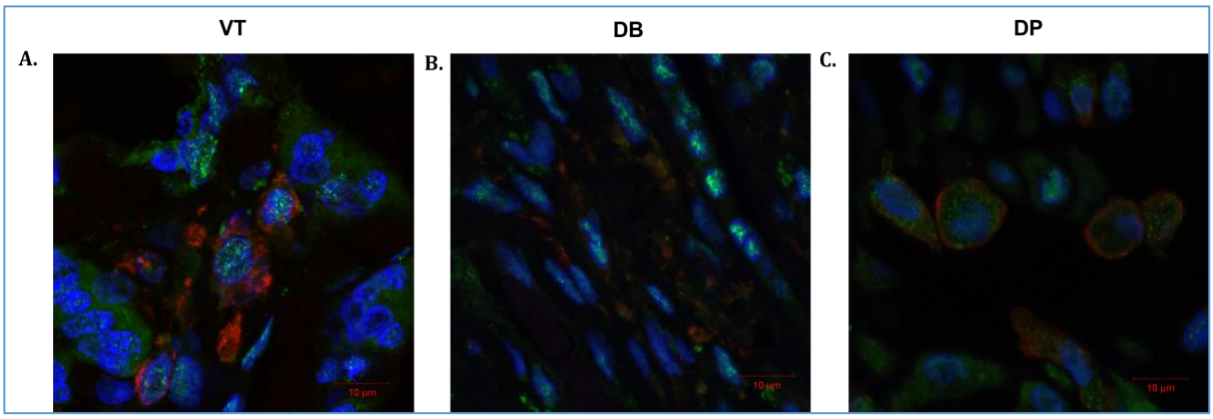

Figure 4.11: Merged images showing co-expression of CD163 (red) and IRF-5 (Green) in (A) Villous Tissue, (B) Decidua Basalis, and (C) Decidua Parietalis.

4.3. Discussion

Upon pathological examination of placentas collected from the women at delivery, it was noted that 3/11 (27%) placentas from women who initiated ART during pregnancy had chorioamnionitis (CA), an acute inflammation of the placental membranes and the chorion due to microbial infections such as *Escherichia coli* and Group B Streptococcus (Czikk et al., 2011, Kawamura et al., 2015) compared to 1/11 (9%) placentas examined from women who were stable on ART prior to pregnancy. Although the pathophysiology of CA is not yet clear, it is believed to be a consequence of disturbed immune homeostasis at the maternal-foetal interface associated with Hofbauer cells (Bracci and Buonocore, 2003). The prevalence of common complications of pregnancy such as CA, gestational diabetes mellitus (GDM); and pre-eclampsia among HIV-infected women in South Africa is not yet known. Numerous studies have reported the dysregulation of placental macrophages leading to adverse birth outcomes in pregnancies complicated by one or more of the above complications (Vinnars et al., 2010, Hung et al., 2006, Toti et al., 2011, Joerink et al., 2011, Sisino et al., 2013).

Apart from the mother's age at birth, all other parameters compared between these two study groups were statistically not significantly different. When gestational age and infant birth weight at delivery was compared between these two ART groups, there was a trend towards preterm birth and low infant birth weight (Fig. 4.1B & Fig. 4.1C)

among women who initiated ART during pregnancy. These few outliers infer that initiating ART during pregnancy may lead to adverse birth outcomes among HIV-infected women initiating ART during pregnancy. That would be contradictory to the findings of Uthman and colleagues who reported that women who initiate ART before pregnancy were significantly more likely to deliver preterm and to have low-birth weight infants compared to those who initiate ART during pregnancy (Uthman et al., 2017). However, it is also important to note that these differences may be due to differences in sample size, population demographics and ART regimens. Although not significant, umbilical cord insertions to the basal plate of the placenta and umbilical cord lengths were either displaced or shorter in placentas from women who were stable on ART before pregnancy, compared with those that initiated ART during pregnancy. What this might mean is not yet known. However, we propose that the conspicuous histological differences in these placentas may have implications for placental functions and subsequent birth outcomes.

In their epidemiological study based on HIV-1 infected South African women, Malaba *et al*., reported no association between the timing of ART initiation (before or during pregnancy) and adverse birth outcomes (Malaba et al., 2017). We posit that the prevalence of adverse birth outcomes among HIV-1 infected women on ART may not be a direct consequence HIV-infection and the timing of ART initiation, but rather be due to the predisposition of HIV-1 infected pregnant women to common complications of pregnancy such as chorioamnionitis, gestational diabetes mellitus, and pre-eclampsia. We therefore hypothesize that these complications of pregnancy are the main drivers of the dysregulation of immune cells at the maternal-foetal interface leading to adverse birth outcomes. In order to fully understand the impact of HIV-infection and the duration of ART exposure on immune mechanisms regulating pregnancy, we propose to further investigate the impact of HIV and/ ART exposure exclusively of common complications of pregnancy and compare these to healthy controls. The major limitations to our current data is the lack of HIV-1 uninfected or healthy participants, a small sample size (n) and the lack of screening of participants for common complications of pregnancy (such as CA, pre-eclampsia, and gestational diabetes mellitus) at enrolment.

5. Identification of Novel Markers of Decidual Macrophages and Hofbauer Cells

5.1. Introduction

Phenotypic characterization of macrophages at the maternal-foetal interface is incomplete. All markers currently used to phenotype decidual macrophages and Hofbauer cells lack specificity and the markers used in most studies to investigate macrophages at the maternal-foetal interface are general markers for other tissue-resident myeloid cells. Cell-specific markers will allow for further characterization of these cells regardless of their placental tissue localization. The aim of this chapter was to identify novel markers that can be used to isolate and further characterize decidual macrophages and Hofbauer cells of the human placenta. A novel approach was developed on the human transcriptome database in the Human Protein Atlas (www.proteinatlas.org) to identify highly expressed protein-encoding genes in the human placenta. Based on their localization, we assigned them to different types of cells making-up the placental tissue. According to the transcriptome analysis of the Human Protein Atlas, 69% (n=13592) of all human proteins (n=19613) are expressed in the placenta. There was an elevated expression of 356 of these genes in the placenta compared to other types of tissue. The 356 genes were categorized into 3 groups summarized in Table 5.1.

Table 5.1: Genes with elevated expression in the placenta compared to other organs.

Category	Number of genes	Description
Group enriched	73	≥ 5-fold higher mRNA levels in a group of 2-7 tissues
Tissue enriched	78	≥ 5-fold higher mRNA levels in a particular tissue as compared to all other tissues
Tissue enhanced	205	≥ 5-fold higher mRNA levels in a particular tissue as compared to average levels in all tissues
Total	356	Total number of elevated genes in placenta

5.2. Methods

We used immunohistochemically stained placental-tissue sections of the Human Protein Atlas (HPA) (https://www.proteinatlas.org) to visualize placental cell localization and expression pattern of the 78 genes defined as tissue-enriched in the placenta (Appendix A: Table 1). The flowchart below details the procedure used to identify novel markers of decidual macrophages and Hofbauer cells in the human placenta. This strategy could be applied to the identification of cellular markers in various other tissues.

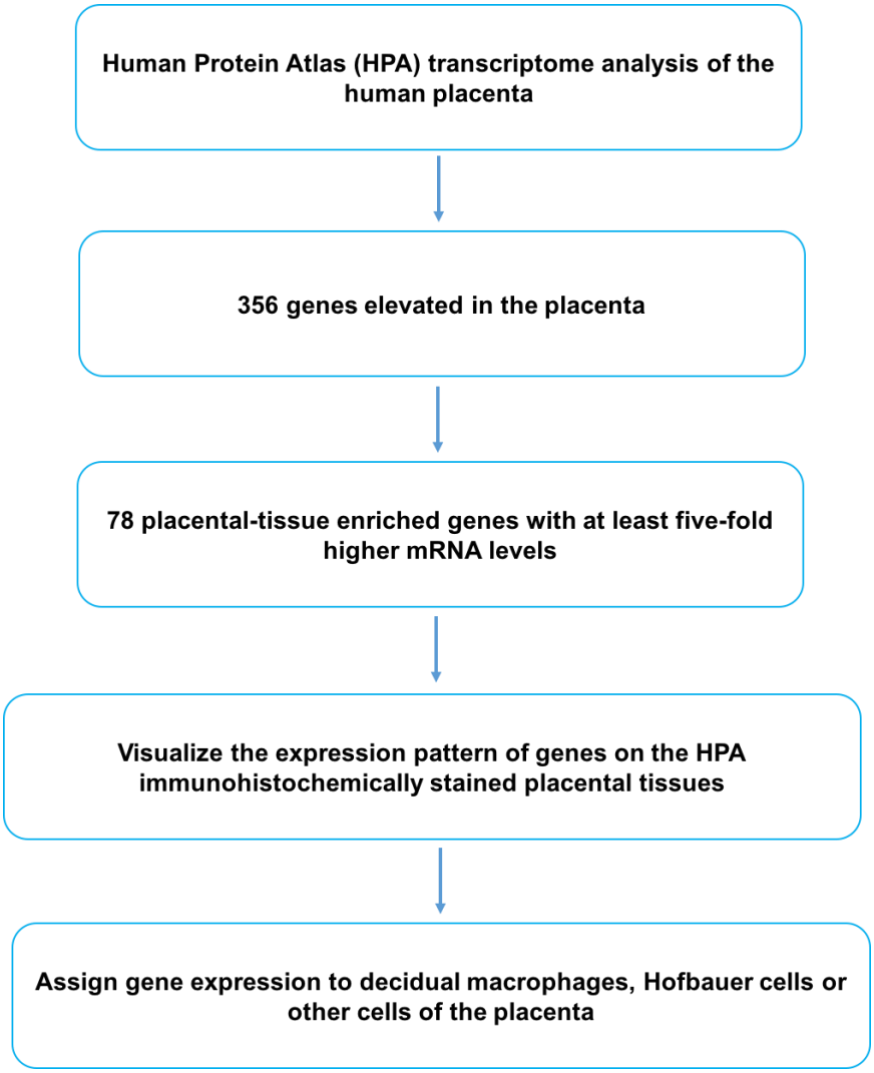

Figure 5.1: Strategy used to identify new markers of decidual macrophages and Hofbauer cells.

5.3. Results

Among the 78 placental-tissue enriched genes with at least five-fold higher mRNA levels, there are 12 genes that had the highest level of expression. The mRNA level (mRNA transcript) and placental cell localization (decidual macrophage, Hofbauer cell or other) of these genes are shown in Table 5.2.

Table 5.2: Placental cell localization and mRNA transcript level of the 12 genes with the highest level of expression in the placenta.

Gene Name	Description	Placental cell localization	mRNA transcript level
HBG2	hemoglobin subunit gamma 2	other cells	18253.8
CSH1	chorionic somatomammotropin hormone 1	Other cells	13487
CSH2	chorionic somatomammotropin hormone 2	other cells	3932.3
XAGE3	X antigen family member 3	other cells	575.4
PSG1	pregnancy specific beta-1-glycoprotein 1	other cells	362.6
PSG2	pregnancy specific beta-1-glycoprotein 2	other cells	343.4
ISM2	isthmin 2	Hofbauer cells & other cells	274.5
CSHL1	chorionic somatomammotropin hormone like 1	Other cells	216.7
GH2	growth hormone 2	other cells	189.1
PSG3	pregnancy specific beta-1-glycoprotein 3	other cells	188.8
PSG5	pregnancy specific beta-1-glycoproetin 5	other cells	157.6
PSG9	pregnancy specific beta-1-glycoprotein 9	other cells	147

We observed that the majority of genes that are highly-enriched on the human placenta localized to other regions of the placenta such as trophoblast cells and endothelial cells. Among the 12/78 (15.3%) genes that had the highest level of expression in the placenta, none localized or were expressed by decidual macrophages. Isthmin 2 (ISM2) was the only protein that localized to Hofbauer cells, although, it was also expressed by other cells within the chorionic villi of the placenta

but not the decidua (Figure 5.2). ISM2 has two alternatively spliced transcript variants that encodes different isoforms. However, very little is known about ISM1 or ISM2 in the human placenta, despite having the highest expression of ISM2 compared to other human tissues (Figure 5.3).

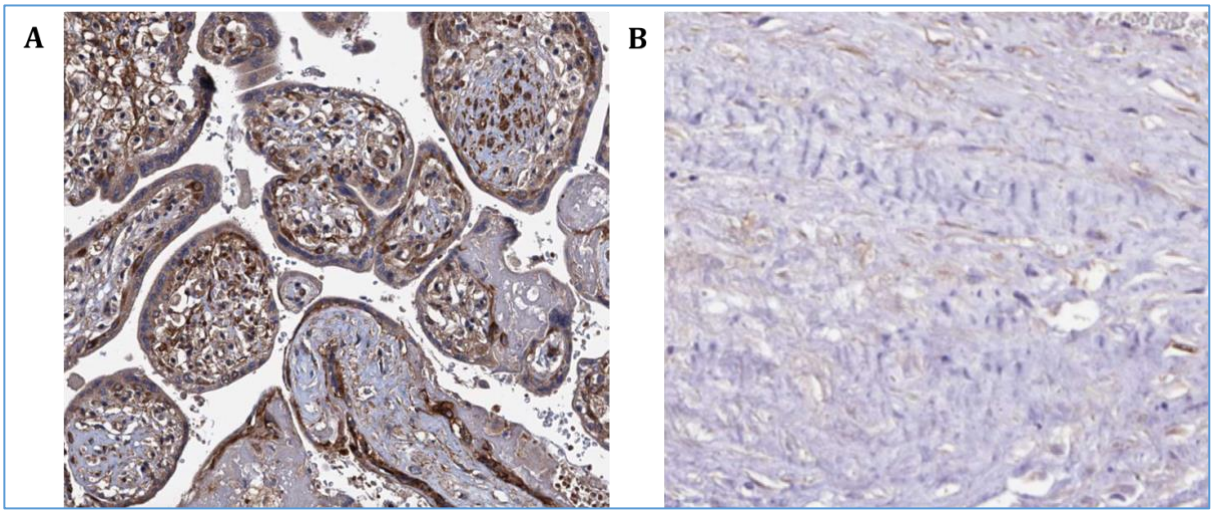

Figure 5.2: The expression of Isthmin 2 (ISM2) in the chorionic villi (A) of the placenta compared to the decidua (B).

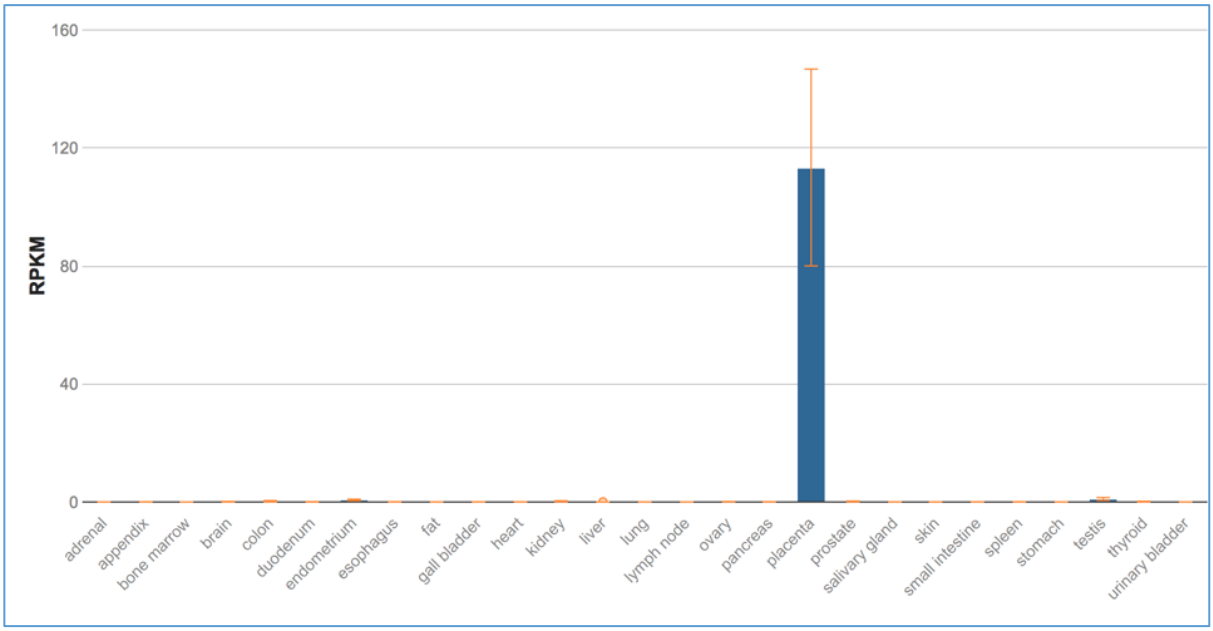

Figure 5.3: Expression of Isthmin 2 in the placenta compared to other human tissues.

Immunohistochemically stained human placental tissue sections revealed that among the 78 genes defined as tissue-enriched in the placenta, 8 (10.3%) were uniquely expressed by Hofbauer cells, 5 (6.4%) were expressed by decidual macrophages, 3 (3.8%) were expressed by both decidual macrophages and Hofbauer cells while the remaining 62 (79.5%) were expressed by other cells of the placenta such as trophoblasts, fibroblasts and endothelial cells (Figure 5.4).

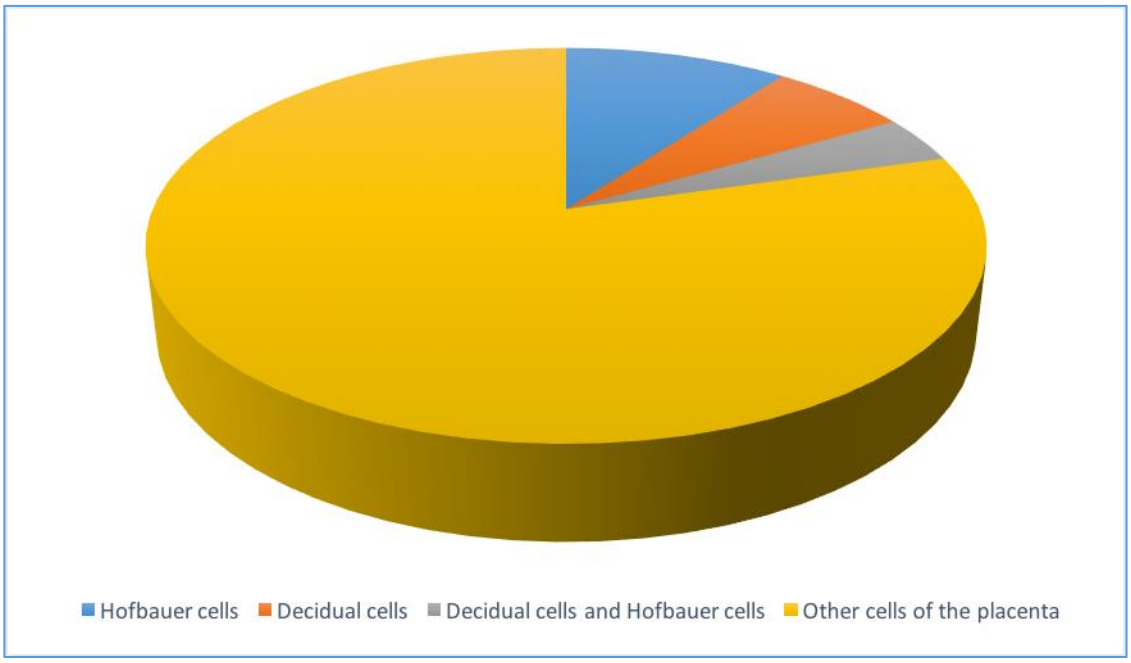

Figure 5.4: Placental localization of the 78 genes enriched in the placenta.

Table 5.3: Genes expressed by Hofbauer cells and their predicted function.

Gene Name	Description	Predicted Function
ISM2	Isthmin 2	For cell adhesion and angiogenesis
EGFL6	EGF like domain multiple 6	Regulates cell cycle, proliferation, and development processes
PLAC1	Placenta-specific 1	Plays a role in normal embryo development
FCGR2B	Fc fragment of IgG receptor IIb	Required for the maintenance of tolerance

HGF	Hepatocyte growth factor	Involve in epithelial cell proliferation, migration and morphogenesis
VGLL1	Vestigial like family member 1	Specific activator of mammalian transcription factors
IL1RL1	Interleukin 1 receptor like 1	Activates MAP kinases
LIN28B	Lin-28 homolog B	Regulates gene expression

Immunohistochemistry stained placental chorionic villi for Lin-28 homolog B (LIN28B) and Placenta-specific-1 (PLAC1) showed high expression of these markers on Hofbauer cells (Figure 5.5). IHC stains of the placental decidua, showed high expression of insulin-like growth factor-2 (IGF2) and Pappaliysin-2 (PAPPA-2) on decidual macrophages (Figure 5.6).

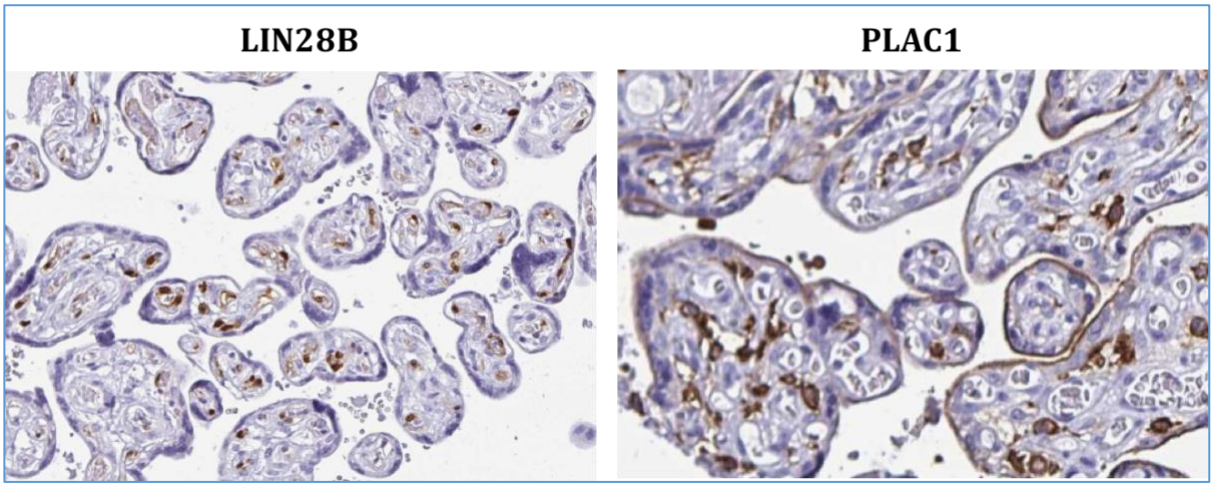

Figure 5.5: Expression of Lin-28 homolog encoded by the LIN28 gene and placenta-specific 1 protein encoded by the PLAC1 gene on Hofbauer cells. (https://www.proteinatlas.org)

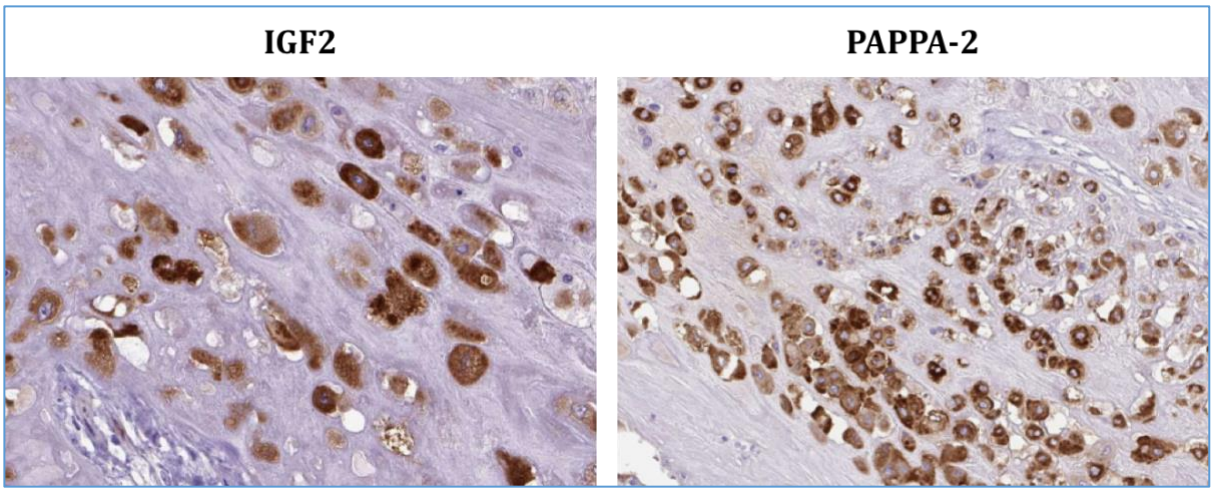

Figure 5.6: Expression of IGF-2 and PAPPA-2 on decidual macrophages of the placenta. (https://www.proteinatlas.org)

A few studies which investigated the human placenta transcriptome reported various other genes as highly enriched in the human placenta (Saben et al., 2014, Sood et al., 2006). The Human Protein Atlas database was used to determine the expression pattern of the genes reported by these studies, Coagulation Factor XIII A1 (FXIIIA1) and Allograft Inflammatory Factor 1 (AIF-1) was highly expressed specific markers for Hofbauer cells (Figure 5.7). Tetratricopeptide Repeat Protein 39B (TTC39B) and Exoribonuclease 1 (ERI1) were highly expressed and specific for decidual macrophages (Figure 5.8). Table 5.4 and Table 5.5 below, details the other genes that were highly expressed by decidual macrophages and Hofbauer cells, respectively. While Figure 5.9 shows the expression pattern of enriched genes that are expressed on both the chorionic villi (Hofbauer cells and other cells) and decidua of the placenta.

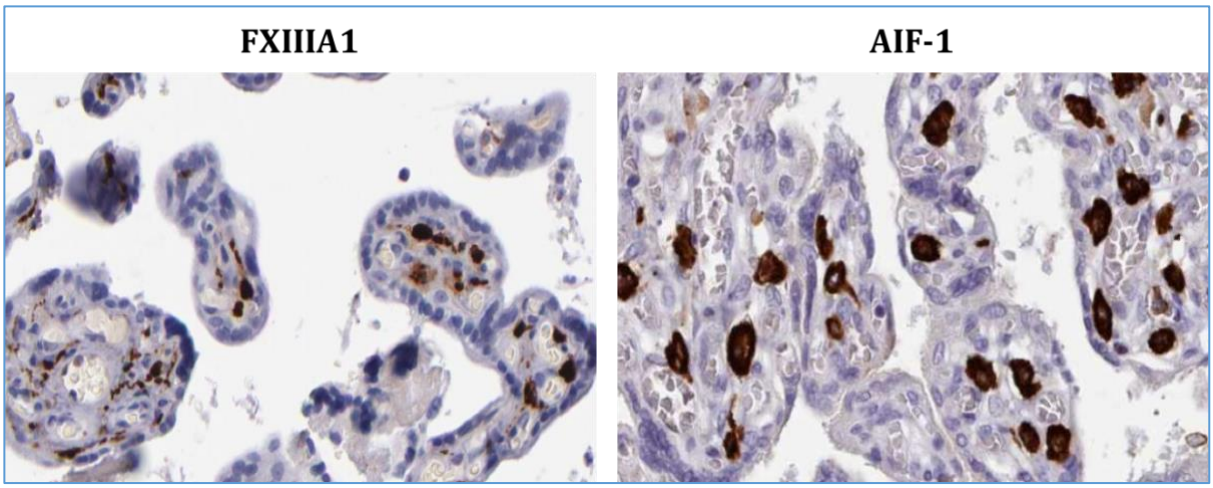

Figure 5.7: Expression of Coagulation Factor XIII A1 (FXIIIA1) and allograft inflammatory factor 1 (AIF-1) on Hofbauer cells. (https://www.proteinatlas.org)

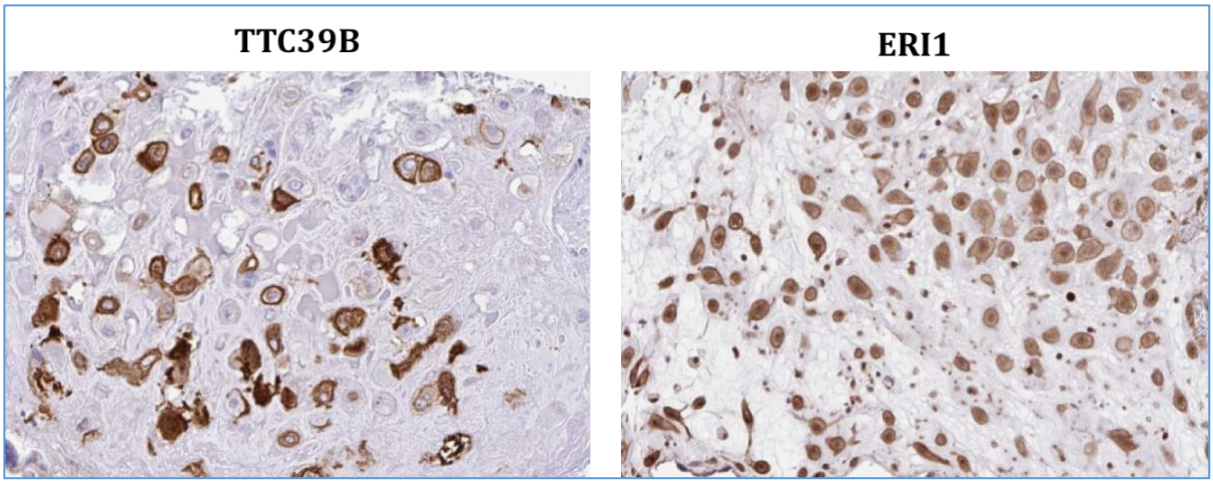

Figure 5.8: The expression of Tetratricopeptide Repeat Protein 39B (TTC39B) and Exoribonuclease 1 (ERI1) on decidual macrophages of the placenta. (https://www.proteinatlas.org)

Table 5.4: Genes expressed by decidual macrophages and their predicted function.

Gene Name	Description	Predicted Function
HLA-G	Major histocompatibility complex, class I, G	Induces the immune tolerance of the foetus
IGF-2	Insulin-like growth factor 2	Promotes placental differentiation and function
SLC13A4	Solute carrier family 13 member 14	Involved in nutrients transport
PAPPA2	Pappalysin 2	Regulates metabolism and nutrient transport
SKP2	S-phase kinase associated protein 2	Promotes vascular smooth muscle cell proliferation

Table 5.5: Genes expressed by both decidual macrophages and Hofbauer cells and their predicted functions

Gene Name	Description	Predicted Function
TRIM64B	Tripartite motif containing protein 64B	Autophagy receptor regulator
ADAMTS18	ADAM metallopeptidase with thrombospondin type 1 motif 18	Involved in extracellular matrix degradation
CHAT	Choline O-acetyltransferase	Plays a role in the synthesis of acetylcholine

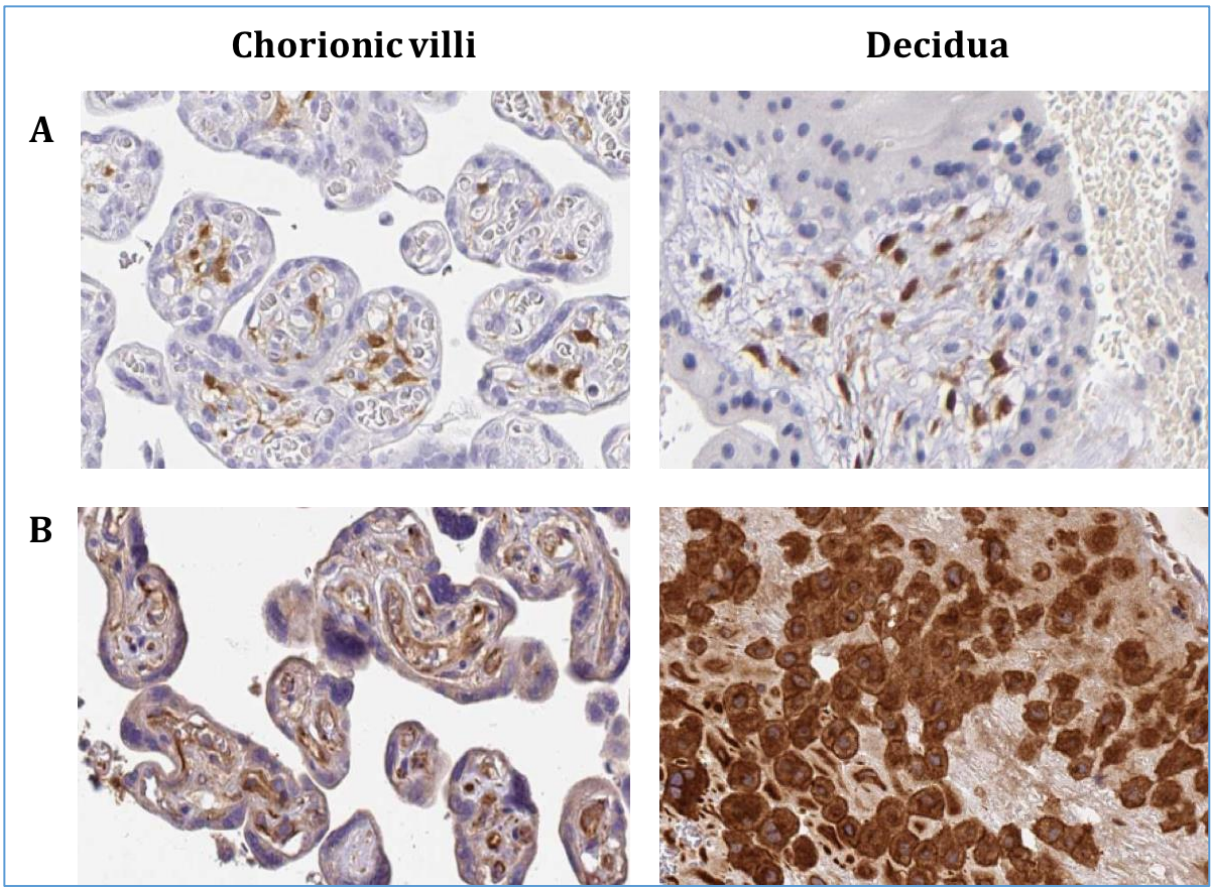

Figure 5.9: Placental-tissue enriched genes that are expressed on both the chorionic villi and decidua of the placenta. The expression of (A) CHAT on the Hofbauer cells of the chorionic villi and decidua, (B) ADAMTS18 on the Hofbauer cells of the chorionic villi and decidua. (https://www.proteinatlas.org)

5.4. Discussion

In this chapter we determined placental tissue localization of a number of expressed genes that have been shown to be highly-enriched in human placental tissues compared to other tissue types. The majority of genes that are highly enriched in human placentas, as determined by The Human Protein Atlas, localized to trophoblasts and endothelial cells of the placenta and fewer expressed genes localized to Hofbauer cells. Those that did included LIN28B, which plays a role in the regulation of gene expression (Viswanathan et al., 2008), Placenta-specific-1 (PLAC-1), which is involved in normal placental and embryonic development (Jackman et al., 2012, Fant et al., 2010), and EGFL6, which is involved in the regulation of the cell

cycle, proliferation, and development processes (Noh et al., 2017). The latter protein, suggests a role of Hofbauer cells in placental and foetal development. Along with expression expression of a potent angiogenesis inducer, Hepatocyte growth factor (HGF) which plays a role in epithelial cell proliferation, migration, and morphogenesis further supports the involvement of Hofbauer cells in placental and foetal development (Kauma et al., 1997).

The genes localizing to the decidual membrane region of the placenta such as insulin-like growth factor 2 (IGF-2) and pregnancy-associated plasma protein A2 (PAPPA-2) are involved in the regulation of metabolism and nutrient transport (Sibley et al., 2004). PAPPA-2 is a metalloproteinase from syncytiotrophoblasts, where it cleaves the complex formed between insulin-like growth factor (IGF) and insulin-like growth factor binding protein (IGFBP). The expression of PAPPA, PAPPA-2 and PLAC-1 in the placenta during pregnancy was recently associated with foetal growth restriction (FGR) (Sifakis et al., 2018). Interestingly, Human Leukocyte Antigen G (HLA-G), a non-classical MHC class I molecule that is specifically expressed by invading Extra-Villous trophoblasts (EVT) earlier on during pregnancy (Kovats et al., 1990) was found to be highly expressed by decidual cells and not by Hofbauer cells in (Human Protein Atlas). Such differential expression of HLA-G suggests a synergy between the induction and maintenance of immune tolerance by foetal (trophoblasts) and maternal cells (decidual cells). Coagulation Factor XIII (FXIII) is a transglutaminase enzyme that circulates in tetrameric form (FXIII-A_2B_2). It is made-up of two A subunits (FXIII-A) and two B subunits (FXIII-B) and catalyzes the cross-linking of fibrin and stabilizes the fibrin clot (Muszbek et al., 2011). It has also been shown to have a role in wound healing, bone metabolism and pregnancy (Shi and Wang, 2017). It has also been reported to be a marker for alternative activation of macrophages (Torocsik et al., 2005). We proposed Coagulation Factor XIIIA chain 1 (FXIIIA1) and Insulin-like growth factor 2 as potential specific markers for Hofbauer cells and decidual macrophages, respectively. However, these markers require further validation.

6. Discovering Placental Macrophage-specific biomarkers of HIV-infection

6.1. Introduction

There is no available data on the interactions of immunological cells of the maternal-foetal interface and the maternal immune system exposed to HIV and/or antiretroviral drugs. Placental macrophages have been reported to play a central role in the establishment and maintenance of immune tolerance towards the semi-allogeneic foetus throughout gestation (Svensson-Arvelund and Ernerudh, 2015, Svensson-Arvelund et al., 2015). Hofbauer cells have also been implicated the vertical transmission of TORCH (Toxoplasmosis, other infections, Rubella, Cytomegalovirus and Herpes Simplex II virus) infections (Quicke et al., 2016, Simoni et al., 2017), while a study by Quillay and colleagues reported that decidual macrophages are permissive to HIV-1 infection, in vitro (Quillay et al., 2015). Therefore, there is a need to identify biomarkers of placental macrophage dysregulation that can be associated with adverse birth outcomes observed in pregnancies complicated by maternal HIV-infection. Identification of these biomarkers would provide a better understanding of HIV pathogenesis amongst pregnant women and provide a foundation for the development of therapeutic interventions required to improve birth outcomes and maternal health. The aim of this chapter was to discover novel decidual macrophage-specific and Hofbauer cell-specific biomarkers of HIV-1 infection.

6.2. Methods

Existing microarray datasets by Cobos *et al*., deposited on GEO Profiles (Superseries GSE55029) was used. In this particular study, polarized macrophages were obtained from healthy, HIV-uninfected donors by the stimulation of isolated primary monocytes with IFN-γ (250 U/ml) in combination with TNF (12.5 ng/ml), IL-4 (50 ng/ml), IL-10 (50 ng/ml) for 5 days. Monocyte-derived macrophages (MDM) were also infected for 24 hours with one of two HIV-1 strains (CCR5- or CXCR4-using HIV-1) or their non-

replicating counterparts (heat inactivated virus). Macrophages not stimulated with cytokines or uninfected with HIV-1 were used as controls. A total of 16 treatment conditions were tested in triplicate, for a total of 48 samples analyzed. To verify protein expression in placental tissue, we used the Human Protein Atlas database (http://www.proteinatlas.org/). The strategy used to discover novel biomarkers of HIV-1 infection is outlined in Figure 6.1 below.

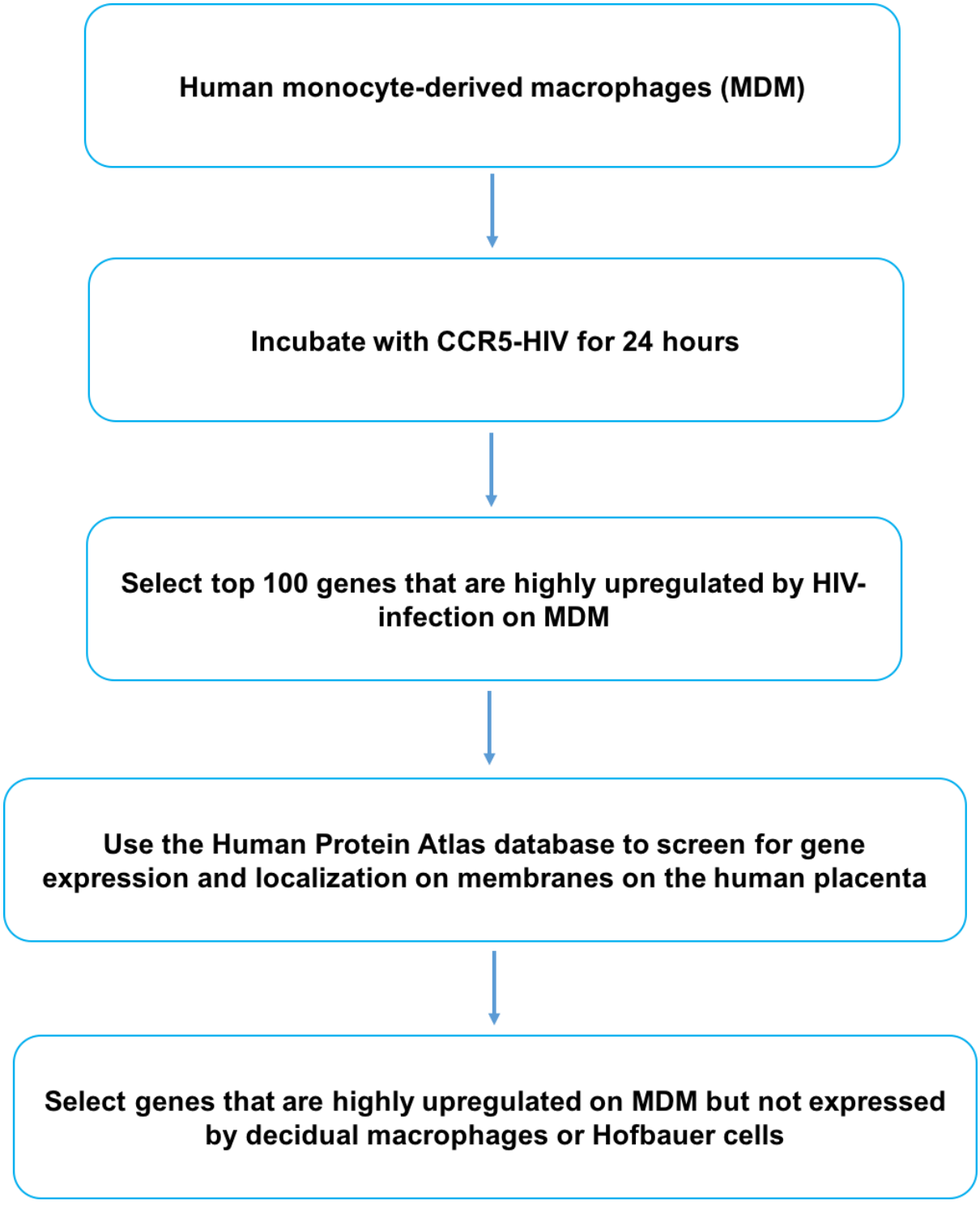

Figure 6.1: Strategy used to discover novel biomarkers of HIV-1 infection.

6.3. Results

Macrophages in the placenta were compared to IL-4, IL-10, HIV-infected, and IFN-γ/TNF-α treated human monocyte-derived macrophages (MDM). For this study, the top 100 genes that were highly upregulated in each treatment group were checked for constitutive expression in the Human Protein Atlas database. Localization of these genes on placental tissue are represented in Appendix A, Tables II-V. To discover novel biomarkers of HIV-1 infection, we focused on genes/markers upregulated by CCR5-HIV-1 infection of MDM after 24 hours. Markers that were highly upregulated by CCR5-HIV-1 infection of MDM after 24 hours but constitutively expressed by decidual macrophages and Hofbauer cells were not regarded as potential biomarkers of HIV-infection (Fig. 6.2). The markers that were highly upregulated on these MDM but not constitutively expressed by decidual macrophages and Hofbauer cells were regarded as potential biomarkers of HIV-1 infection in these cells. Markers Identified as potential biomarkers of HIV-1 infection on decidual macrophages and Hofbauer cells are shown in Figure 6.3.

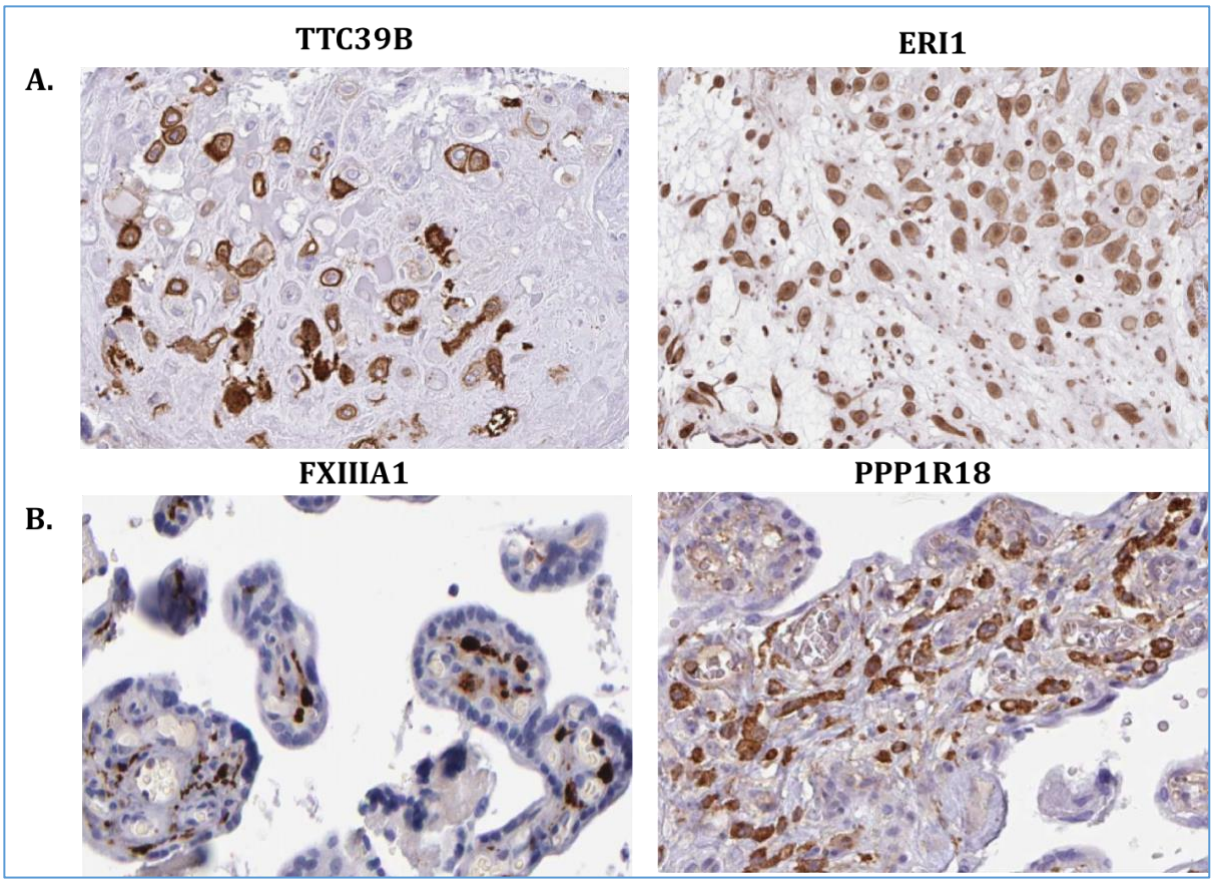

Figure 6.2: IHC images of markers encoded by genes that are highly upregulated by 24hr CCR5-HIV infection of MDM. These markers do not represent potential biomarkers of HIV infection. (A) Expression of Tetratricopeptide repeat domain 39B (TTC39B) and Exoribonuclease 1 (ERI) on decidual cells and, (B) Expression of Human Coagulation Factor XIIIA1 (FXIIIA1) and Protein phosphatase 1 regulatory subunit 18 (PPP1R18) on Hofbauer cells. (https://www.proteinatlas.org)

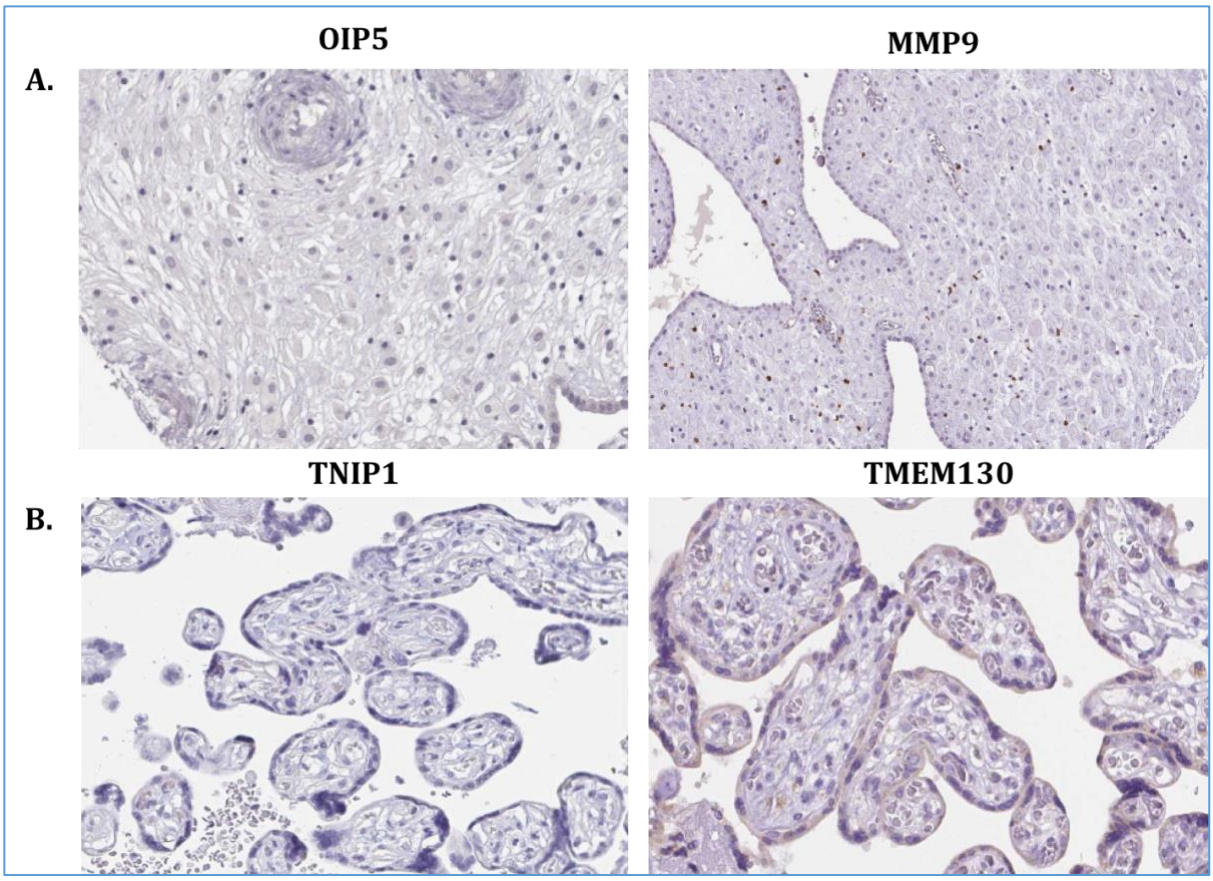

Figure 6.3: IHC images of markers that are highly upregulated by 24hr CCR5-HIV infection of MDM but not constitutively expressed by placental macrophages. These represent potential biomarkers of Maternal HIV-infection, (A) Opa Interacting Protein 5 (OIP5) and Matrix Metallopeptidase 9 (MMP9) in decidual cells and (B) TNF-α-induced protein 3-interacting protein 1 (TNIP1) and Transmembrane protein 130 (TMEM130) on Hofbauer cells. (https://www.proteinatlas.org)

6.4. Discussion

In this Ph.D., a novel approach was used to discover new markers that are highly upregulated by HIV-1 infection on monocyte-derived macrophages (MDMs) and could be used as biomarkers of HIV-1 infection in placental macrophages. Upon HIV-infection of MDMs, there was an upregulation of Tetratricopeptide repeat domain 39B (TTC39B) and Exoribonuclease 1 (ERI) which both localized to decidual macrophages; and protein phosphate 1 regulatory subunit 18 (PPP1R18) and

Coagulation Factor XIII A chain 1 (F13A1) which localized to Hofbauer cells of the placenta chorionic villi. Using the Human Protein Atlas database, it was determined that these genes are constitutively expressed on decidual macrophages and placental Hofbauer cells, and therefore could not be used as potential biomarkers of HIV-1 infection in these cells. We identified Opa Interacting Protein 5 (OIP5) and matrix metalloproteinase 9 (MMP9) as potential biomarkers of HIV-1 infection in Hofbauer cells; and TNF-α-induced protein 3-interacting protein 1 (TNIP1) and Transmembrane protein 130 (TMEM130) as potential biomarkers of HIV-1 infection in decidual macrophages as these markers were highly upregulated by HIV-1 infection but not constitutively expressed by these macrophages. MMP9 is a major matrix metalloproteinase produced by macrophages, but its regulation is not yet known (Brown et al., 1995). It cleaves basement membrane collagens type IV and V and gelatin, elastin and fibronectin (Vu et al., 1998). The significance of these markers on pregnancy is not yet known. As an extension to this work, freshly-isolated placental macrophages from HIV-1 exposed and unexposed placentas will be stained to determine the expression of these markers. We will also determine the permissiveness of placenta-isolated decidual macrophages and Hofbauer cells to HIV-1 infection, *in vitro*. Due to the ambiguity in the ontogeny of these cells, it is not known whether monocyte-derived macrophages are a good model for decidual macrophages or Hofbauer cells.

7. Conclusions and Limitations

The work presented in this thesis focused on understanding the effect of maternal HIV infection and the duration of ARV drug exposure on the expression of markers associated with M1 and M2 polarisation of macrophages by placental macrophages, decidual macrophages and foetal Hofbauer cells. The immunohistochemistry data presented in this thesis showed histological differences between placental membranes (VT, DB and DP) making-up the maternal-foetal interface. Although similar to one another, the maternal-derived placental membranes, the decidua basalis and decidua parietalis, are different in cellular composition and architecture from the foetal-derived, chorionic villous tissue (VT).

Due to lack of M1 macrophage-specific markers, in this study, M1 macrophages (classically activated) were defined as cells expressing the pan-macrophage marker, CD68 while lacking the expression of M2 macrophage-associated markers, CD163, CD206 and CD209. The data presented in this thesis showed that the expression of CD68 is higher on Hofbauer cells compared to decidual macrophages (DB and DP) in placentas from HIV-1 infected mothers. The expression of CD206 and CD209, but not CD163, was also higher on Hofbauer cells compared to decidual macrophages. These data suggest that decidual macrophages and Hofbauer cells in HIV-1 infected women are not polarized in accordance with the M1/M2 paradigm of macrophage polarization, but rather express markers associated with both inflammation (M1) and immune regulation (M2). This is supported by the immunofluorescence imaging data which showed the co-expression of IRF-5, a transcription factor associated with classical (M1) activation; and CD163, a marker associated with alternative (M2) activation, on both Hofbauer cells and decidual macrophages of placentas from HIV-1 infected mothers. There were no significant differences in the expression of these markers by Hofbauer cells, decidual macrophages of the DB and decidual macrophages of the DP in placentas from HIV-1 infected women who initiated ART before pregnancy and those who initiated ART during pregnancy. Based on these results, we conclude that the duration of ART exposure has no effect on the expression of macrophage markers associated with classical and alternative activation of Hofbauer cells and decidual macrophages from HIV-1 infected mothers. However, a major limitation to these data

is the lack of immunofluorescence images quantification tool. Therefore, these conclusions are only based on qualitative assessment of the IF images.

Immunohistochemistry (IHC) is a routine diagnostic and basic research technique, however, it does have limitations. The number of steps involved in IHC such as tissue preparation and fixation, paraffin block preparation, antigen retrieval methods, reagents and antibody specificities, incubation, washing steps and counterstaining may introduce a variety of confounders. There are other techniques available with the same principle as IHC, such as the EnVision system (DAKO, Hamburg, Germany). The EnVision system is a highly sensitive two-step immunohistochemical technique that takes less time to prepare than regular IHC (Kammerer et al., 2001). Apart from advances in experimental protocols which can now allow for the visualization of several antigens in a single multiplexed IHC experiment, imaging technology has also advanced tremendously. In recent years, a number of high resolution microscopes have been developed. However, a tissue-specific method to quantify antigen expression is a major limitation, especially in resource-limited settings.

We identified Coagulation Factor XIIIA1 (FXIIIA1) and Insulin-like growth factor 2 (IGF-2) as potential constitutive markers for Hofbauer cells and decidual macrophages respectively. Using a microarray dataset of Cobos *et al*., and the Human Protein Atlas database, we also identified OIP5 and MMP9 as potential biomarkers of HIV-1 infection in Hofbauer cells, and TNIP1 and TMEM130 as potential biomarkers of HIV-1 infection in decidual macrophages. Although the strategy of identifying new markers for tissue-resident cells is yet to be validated, this approach was used to identify novel markers for placental macrophage populations. A further limitation in this Ph.D. was to validate this strategy using placenta-isolated macrophages from placentas collected in the cohort study using multicolor flow cytometry, IHC and immunofluorescence staining. One potential limitation of using MDM is that these cells do not represent HC in the placenta and may have a different transcriptomic profile. However, this was outside the scope of this PhD and may need to be addressed in future studies. To further investigate the effect of HIV and or ART exposure on immune mechanisms associated with macrophages of the maternal-foetal interface, this work will be extended to compare differences in expression of these novel macrophage markers with those in HIV-unexposed, healthy pregnancies.

8. References

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9. Appendices

9.1. Appendix A: Gene localization to the human placental cells

Table 1: The localization of the 78 placental tissue enriched genes (with at least five-fold higher mRNA levels) on the Human Protein Database.

Gene Name	Description	Placental cell localization
ADAM12	ADAM metallopeptidase domain 12	Other cells
ADAMTS18	ADAM metallopeptidase with thrombospondin type 1 motif 18	Hofbauer Cells & Decidual macrophages
C4orf26	Chromosome 4 open reading frame 26	Other cells
CGA	Glycoprotein hormones, alpha polypeptide	Other cells
CGB3	Chorionic gonadotropin beta subunit 3	Other cells
CGB5	Chorionic gonadotropin beta subunit 5	Other cells
CGB8	Chorionic gonadotropin beta subunit 8	Other cells
CHAT	Choline O-acetyltransferase	Hofbauer cells & decidual macrophages
CLEC1A	C-type lectin domain family 1 member A	Other cells
COL11A1	Collagen type XI alpha 1 chain	Other cells

CSH1	Chorionic somatomammotropin hormone 1	Other cells
CSH2	Chorionic somatomammotropin hormone 2	Other cells
CSHL1	Chorionic somatomammotropin hormone like 1	Other cells
CYP19A1	Cytochrome P450 family 19 subfamily A member 1	Other cells
EBI3	Epstein-Barr virus induced 3	Other cells
EGFL6	EGF like domain multiple6	Hofbauer cells
EPYC	Epiphycan	Other cells
ERVV-1	Endogenous retrovirus group V member 1	Other cells
ERVV-2	Endogenous retrovirus group V member 2	Other cells
ERVW-1	Endogenous retrovirus group W member 1	Other cells
FAM26D	Family with sequence similarity 26 member	Other cells
FBN2	Fibrillin 2	Other cells
FCGR2B	Fc fragment of IgG receptor IIb	Other cells
FLT1	Fms related tyrosine kinase 1	Other cells
FOXI3	Fork head box I3	Other cells
GCM1	Glial cells missing homolog 1	Other cells
GH2	Growth Hormone 2	Other cells

GNGT1	G protein subunit gamma transducing 1	Other cells
GPC3	Glycan	Other cells
HAPLN1	Hyaluronic and proteoglycan link protein 1	Other cells
HBG1	Hemoglobin subunit gamma 1	Other cells
HBG2	Hemoglobin subunit gamma 2	Other cells
HGF	Hepatocyte growth factor	Hofbauer cells
HLA-G	Major histocompatibility complex, class I, G	Decidual macrophages
HSD17B1	Hydroxylated 17-beta dehydrogenase 1	Other cells
HSD3B1	Hydroxy-delta-5-steriod dehydrogenase, 3 beta- and steroid delta-isomerase 1	Other cells
HTRA4	HTRA serine peptidase 4	Other cells
IGF2	Insulin-like growth factor 2	Decidual macrophages
IL1RL1	Interleukin 1 receptor like 1	Hofbauer cells
INSL4	Insulin like 4	Other cells
ISM2	Isthmin 2	Hofbauer cells
KISS1	Kiss-1 metastasis-suppressor	Other cells
KRTAP26-1	Keratin associated protein 26-1	Other cells
LGALS13	Galectin 13	Other cells
LGALS14	Galectin 14	Other cells
LGALS16	Galectin 16	Other cells
LIN28B	Lin-28 homolog B	Hofbauer cells

MAGEA8	MAGE family member A8	Other cells
MEST	Mesoderm specific transcript	Other cells
MSMP	Macroseminoprotein, prostate associated	Other cells
NFE4	Nuclear factor, erythroid 4	Other cells
NOTUM	NOTUM, palmitoyl-protein carboxylesterase	Other cells
PAPPA	Pappalysin 1	Other cells
PAPPA2	Pappalysin 2	Decidual macrophages
PHLDA2	Pleckstrin homology like domain family A member 2	Other cells
PLAC1	Placenta specific 1	Hofbauer cells
PLAGL1	PLAG1 like zinc finger 1	Other cells
PRG2	Proteoglycan 2, pro eosinophil major basic protein	Other cells
PSG1	Pregnancy specific beta-1-gycoprotein 1	Other cells
PSG11	Pregnancy specific beta-1-glycoprotein 11	Other cells
PSG2	Pregnancy specific beta-1-glycoprotein 2	Other cells
PSG3	Pregnancy specific beta-1-glycoprotein 3	Other cells
PSG5	Pregnancy specific beta-1-glycoprotein 5	Other cells
PSG6	Pregnancy specific beta-1-glycoprotein 6	Other cells
PSG8	Pregnancy specific beta-1-glycoprotein 8	Other cells

PSG9	Pregnancy specific beta-1-glycoprotein 9	Other cells
SERPINE2	Serpin family E member 2	Other cells
SIGLEC6	Sialic acid binding Ig like lectin 6	Other cells
SKP2	S-phase kinase associated protein 2	Decidual macrophages
SLC13A4	Solute carrier family 13 member 4	Decidual macrophages
TAC3	Tachykinin 3	Other cells
TFPI2	Tissue factor pathway inhibitor 2	Other cells
TRIM64	Tripartite motif containing 64	Other cells
TRIM64B	Tripartite motif containing 64B	Hofbauer cells & decidual macrophages
VGLL1	Vestigial like family member 1	Hofbauer cells
WNT3A	Wnt family member 3A	Other cells
XAGE2	X antigen family 2	Other cells
XAGE3	X antigen family 3	Other cells

Table II: Placenta localization of proteins encoded by genes highly regulated by HIV-1 infection of MDM.

Gene Symbol	Gene Name	Placental cell localization
ABCA1	ATP binding cassette subfamily A member 1	Decidual macrophages
ABHD6	abhydrolase domain containing 6	Decidual macrophages
ADAMDEC1	ADAM like decysin 1	Other cells
ADORA2A	adenosine A2a receptor	Other cells
AKR1B1	aldo-keto reductase family 1 member B	Other cells
AMPD3	adenosine monophosphate deaminase 3	Other cells
ASF1B	anti-silencing function 1B histone chaperone	Other cells
BCAT1	branched chain amino acid transaminase 1	Other cells
BOK	BOK, BCL2 family apoptosis regulator	Other cells
C15orf48	chromosome 15 open reading frame 48	Other cells
C15orf48	chromosome 15 open reading frame 48	Other cells
CACNB4	calcium voltage-gated channel auxiliary subunit beta 4	Other cells
CCDC85C	coiled-coil domain containing 85C	Decidual macrophages
CCND1	cyclin D1	Other cells
CDC20	cell division cycle 20	Hofbauer cells
CDK1	cyclin dependent kinase 1	Decidual macrophages

CENPM	centromere protein M	Other cells
CEP55	centrosomal protein 55	Other cells
CHCHD7	coiled-coil-helix-coiled-coil-helix domain containing 7	Other cells
COQ2	coenzyme Q2, polyprenyltransferase	Other cells
CTNNAL1	catenin alpha like 1	Other cells
CTSD	cathepsin D	Other cells
CXCL8	C-X-C motif chemokine ligand 8	Other cells
CXCL8	C-X-C motif chemokine ligand 8	Other cells
DACT1	dishevelled binding antagonist of beta catenin 1	Other cells
DLGAP5	DLG associated protein 5	Other cells
DLGAP5	DLG associated protein 5	Other cells
DNASE1L3	Deoxy-ribonuclease 1 like 3	Both Hofbauer cells & decidual macrophages
DRAM1	DNA damage regulated autophagy modulator 1	Other cells
EBI3	Epstein-Barr virus induced 3	Other cells
ECHDC3	enoyl-CoA hydratase domain containing 3	Other cells
EHD1	EH domain containing 1	Other cells
EMP1	epithelial membrane protein 1	Other cells
ERI1	exoribonuclease 1	Decidual macrophages
F13A1	coagulation factor XIII A chain	Hofbauer cells
FCHO1	FCH domain only 1	Other cells

FN1	fibronectin 1	Hofbauer cells
GK	glycerol kinase	Other cells
GPSM2	G-protein signaling modulator 2	Other cells
GRAMD1A	GRAM domain containing 1A	Other cells
HADHB	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	Other cells
HINT3	histidine triad nucleotide binding protein 3	Decidual macrophages
HMMR	hyaluronan mediated motility receptor	Other cells
IER3	immediate early response 3	Other cells
INSIG1	insulin induced gene 1	Other cells
IRAK2	interleukin 1 receptor associated kinase 2	Decidual macrophages
IRF1	interferon regulatory factor 1	Other cells
ITGAV	integrin subunit alpha V	Other cells
KIAA0101	KIAA0101	Other cells
KIF20B	kinesin family member 20B	Decidual macrophages
KLF4	Kruppel-like factor 4	Hofbauer cells
MAP1S	microtubule associated protein 1S	Other cells
MELK	maternal embryonic leucine zipper kinase	Other cells
MMP9	matrix metallopeptidase 9	Other cells

MTF1	metal regulatory transcription factor 1	Other cells
NFKB2	nuclear factor kappa B subunit 2	Other cells
OGFRL1	opioid growth factor receptor like 1	Other cells
OIP5	Opa interacting protein 5	Other cells
OSBPL1A	oxysterol binding protein like 1A	Other cells
PCM1	pericentriolar material 1	Other cells
PCSK5	Pro-protein convertase subtilisin/kexin type 5	Other cells
PDPN	podoplanin	Other cells
PNKD	paroxysmal nonkinesigenic dyskinesia	Other cells
POLQ	DNA polymerase theta	Other cells
PPP1R14A	protein phosphatase 1 regulatory inhibitor subunit 14A	Hofbauer cells
PPP1R18	protein phosphatase 1 regulatory subunit 18	Hofbauer cells
PRC1	protein regulator of cytokinesis 1	Other cells
PTGFRN	prostaglandin F2 receptor inhibitor	Other cells
RAB3IL1	RAB3A interacting protein like 1	Other cells
RAMP1	receptor activity modifying protein 1	Other cells
RBM11	RNA binding motif protein 11	Other cells
RRP1B	ribosomal RNA processing 1B	Both Hofbauer cells & decidual macrophages

SASH3	SAM and SH3 domain containing 3	Other cells
SIGLEC8	sialic acid binding Ig like lectin 8	Other cells
SLC22A15	solute carrier family 22 member 15	Other cells
SLC2A6	solute carrier family 2 member 6	Other cells
SLC43A3	solute carrier family 43 member 3	Decidual macrophages
SNTB1	syntrophic beta 1	Decidual macrophages
SOD2	superoxide dismutase 2, mitochondrial	Other cells
STARD10	StAR related lipid transfer domain containing 10	Other cells
TMEM130	transmembrane protein 130	Other cells
TNFAIP3	TNF alpha induced protein 3	Decidual macrophages
TNFSF15	tumor necrosis factor superfamily member 15	Decidual macrophages
TNFSF9	tumor necrosis factor superfamily member 9	Other cells
TNIK	TRAF2 and NCK interacting kinase	Other cells
TNIP1	TNFAIP3 interacting protein 1	Other cells
TOP2A	topoisomerase (DNA) II alpha	Other cells
TPX2	TPX2, microtubule nucleation factor	Other cells
TRAF5	TNF receptor associated factor 5	Other cells

TREM2	triggering receptor expressed on myeloid cells 2	Other cells
TTC39B	tetratricopeptide repeat domain 39B	Decidual macrophages
UBE2C	ubiquitin conjugating enzyme E2 C	Decidual macrophages
UHRF1	ubiquitin like with PHD and ring finger domains 1	Other cells
WFS1	wolframin ER transmembrane glycoprotein	Other cells
ZC3H12A	zinc finger CCCH-type containing 12A	Other cells
ZDHHC19	zinc finger DHHC-type containing 19	Other cells
ZSWIM4	zinc finger SWIM-type containing 4	Other cells

Table III: Placenta localization of proteins encoded by genes highly regulated by IL-4 on MDM.

Gene Symbol	Gene Name	Placental cell localization
ACAA2	acetyl-CoA acyltransferase 2	Other cells
ACTN1	actinin, alpha 1	Other cells
ADSL	adenylosuccinate lyase	Other cells
AIF1	allograft inflammatory factor 1	Hofbauer cells
ALDH1A2	aldehyde dehydrogenase 1 family, member A2	Other cells

ALOX5AP	Arachidonate 5-lipoxygenase-activation protein	Both Hofbauer cells & decidual macrophages
ANXA4	annexin A4	Other cells
APOC2	Apopoliprotein C-II	Other cells
APOE	apopoliprotein E	Other cells
AQP9	Aquaporin 9	Other cells
ATP5C1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	Other cells
BCAR3	Breast cancer anti-estrogen resistance 3	Other cells
CBR1	carbonyl reductase 1	Other cells
CD14	CD14 Molecule	Both Hofbauer cells & decidual macrophages
CD37	CD37 molecule	Other cells
CFL1	cofilin 1 (non-muscle)	Hofbauer cells
CH25H	Cholesterol 25-hydroxylase	Other cells
CISH	Cytokine inducible SH2-containing protein	Other cells
CLTA	clathrin, light chain A	Both Hofbauer cells & decidual macrophages
COTL1	coactosin-like 1 (Dictyostelium)	Hofbauer cells
COX6B1	cytochrome c oxidase subunit Vib polypeptide 1	Other cells
CSTB	cystatin B (stefin B)	Other cells
CTSA	cathepsin	Decidual macrophages
CTSD	cathepsin D	Other cells
CTSS	cathepsin S	Other cells

CXCL2	Chemokine (C-X-C motif) ligand 2	Other cells
CYBB	cytochrome b-245, beta polypeptide	Hofbauer cells
CYFIP1	cytoplasmic FMR1 interacting protein 1	Other cells
DBI	diazepam binding inhibitor (GABA receptor modulator)	Other cells
DDOST	dolichyl-diphosphooligosaccharide-proetin glycosltransferase	Other cells
DPYSL2	dihydropyrimidinase-like 2	Hofbauer cells
DUSP6	Dual specificity phosphatase 6	Other cells
EIF4E	eukaryotic translation initiation factor 4E	Other cells
EIF5A	eukaryotic translation initiation factor 5A	Decidual macrophages
ESD	esterase D	Other cells
ESYT1	extended synaptotagmin-like proetin 1	Other cells
GARS	glycyl-tRNA sythetase	Other cells
GLB1	galactosidase, beta 1	Other cells
GLS	glutaminase	Other cells
GRB2	growth factor-bound protein 2	Hofbauer cells
GRN	granulin	Decidual macrophages
GSR	glutathione reductase	Other cells
GUSB	glucurinidase, beta	Other cells
HIST1H1C	histone cluster 1, H1c	Both Hofbauer cells & decidual macrophages
HK3	hexokinase 3 (white cell)	Hofbauer cells

HMOX2	heme oxygenase (decycling) 2	Decidual macrophages
HSPA9	heast shock 70kDa proetin (mortalin)	Decidual macrophages
IFITM3	Interferon induced transmembrane proetin 3	Other cells
IRF4	Interferon regulatory factor 4	Other cells
ITGAM	integrin, alpha M (complement comonent 3 receptor 3 subunit)	Decidual macrophages
KLF4	Kruppel-like factor 4 (gut)	Both Hofbauer cells & decidual macrophages
KLHL6	Kelch-like 6	Other cells
KRT1	keratin 1	Other cells
LPAR6	Lysophosphatidic acid receptor	Other cells
LRPAP1	low density lipoproetin receptor-related proetin associated proetin 1	Decidual macrophages
MRC1	Mannose receptor, C type 1	Both Hofbauer cells & decidual macrophages
MYC	v-myc myelocytomatosis viral oncogene homolog	Other cells
NAGA	N-acetylgalactosominidase, alpha	Hofbauer cells
NCL	nucleolin	Both Hofbauer cells & decidual macrophages
NDUFA4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9kDa	Other cells
NUCB1	nucleobinding 1	Other cells

OTUB1	OUT domain, ubiquitin aldehyde binding 1	Other cells
PDCD1LG2	Programmed cell death 1 ligand 2	Other cells
PLD3	phospholipase D family, member 3	Other cells
PPIA	peptidylprolyl isomerase A (cyclophilin A)	Other cells
PPT1	palmitoyl-protein thioesterase 1	Other cells
PRDX1	peroxiredoxin 1	Hofbauer cells
PRDX3	peroxiredoxin 3	Other cells
PSMD2	proteosome 26S subunit, non-ATPase, 2	Other cells
PTGS1	Prostaglandin-endoperoxidase synthase 1	Other cells
PTK2B	PTK2B protein tyrosine 2 beta	Other cells
PTPRC	protein tyrosine phosphatase, receptor type C	Other cells
PYCARD	PYD and CARD domain containing	Hofbauer cells
RGL1	Ral guanin nucleotide dissociation stimulator-like 1	Other cells
RPL4	ribosomal protein L4	Other cells
RPS10	ribosomal protein S10	Other cells
RPS15	ribosomal protein S15	Decidual macrophages
RPS23	ribosomal protein S23	Other cells
S100A11	S100 calcium binding protein A11	Other cells

S100A8	S100 calcium binding protein A8	Other cells
SEPHS2	Selenophosphate synthetase 2	Other cells
SEPP1	selenoprotein P, plasma 1	Hofbauer cells
SERPINB6	serpin peptide inhibitor, clade B, member 6	Other cells
SHMT2	Serine hydroxymethlytransferase 2	Decidual macrophages
SLA	Src-like-adapter	Other cells
SLC9A9	Solute carrier family 9, member 9	Other cells
SNX2	sorting nexin 2	Both Hofbauer cells & decidual macrophages
SOCS1	Suppressor of cytokine signaling 1	Other cells
SORL1	Sortilin-related receptor, L(DLR class) A repeats containing	Other cells
SPARC	secreted protein, acidic, cysteine-rich	Other cells
TAPBP	TAP binding protein (tapasin)	Other cells
TBCB	tubulin folding cofactor B	Other cells
TFRC	transferrin receptor (p90, CD71)	Decidual macrophages
TGM2	Transglutaminase 2	Other cells
TXNRD1	thioredixin reductase 1	Other cells
UBA1	ubiquitin-like modifier activating enzyme 1	Decidual macrophages
UBE2V1	ubiquitin-conjugating enzyme E2 variant 1	Other cells

VPS35	vacuolar protein sorting 35 homolog (S. cerevisiae)	Other cells

Table IV: Placenta localization of proteins encoded by genes highly-regulated by IL-10 stimulation on MDM.

Gene Symbol	Gene Name	Placental cell localization
ACADVL	Acyl-CoA dehydrogenase, very long chain	Other cells
ACAT1	Acetyl-CoA acetyltransferase 1	Both Hofbauer cells & decidual macrophages
ACTL6A	Actin like 6A	Other cells
ADORA2A	Adenosine A2a receptor	Other cells
AKR1B1	Aldo-keto reductase family 1 member B	Other cells
APOPT1	Apoptogenic 1, mitochondrial	Other cells
ATG4C	Autophagy related 4C cysteine peptidase	Other cells
C12orf57	chromosome 12 open reading frame 57	Other cells
C16orf59	Chromosome 16 open reading frame 59	Other cells
C1S	Complement component 1, s subcomponent	Other cells
C3orf38	Chromosome 3 open reading frame 38	Other cells
CBWD5	COBW domain containing 5	Hofbauer cells
CD274	CD274 molecule	Other cells
CDK1	Cyclin dependent kinase 1	Decidual macrophages
CENPM	Centromere protein M	Other cells

CHCHD7	Coiled-coil-helix-coiled-coil-helix domain containing 7	Other cells
CHD8	Chromodomain helicase DNA binding protein 8	Other cells
CHMP3	Charged multi-vesicular body protein 3	Other cells
CNIH1	Cornichon family AMPA receptor auxiliary protein 1	Other cells
COQ2	coenzyme Q2, polyprenyltransferase	Other cells
COX20	COX20, cytochrome c oxidase assembly factor	Other cells
DLGAP5	DLG associated protein 5	Other cells
DNASE2B	Deoxyribonuclease 2 beta	Other cells
DYNC1H1	dynein cytoplasmic 1 heavy chain 1	Other cells
ECI2	Enoyl-CoA delta isomerase 2	Other cells
ERAP2	Endoplasmic-reticulum aminopeptidase 2	Decidual macrophages
ESD	Esterase D	Other cells
ETNK1	Ethanolamine kinase 1	Other cells
FBXO8	F-box protein 8	Other cells
FGD5	FYVE, RhoGEF and PH domain containing 5	Other cells
FOXO3	Fork head box O3	Other cells
GNB4	G protein subunit beta 4	Other cells
GPSM2	G-protein signaling modulator 2	Other cells
GTSE1	G2 and S-phase expressed 1	Other cells

H2AFZ	H2A histone family member Z	Both Hofbauer cells & decidual macrophages
HADH	Hydroxyacyl-CoA dehydrogenase	Hofbauer cells
HINT3	Histidine triad nucleotide binding protein 3	Decidual macrophages
HMGB2	High mobility group box 2	Other cells
HMGN1	High mobility group nucleosome binding domain 1	Both Hofbauer cells & decidual macrophages
HMMR	Hyaluronic mediated motility receptor	Other cells
IRF1	Interferon regulatory factor 1	Other cells
ITGAV	Integrin subunit alpha V	Decidual macrophages
KIAA0101	KIAA0101	Other cells
KIF15	Kinesin family member 15	Other cells
KIF20B	kinesin family member 20B	Hofbauer cells
KPNA6	Karyopherin subunit alpha 6	Other cells
LBR	Lamin B receptor	Both Hofbauer cells & decidual macrophages
LOC101926963	Uncharacterized LOC101926963	Other cells
LRRFIP1	LRR binding FLII interacting protein 1	Other cells
LSS	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	Other cells
LYPLAL1	Lysophospholipase like 1	Decidual macrophages
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	Other cells

MAP3K7	Mitogen-activated protein kinase kinase kinase 7	Other cells
MAPK3	Mitogen-activated protein kinase 3	Both Hofbauer cells & decidual macrophages
MAT2B	Methionine adenosyltransferase 2B	Other cells
MAX	MYC associated factor X	Decidual macrophages
MDFIC	MyoD family inhibitor domain containing	Other cells
MMP12	Matrix metallopeptidase 12	Other cells
MMP14	Matrix metallopeptidase 14	Hofbauer cells
MORC3	MORC family CW-type zinc finger 3	Hofbauer cells
MRPL18	mitochondrial ribosomal protein L18	Decidual macrophages
MTMR11	Myotubularin related protein 11	Decidual macrophages
NUSAP1	Nucleolar and spindle associated protein 1	Other cells
OIP5	Opa interacting protein 5	Other cells
OIP5	Opa interacting protein 5	Other cells
OPLAH	5-oxoprolinase (ATP-hydrolysing)	Other cells
PGK1	phosphoglycerate kinase 1	Other cells
PLXDC2	Plexin domain containing 2	Other cells
POLQ	DNA polymerase theta	Other cells
PPA1	Pyrophosphatase (inorganic) 1	Other cells

PPIAL4A	peptidylprolyl isomerase A like 4A	Other cells
PRDX3	Peroxiredoxin 3	Hofbauer cells
RAD51AP1	RAD51 associated protein 1	Other cells
RFC4	Replication factor C subunit 4	Both Hofbauer cells & decidual macrophages
ROCK2	Rho associated coiled-coil containing protein kinase 2	Other cells
RRM1	Ribonucleotide reductase catalytic subunit M1	Other cells
SEMA3E	Semaphorin 3E	Decidual macrophages
SH3BGR	SH3 domain binding glutamate rich protein	Other cells
SLC44A1	Solute carrier family 44 member 1	Other cells
SRP9	signal recognition particle 9	Other cells
STARD10	StAR related lipid transfer domain containing 10	Other cells
STMN1	Stathmin 1	Hofbauer cells
TAGLN3	Transgelin 3	Other cells
TCEAL8	Transcription elongation factor A like 8	Other cells
TNFSF15	Tumor necrosis factor superfamily member 15	Decidual macrophages
TNFSF9	Tumor necrosis factor superfamily member 9	Other cells
TOP2A	Topoisomerase (DNA) II alpha	Other cells
TP53BP2	Tumor protein p53 binding protein 2	Decidual macrophages

TPX2	TPX2, microtubule nucleation factor	Decidual macrophages
TRIM16L	Tripartite motif containing 16-like	Other cells
TROVE2	TROVE domain family member 2	Other cells
TYW3	tRNA-yW synthesizing protein 3 homolog	Other cells
UBE2C	Ubiquitin conjugating enzyme E2 C	Decidual macrophages
XPNPEP3	X-prolyl aminopeptidase 3	Other cells
ZC3H12A	Zinc finger CCCH-type containing 12A	Other cells
ZFAND6	Zinc finger AN1-type containing 6	Other cells
ZMAT3	Zinc finger matrin-type 3	Other cells
ZNF394	Zinc finger protein 394	Other cells
ZWINT	ZW10 interacting kinetochore protein	Other cells

Table V: Hofbauer Cell and decidual macrophage localization of 100 proteins encoded by genes highly regulated by TNF-α (12.5 ng/ml) and IFN-γ (250 U/ml) on MDM.

Gene Symbol	Gene Name	Placental cell localization
ACADVL	acyl-CoA dehydrogenase, very long chain	Other cells
ANLN	anillin actin binding protein	Other cells
APBA3	amyloid beta precursor protein binding family A member 3	Other cells
APOBR	apolipoprotein B receptor	Other cells

ARAP3	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3	Other cells
ARRB1	arrestin beta 1	Other cells
ASF1B	anti-silencing function 1B histone chaperone	Other cells
ASPM	abnormal spindle microtubule assembly	Other cells
ATP2A3	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 3	Other cells
AURKB	aurora kinase B	Other cells
BIRC5	baculoviral IAP repeat containing 5	Other cells
BUB1	BUB1 mitotic checkpoint serine/threonine kinase	Other cells
C12orf57	chromosome 12 open reading frame 57	Other cells
C16orf59	chromosome 16 open reading frame 59	Other cells
CAMSAP2	calmodulin regulated spectrin associated protein family member 2	Decidual macrophages
CARHSP1	calcium regulated heat stable protein 1	Other cells
CASC3	cancer susceptibility candidate 3	Other cells
CCDC14	coiled-coil domain containing 14	Other cells
CCM2	CCM2 scaffolding protein	Decidual macrophages
CCNA2	cyclin A2	Other cells
CCNB1	cyclin B1	Other cells

CCNB2	cyclin B2	Other cells
CCNF	cyclin F	Other cells
CD6	CD6 molecule	Other cells
CDC20	cell division cycle 20	Other cells
CDCA3	cell division cycle associated 3	Other cells
CDCA5	cell division cycle associated 5	Other cells
CDK1	cyclin dependent kinase 1	Decidual macrophages
CENPE	centromere protein E	Other cells
CENPM	centromere protein M	Other cells
CENPU	centromere protein U	Other cells
CEP55	centrosomal protein 55	Other cells
CKAP2L	cytoskeleton associated protein 2 like	Hofbauer cell
CKS2	CDC28 protein kinase regulatory subunit 2	Other cells
CLIC2	chloride intracellular channel 2	Other cells
DLGAP5	DLG associated protein 5	Other cells
E2F2	E2F transcription factor 2	Other cells
FDFT1	farnesyl-diphosphate farnesyltransferase 1	Other cells
FGD5	FYVE, RhoGEF and PH domain containing 5	Other cells
GTSE1	G2 and S-phase expressed 1	Other cells
H2AFZ	H2A histone family member Z	Hofbauer cells & decidual macrophages
HELLS	helicase, lymphoid-specific	Other cells
HIST1H4C	histone cluster 1, H4c	Other cells

HJURP	Holliday junction recognition protein	Other cells
HMGB2	high mobility group box 2	Other cells
HMMR	hyaluronan mediated motility receptor	Other cells
HVCN1	hydrogen voltage gated channel 1	Other cells
INSIG1	insulin induced gene 1	Other cells
IRF1	interferon regulatory factor 1	Other cells
KIAA0101	KIAA0101	Other cells
KIF11	kinesin family member 11	Other cells
KIF14	kinesin family member 14	Decidual macrophages
KIF15	kinesin family member 15	Other cells
KIF20A	kinesin family member 20A	Hofbauer cells
KIF20B	kinesin family member 20B	Hofbauer cells
KIF23	kinesin family member 23	Other cells
LDHB	lactate dehydrogenase B	Other cells
LOC101926963	uncharacterized LOC101926963	Other cells
LSM10	LSM10, U7 small nuclear RNA associated	Other cells
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	Other cells
MCM10	minichromosome maintenance 10 replication initiation factor	Other cells
MELK	maternal embryonic leucine zipper kinase	Other cells
MTMR10	myotubularin related protein 10	Other cells

NCAPG	non-SMC condensin I complex subunit G	Decidual macrophages
NDC80	NDC80, kinetochore complex component	Other cells
NUSAP1	nucleolar and spindle associated protein 1	Other cells
OIP5	Opa interacting protein 5	Other cells
OPLAH	5-oxoprolinase (ATP-hydrolysing)	Other cells
PARPBP	PARP1 binding protein	Other cells
PLK4	polo like kinase 4	Other cells
PLXDC2	plexin domain containing 2	Other cells
POLQ	DNA polymerase theta	Other cells
POLQ	DNA polymerase theta	Other cells
PRC1	protein regulator of cytokinesis 1	Other cells
PRKCDBP	protein kinase C delta binding protein	Other cells
RFC4	replication factor C subunit 4	Other cells
RILP	Rab interacting lysosomal protein	Other cells
SASH1	SAM and SH3 domain containing 1	Hofbauer cells & decidual macrophages
SKP2	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	Decidual macrophages
SLC18B1	solute carrier family 18 member B1	Other cells
STIL	SCL/TAL1 interrupting locus	Other cells
STMN1	stathmin 1	Hofbauer cells

SUZ12	SUZ12 polycomb repressive complex 2 subunit	Other cells
TK1	thymidine kinase 1	Other cells
TOP2A	topoisomerase (DNA) II alpha	Other cells
TPX2	TPX2, microtubule nucleation factor	Other cells
TROAP	trophinin associated protein	Other cells
TTK	TTK protein kinase	Other cells
TYMS	thymidylate synthetase	
UBE2C	ubiquitin conjugating enzyme E2 C	Decidual macrophages
ZNF385A	zinc finger protein 385A	Other cells
ZNF428	zinc finger protein 428	Other cells

9.2. Appendix B: Protocol for leukocyte isolation from the maternal-foetal interface

1. For decidua parietalis macrophage-isolation, dissect a piece of the chorioamniotic membrane. Remove amnion and carefully scrape the DP from the chorion using fine-point forceps. Place DP tissue into a 50ml falcon tube.
2. For decidua basalis macrophage-isolation, dissect chorionic villi from the basal plate of the placenta and carefully scrape off the villi from the foetal side. Place the remaining DB tissue into a 50ml falcon tube.
3. For Hofbauer cell isolation, dissect the chorionic villi of the placenta, free of contamination by the decidua basalis and place into a 50ml falcon tube.
4. Fill the falcon tubes containing decidual tissue and chorionic villi with 1x PBS to remove blood clots, and mince the tissues into small pieces using scissors.
5. When the supernatant is clear, fill the tubes with RPMI medium containing 1x penicillin-streptomycin (P/S) and wash once (1000 rpm for 1 minute).
6. Remove the supernatant and digest the remaining placental tissue in 1% collagenase IV + 0.1% DNase I in RPMI medium (10 ml per 5ml of tissue) and incubate for 75 minutes at 37^{o}C in a shaking water bath.
7. Prepare the following Percoll solutions, 70% in RPMI 1640 medium with 1xP/S, 45% in 1xPBS, 50% in RPMI 1640 medium with 1xP/S). Prepare the Percoll gradients in a 50ml tube (1 gradient per 5ml of original sample) as follows:
    a) 10 ml of 70% Percoll solution
    b) Carefully pipette 15 ml of the 45% Percoll solution on top to form 2 phases. Keep in 4^{o}C until sample is ready.
8. After digestion, fill up the sample tubes with RPMI containing 10% FCS and 1x P/S and wash once (1800 rpm, 7 min).
9. Remove the supernatant and re-suspend the remaining pellet in 15-20 ml of serum free RPMI medium containing 1x P/S and 0.1% DNase I.
10. Filter the sample first in the metallic strainer to the big petri plate and then collect the filtrate and filter in 3 successive filters - 100µm pore, 70µm pore

and 40 µm pore into 50ml Falcon tubes. Use a bulb pipette to stir the tissue in the filter, and add media during the process to help the filtration.

11. Fill up the last collection tube with serum free RPMI medium containing 1x P/S and wash once (1800rpm, 7 min).
12. After washing, remove the supernatant and re-suspend the sample in 10 ml of serum free RPMI medium containing 1x P/S (per 5ml of original sample), and add 10ml of 50% Percoll solution (per 5ml of original sample), to obtain a 25% solution.
13. Carefully pipette 20ml of the mixture of sample, media and 50% Percoll solution on top of the 45% Percoll phase per tube.
14. Carefully pipette 5ml of PBS on top of the sample phase.
15. Centrifuge at 2000 rpm, 25 min without acceleration and deceleration. After spinning, the tube will look like Figure 3.2.1.
16. Remove the top layer with the junk, and use bulb pipettes to collect the cell rings to 50ml Falcon tubes. Use more tubes per sample if collecting a lot of Percoll. Fill the tubes with serum free RPMI medium containing 1x P/S and wash once (7 min, 2000 rpm).
17. Combine the tubes from the same samples into one, refill with RPMI medium containing 1x P/S and wash again (7 min, 1800 rpm).
18. Placenta-isolated cells are now ready for subsequent experiments.

9.3. Appendix C: Protocol for tissue processing using Leica TP 1020 Processor

Formalin-fixed placental tissues were trimmed and placed onto labeled-cassettes for processing. Each sample had to go through the following reactions on the processor:

1. 10% formalin - Fixative
2. 10% formalin – 2 hours
3. 70% ethanol – 2 hours
4. 96% ethanol – 2 hours
5. 96% ethanol – 2 hours
6. 100% ethanol – 2 hours
7. 100% ethanol – 2 hours
8. 100% ethanol – 2 hours
9. 100% ethanol – 2 hours
10. Xylene – 2 hours
11. Xylene – 2 hours
12. Wax (55-60^{o}C) with vacuum – 2 hours
13. Wax (55-60^{o}C) with vacuum – 2 hours
14. Samples are now ready for embedding on the Leica EG1140H.

9.3.1. Mayer's Hematoxylin and Eosin (H&E) staining Protocol

Preparing Mayer's Hematoxylin stock solution:

1. Add 1g of Mayer's Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) into 1 Liter of distilled water and allow to dissolve by stirring gently.
2. Add 50g of Potassium Sulphate (Sigma-Aldrich, St. Louis, MO, USA).
3. Add 0.2g of Sodium Iodate (Sigma-Aldrich, St. Louis, MO, USA).
4. Add 1g Citric acid (Sigma-Aldrich, St. Louis, MO, USA) and allow the solvents to dissolve by stirring gently.
5. Add 50g Chloral hydrate (Saarchem Merck Chemicals, Gauteng, SA).

6. Mix well and allow solution to cool. Filter and store in the dark at room temperature.

Preparing Eosin Y stock solution:

1. Add 1% Eosin Y aqueous (Sigma-Aldrich, St. Louis, MO, USA) and 1g Eosin Y dye (Sigma-Aldrich, St. Louis, MO, USA) into 100ml distilled water.
2. Add 1% of Phloxine B (Sigma-Aldrich, St. Louis, MO, USA).
3. Mix well and store in the dark at room temperature.

Staining Method:

1. Dip slides into 3x Xylene and 3x absolute alcohol solutions for 1 minutes in each.
2. Dip slides into 96% ethanol for 2 minutes and then 70% ethanol for 2 minutes.
3. Rinse slides using tap water.
4. Dip slides into Mayer's Hematoxylin solution for 9 minutes.
5. Rinse slides using tap water.
6. Dip slides into Scott's water (Sigma-Aldrich, St. Louis, MO, USA) for 3 minutes.
7. Dip slides into 1% Eosin solution for 3 minutes.
8. Rinse slides using tap water.
9. Dip slides into 70% and 100% ethanol for 3 minutes respectively.
10. Mount slides using Entellan and store in the dark at room temperature until image acquisition.

9.3.2. Immunohistochemistry staining protocol

1. Place formalin-fixed paraffin-embedded slides in an incubator at 56^0C for at least 2 hours.
2. Dip slides into 3x Xylene and 3x absolute alcohol solutions for 1 minutes in each.
3. Antigen retrieval, for CD68 (Abcam 49777 mouse monoclonal antibody) and CD209 (Abcam 5715 rabbit polyclonal) staining, incubate slides in a pre-heated pressure cooker containing 10mM Tris/EDTA buffer (pH9) for 2 minutes. For CD163 (Abcam 189915 rabbit monoclonal) and CD206 (Abcam 1176441

mouse monoclonal) staining, incubate slides in a pre-heated pressure cooker containing 10nM Citrate buffer (pH6) for 2 minutes.

4. Cool slides to room temperature using tap water.
5. Wash slides using PBST.
6. For CD68 and CD163, incubate slides with primary antibodies at 1:100 and 1:500 dilutions respectively for 90 minutes at room temperature. For CD206: incubate slides with CD206 primary antibody at 1:100 dilutions overnight at 4^{o}C. For CD209: incubate slides with CD209 primary antibody at 1:100 dilutions for 90 minutes at room temperature.
7. Wash slides using PBST.
8. For CD68 and CD206, incubate slides with Dako anti-mouse Envision (Dako K4001) for 30 minutes at room temperature. For CD163 and CD209, incubate slides with Dako anti-Rabbit Envision (Dako K4003) for 30 minutes at room temperature.
9. Wash slides using PBST.
10. Detect by staining with the DAB Substrate (Dako K3468) for 10 minutes.
11. Wash slides using tap water.
12. Counterstain slides with Mayer's Hematoxylin for 4 minutes.
13. Wash slides with tap water.
14. Dip slides into 3x clean tap water and 3x xylene solution for 1 minutes in each.
15. Coverslip using Entallan, seal and keep in the dark at room temperature until image acquisition.

9.3.3. Immunofluorescence staining protocol

1. Place formalin-fixed paraffin-embedded slides in an incubator at 56^{0}C for at least 2 hours.
2. Dip slides in 3x Xylene and 3x absolute alcohol solutions for 1 minute in each.
3. For antigen retrieval, place slides in a pre-heated pressure-cooker containing 10nM Citrate buffer (pH6) and allow pressure to build-up for 2 minutes.
4. Cool slides to room temperature using tap water.
5. Block non-specific binding of antibodies using 5% normal goat serum and incubate in a moist chamber for 60 minutes.

6. Wash slides with PBST.
7. Dry the slides and mark the location of your tissue using the hydrophobic Novo Pen (Leica Biosystems, Wetzler, Germany).
8. Incubate slides with primary antibodies in the dark at room temperature for 90 minutes.
9. Wash slides 3x with PBST.
10. Incubated slides with secondary antibodies in the dark at room temperature for 30 minutes.
11. Wash slides with PBST.
12. For intracellular staining, Permeabilize cells on the tissue section using 0.1% Triton X100. For 10 minutes.
13. Wash slides 3x with PBST.
14. Incubate slides with the second primary antibody and incubate in the dark at 4^{o}C overnight.
15. The following day, wash the slide with PBST. if the second primary antibody is not conjugated to a fluorophore. Detect accordingly using an appropriate secondary antibody for 30 minutes.
16. Wash the slides using PBST.
17. Stain with DAPI and incubate at room temperature for 20 minutes.
18. Wash the slides using PBST.
19. To quench tissue autofluorescence, incubate slides with 0.1% Sudan Black B in 70% ethanol in the dark for 10 minutes.
20. Wash slides using PBST.
21. Mount slides using fluorescence mounting medium.
22. Seal slides and store at 4^{o}C until acquisition.

9.4. Appendix D: Original Review Manuscript

Review Article

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The Elusive Role of Placental Macrophages: The Hofbauer Cell

Michael Z. Zulu[a] Fernando O. Martinez[b] Siamon Gordon[c, d] Clive M. Gray[a, e]

[a]Institute of Infectious Disease and Molecular Medicine, Division of Immunology, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa; [b]Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK; [c]Chang Gung University, Graduate Institute of Biomedical Sciences, College of Medicine, Taoyuan City, Taiwan; [d]Sir William Dunn School of Pathology, University of Oxford, Oxford, UK; [e]National Health Laboratory Services/Groote Schuur Hospital, Cape Town, South Africa

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Abstract
In this review, we discuss the often overlooked tissue-resident fetal macrophages, Hofbauer cells, which are found within the chorionic villi of the human placenta. Hofbauer cells have been shown to have a phenotype associated with regulatory and anti-inflammatory functions. They are thought to play a crucial role in the regulation of pregnancy and in the maintenance of a homeostatic environment that is crucial for fetal development. Even though the numbers of these macrophages are some of the most abundant immune cells in the human placenta, which are sustained throughout pregnancy, there are very few studies that have identified their origin, their phenotype, and functions and why they are maintained throughout gestation. It is not yet understood how Hofbauer cells may change in function throughout normal pregnancy, and especially in those complicated by maternal gestational diabetes, preeclampsia, and viral infections, such as Zika, cytomegalovirus, and human immunodeficiency virus. We review what is known about the origin of these macrophages and explore how common complications of pregnancy dysregulate these cells leading to adverse birth outcomes in humans. Our synthesis sheds light on areas for human studies that can further define these innate regulatory cells.

Introduction

Macrophages are tissue-resident immune cells in the same way as fibroblasts, MAIT cells, and Langerhans cells and perform crucial immunological functions such as antigen presentation, phagocytosis, cytokine secretion, and coordination of innate and adaptive immune responses [1]. Macrophages play a role in virtually every aspect of an organism's biology, from development and homeostasis to tissue repair, as well as immune responses to pathogens. They are a heterogeneous population of immune

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E-Mail karger@karger.com
www.karger.com/jin

Clive M. Gray
Institute of Infectious Disease and Molecular Medicine
Division of Immunology, Department of Pathology, University of Cape Town
Anzio Road, Level 3, Falmouth Building, Cape Town 7925 (South Africa)
E-Mail clive.gray@uct.ac.za

cells that constantly change their functional state in response to changes in tissue physiology or environmental challenges [2]. The plasticity of these cells allows them to respond to various environmental signals and change their phenotype and physiology in response to cytokines and microbial signals [3]. Historically, tissue-resident macrophages were believed to be continuously replenished by blood-circulating monocytes that originate from progenitors in adult bone marrow. This concept was central in defining the "mononuclear phagocyte system" that grouped together precursors of monocytes in the bone marrow, monocytes in peripheral blood, and macrophages in the tissues [4, 5]. However, advanced techniques for studying cellular ontogeny showed that the homeostatic contribution of peripheral blood monocytes to tissue-resident macrophage populations may be confined to specific tissues such as gut, dermis, and the heart with tissue-specific turnover rates. Alternatively, most tissue-resident macrophages arise from embryonic precursors prior to birth and maintain themselves locally throughout adulthood, independent of circulating monocytes [6, 7].

The chorionic villi of placental mammals has conspicuous macrophages known as the Hofbauer cells, which are considered fetal in origin [8]. Three-dimensional studies and histological analyses of human placental villous tissue show that Hofbauer cells are large (10–30 μm), pleomorphic, highly vacuolated with granular cytoplasm [9–11]. They are found within the fetal villi of the placenta from the first trimester of pregnancy until birth [12], and histological analyses show their location to be close to fetal vessels and trophoblasts, making them likely candidates for placental development and homeostasis [13]. These macrophages were named after an American gynecologist, J. Isfred Isidore Hofbauer (1879–1961); however, little is known about his research or work. (online suppl. Fig. 1 shows the only known portrait of J. Isfred Isidore Hofbauer, courtesy of Rubenstein Rare Book and Manuscript Library of Duke University, for all online suppl. material, see www.karger.com/doi/10.1159/000/497416). Although their origin has yet to be fully established, some studies have proposed that during the first trimester of pregnancy, they originate from mesenchymal progenitor cells [14–16], while the existence of transitional forms between monocytes and macrophages in the second and third trimester suggests that they differentiate from circulating monocytes of the fetus [17, 18]. Takahashi and colleagues reported that primitive macrophages appear as early as day 10 of gestation in the blood vessels of the chorionic villi of the mouse placenta, and that these macrophages enter the chorionic villous mesenchymal stroma and ingest fluid-like stromal materials to transform into Hofbauer cells [19]. Using chromogenic in situ hybridization for Y-chromosome (DYZ1), Kim and colleagues showed that placental villous tissues from male neonates had chromogenic in situ hybridization+ signals from Y chromosome in most macrophages, but not in lymphocytes, suggesting that most macrophages were of fetal origin [20].

Hofbauer cells are targets of a number of viruses and other pathogens at the maternal–fetal interface [21–23]. Better characterized are decidual macrophages, which are the second most abundant immune cell type in the uterine decidua after decidual natural killer cells [24, 25]. No studies appear to exist that directly compare the numbers and function of HCs and decidual macrophages. In fact, characterizing the role of Hofbauer cells in protecting the fetus and maintaining tolerance has somehow been neglected, especially when there is an adverse pregnancy outcome.

Results

Classical and Alternative Polarization of Hofbauer Cells

The functional maturation of macrophages was described in a manner similar to the well-characterized concept of T helper type 1and type 2 polarization of effector T cells [26, 27]. They have been classified as M1 (classically activated) and M2 (alternatively activated) macrophages [28, 29], with M1-like macrophages secreting proinflammatory cytokines and mediating resistance to pathogens, but also contributing to tissue destruction; while M2 macrophages secrete anti-inflammatory cytokines and promote tissue repair and remodeling [29, 30]. However, this paradigm of macrophage activation is simplistic, especially in humans [2]. This definition only considered the effects of particular stimuli on macrophage polarization, neglecting complex mechanisms that leads to macrophage activation in different tissues. A great variety of intermediates have been described based on their plasticity and adaptability [3] so that this concept may need to be revised. However, a comprehensive classification that will consider in vitro stimulus activation, cell origin, tissue microenvironment, pathology, and time is still required [2]. One M1-like phenotype has been described and compared to several M2 phenotypes, such as M2a, M2b, M2c, and M2d [31, 32], where M2b macrophages share characteristics with M1-like macrophages [33]. Each of the M2 macrophage subtypes differs in a

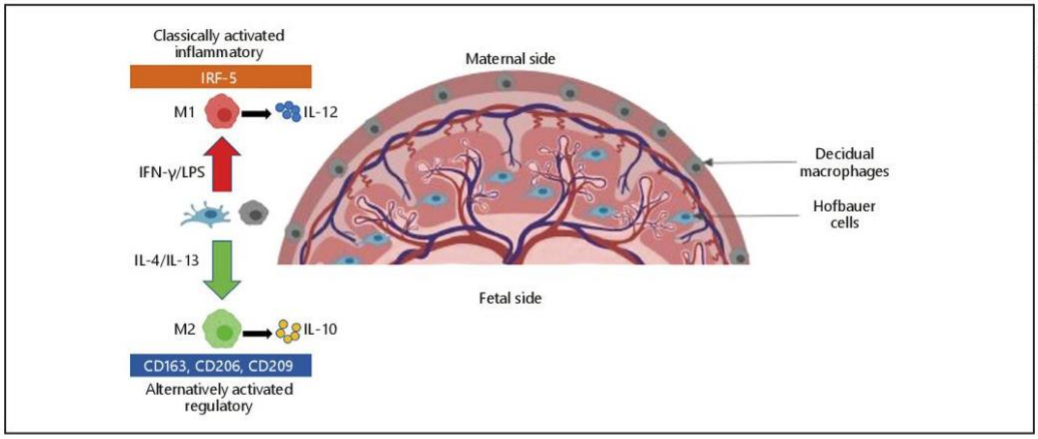

Fig. 1. Anatomical location of decidual macrophages and Hofbauer cells in the placenta. M1 macrophages are distinguished by the lack of expression of M2 markers. M1 macrophages secrete high levels of IL-12 while M2 macrophages secrete high levels of the IL-10 cytokine upon polarization.

number of aspects such as expression of certain surface molecules, cytokine secretion, and function [3]. Although oversimplified, the M1/M2 paradigm provides a useful framework, especially for specific immune responses. Defining the phenotype of HCs within this spectrum of macrophage polarity will illuminate the functional role of these cells in the placenta. Do HCs express M1-like markers, as they protect the fetus from maternally transmitted infections, or do they express an M2-like phenotype, to promote tolerance? Or, do these cells coexpress M1/M2 markers depending on the role being played at any given time?

Figure 1 shows the location of Hofbauer cells and decidual macrophages in the chorionic villi and the decidua of the placenta, respectively. Based on the M1/M2 macrophage polarization paradigm, IL-4 or IL-13 would polarize these macrophages toward a regulatory, M2 phenotype that is characterized by the expression of the scavenger receptor, CD163; mannose receptor, CD206; DC-SIGN, CD209; and secretion of IL-10. Whereas, IFN-γ or LPS would polarize these macrophages toward a proinflammatory, M1 phenotype that is characterized by the lack of expression of markers associated with the M2 phenotype, IRF-5 expression and high secretion of proinflammatory cytokines, such as IL-12 [34–37]. Apart from placental-tissue localization, there are no specific-markers to distinguish decidual macrophages from HCs and all other human tissue macrophages. To understand the developmental nature of macrophages in the placenta, and hence how they contribute to maternal–fetal tolerance, there is a need to distinguish between these macrophage populations.

The Role of Hofbauer Cells in Pregnancy

During pregnancy, the fetus with both maternal and paternal alleles develops within an active maternal immune system without succumbing to immunological rejection [38, 39]. Medawar [40] proposed the presence of immunological tolerance toward the semi-allogeneic fetus that facilitates pregnancy; however, this mechanism is yet to be fully understood. Macrophages have been shown to play a role in regulating pregnancy [41], promote tolerance toward the semi-allogeneic fetus [42], and maintain a homeostatic environment crucial for normal fetal development [38, 43]. In a study aimed at investigating the antigenic phenotype of Hofbauer cells from first- and third-trimester placentas, Goldstein and colleagues showed that these villous stromal macrophages were numerically higher than other villous stromal mononuclear cells and that their phenotype was different from that of macrophages from other tissues. They also showed that the Hofbauer cells possessed surface expression of CD4 and suggested that these cells may serve as a portal of entry, or a reservoir, of human immunodeficiency virus (HIV) in placentas from viremic mothers [44], as will be discussed more in depth.

Hofbauer cells, like other macrophages, express the 3 IgG Fcγ receptors (FcγR): FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) [45] and also the pan-macrophage marker, CD68 [46]. They are thought to have an immunoregulatory phenotype consistent with that of M2 anti-inflammatory macrophages, and several studies have shown that Hofbauer cells can be stimulated by glucocorticoids [47] and IL-10 [43] to express CD163, CD206, and CD209 [43], while secreting IL-10 and TGF-β [48]. Hofbauer cells have also been reported to constitute a mixture of M2a, M2b, and M2c macrophages that differ in marker surface expression, cytokine secretion and functions [49], reinforcing the concept of a regulatory rather than inflammatory role of these cells. There is limited information on the role of these cells in placental physiology; however, there have been suggestions that they may play a role in transport of nutrients within the villous stroma and in transmission of antibodies from the mother to fetus through the surface expression of Fc receptors [50–52]. Hofbauer cells have also been associated with numerous complications of pregnancy such as chorioamnionitis (CA), miscarriage, and preterm birth [53]. The potential role of HCs in the pathophysiology of complications of pregnancy such as villitis of unknown etiology and histological CA, has been discussed by Tang et al. [53]; however, there is very little information on the effect of other common complications of pregnancy and maternal infections on HC phenotypic polarity and gene expression. The polarity and function of maternal decidual macrophages have been reviewed by Brown et al. [54] and others [55] and will not be featured in this review. Rather, we will focus on the impact of common complications of pregnancy; CA, preeclampsia (PE), and gestational diabetes mellitus (GDM) on the phenotype and function of Hofbauer cells and their role in the vertical transmission of congenital viral infections such as Zika virus (ZIKV), HIV, and cytomegalovirus (CMV). We also highlight gaps in knowledge about Hofbauer cells and contradictory findings of studies on their dysregulation in pathological pregnancies.

Impact of Common Complications of Pregnancy on Hofbauer Cells

Chorioamnionitis

CA is an acute inflammation of placental membranes and the chorion due to microbial infections such as *Escherichia coli* and *Group B Streptococcus* [56–58]. Elevated levels of proinflammatory cytokines during CA-specific immune responses are associated with disease in the fetal pulmonary system and brain damage, making CA a risk factor for neonatal morbidity and mortality [59]. Although the pathophysiology of CA is yet to be elucidated, it is believed to be a consequence of the disturbed immune homeostasis in the placenta associated with Hofbauer cells.

There is a contradictory evidence for the effect of CA on the polarization of Hofbauer cells. Joerink et al. [60], suggested that maternal allergen sensitization and the presence of CA have no effect on the phenotype of Hofbauer cells; however, phenotypic and genetic studies have reported impaired function of Hofbauer cells during CA infection [61]. In line with this study, Vinnars et al. [62] had earlier reported that there is a drastic decrease in the number of CD68+ (pan-macrophage marker) Hofbauer cells in the presence of CA compared to healthy controls. Conversely, Hung et al. [63] and Toti et al. [64] showed that the number of CD68+ Hofbauer cells of placentas complicated by CA is increased. We suggest that the discrepancies may be due to differences in quantification and propose that a stringent standardized method should be used to quantify immunohistochemistry and immunofluorescence data. There is also very little information on the impact of altered numbers and/or regulation of selected macrophage markers by CA.

Gestational Diabetes Mellitus

GDM is maternal hyperglycemia due to insulin resistance that develops during pregnancy to create a glucose gradient necessary for the supply of energy and nutrients to the developing fetus [65, 66]. GDM prevalence ranges from 3 to 20% in pregnant women [67] and is associated with chronic low-grade inflammation of the placenta [68, 69]. Sisino et al. [70] reported that in pregnant rats receiving streptozotocin during pregnancy, diabetes alters the normal phenotype of HCs from an M2 anti-inflammatory regulatory phenotype to a proinflammatory phenotype. A recent pilot study by Schliefsteiner et al. [71] contradicts these findings as they reported that HCs maintain an anti-inflammatory M2 phenotype despite the presence of GDM in humans. During inflammatory diseases of the placenta, such as villitis of unknown etiology, there is increased infiltration of the chorionic villi by Hofbauer cells [20, 72], a phenomenon similarly reported in pregnancies complicated by GDM [73]. However, the role and function that Hofbauer cells play or are involved in these conditions are unclear.

Preeclampsia

PE is a placental disorder of unknown etiology, and it is likely that aberrant immune activation plays a role in its pathogenesis [74]. It is characterized by hyperten-

sion (>140/90 mm Hg) after 20 weeks of gestation and proteinuria (>300 mg/L per day) [75]. Its prevalence ranges from 5 to 10% globally, making it one of the leading causes of morbidity and mortality in mother and child [76]. Przybyl et al. [77] reported that CD74, a human leukocyte antigen class II histocompatibility antigen-γ chain, is downregulated on Hofbauer cells in the presence of PE. They suggested that this downregulation alters macrophage polarization from an immunoregulatory M2 phenotype toward a proinflammatory signature affecting essential macrophage-trophoblast crosstalk [77]. It has been reported that the number of Hofbauer cells is significantly decreased in the presence of PE and that the CD209+ HCs from pregnancies with PE secrete less IL-10 than those from normal pregnancies [78]. These data suggest that PE affects an essential immune regulatory role of Hofbauer cells in maternal–fetal tolerance. More studies are needed to investigate the bidirectional effects of PE and Hofbauer cells, and the role of these cells in regulating pregnancies is complicated by PE.

Viral Permissiveness of Hofbauer Cells to ZIKV

Vertical transmission of a number of viruses such as ZIKV, rubella, CMV, herpes simplex virus, and HIV-1, from an infected mother to her developing fetus, suggests viral tropism for placental cells [79]. ZIKV is a mosquito-borne flavivirus, which can be vertically transmitted from an infected mother to her fetus [80, 81], causing adverse birth outcomes such as fetal brain abnormalities and microcephaly [82]. ZIKV-specific antigen was detected in Hofbauer cells and histiocytes in the intervillous space of a placenta from a mother with ZIKA [83]. During pregnancy, increased levels of proinflammatory cytokines such as IL-6, CXCL8 (IL-8), and TNF have been reported in the amniotic fluid, decidua, fetal membranes, and maternal serum [84, 85]. The mechanism by which ZIKV breaches the placental barrier is not clear, and ZIKV RNA has been detected in amniotic fluid and in fetal and newborn brain tissue [83, 86, 87]. Vertical transmission of the ZIKV to the human fetus is mediated through productive infection of Hofbauer cells [88, 89], leading to cell proliferation and hyperplasia of these cells in the second and third trimester [90]. Thus, placental inflammatory abnormalities are not a component of vertical transmission of the ZIKV per se.

Quicke et al. [88] demonstrated that human placental Hofbauer cells are much more susceptible to productive ZIKV infection than autologous cytotrophoblasts. They showed that, upon infection, Hofbauer cells induce secretion of type I interferon, other proinflammatory cytokines, and upregulation of antiviral gene expression [88]. Their findings corresponded to those of Jurado et al. [89], Simoni et al. [91], and others [92]. However, these studies did not demonstrate the phenotypic changes or shift in activation status of Hofbauer cells that may be associated with abnormal functions of Hofbauer cells upon ZIKV infection. ZIKV infection has been shown to be enhanced by preexisting anti-flavivirus immunity through IgG engagement of the FcγR [93]. This antibody-dependent enhancement of infection has also been reported in other flaviviridae, such as dengue virus [94, 95]. Although there is evidence of in vitro Hofbauer cells infection by ZIKV, the mechanisms by which the virus reaches these cells in the villi and how it is further transmitted to the fetal circulation needs further investigation. Phenotypic and functional changes of Hofbauer cells upon ZIKV infection have not yet been fully elucidated [90, 91, 96].

Human Immunodeficiency Virus-1

The large burden of HIV infection in sub-saharan Africa, where adolescent girls and young women between the ages of 15 and 24 years are bearing the brunt of the epidemic [97], has resulted in most HIV-1-infected pregnant women being placed on combination antiretroviral therapy (ART). These women show a high prevalence of aggravated PE [98] and adverse birth outcomes, including fetal death, preterm births [99, 100], and small-for-gestational age infants [100–102]. Whether Hofbauer cells are determinants of these adverse birth outcomes is not understood. The phenotype of Hofbauer cells favors productive infection with HIV-1 [52] and the 15–30% [103] vertical transmission rate, prior to prevention of mother-to-child transmission with ART, is testimony to this. In vivo [104] and in vitro [48] studies confirmed that Hofbauer cells are indeed targets of HIV-1.

During maternal HIV-1 infection, antibody- and cell-associated virions, cell-free virions, and maternal broadly neutralizing antibodies cross the placental barrier to interact with Hofbauer cells prior to entering the fetal circulation [105]. However, whether Hofbauer cells are involved in facilitating *in utero* HIV infection is unclear as these cells have proved to be invariably infected in vitro [106], where it has been shown that they can limit HIV-1 replication by the induction of immunoregulatory cytokines [48]. These cells also possess intrinsic adaptations that facilitate the sequestration of HIV-1 that may serve as a protective viral reservoir allowing for antiretroviral (ARV) drug entry in utero and

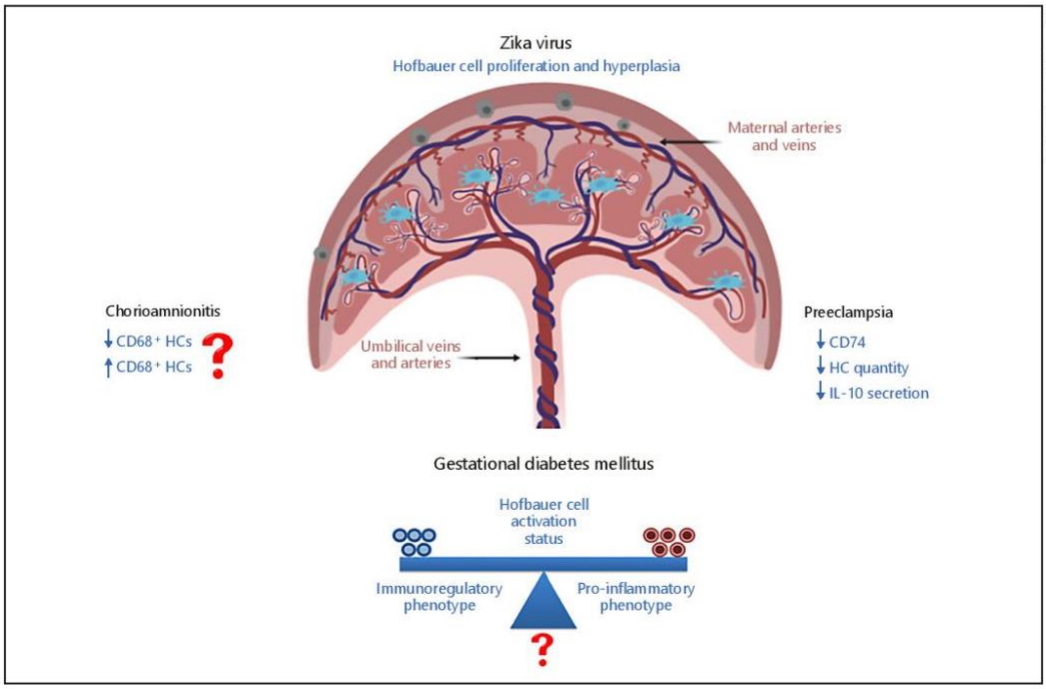

Fig. 2. The impact of common complications (Preeclampsia, Gestational Diabetes Mellitus [GDM] and Chorioamnionitis [CA]) of pregnancy and viral infections (Zika) on the quantity, phenotype and function of Hofbauer cells. "?" denotes unknown or poorly defined and arrows represent either up- or down-regulated.

potentially the inactivation of the virus [106]. Therefore, how Hofbauer cells play a role in utero in protecting or mediating HIV-1 transmission is unclear. Various studies have demonstrated the permissiveness of Hofbauer cells to HIV-1 infection [107]; however, there are few data on the effect of HIV-1 and/or ARV drugs on the phenotype and function of these cells. Further investigation of the involvement of Hofbauer cells in HIV infection may shed light on the ability of these macrophages to inhibit or facilitate viral transmission, which can be relevant to understand other maternally acquired viruses.

Now with successful prevention of mother-to-child transmission programs, where viral transmission has been reduced to 1–2% [108, 109], there is concern that maternal–fetal tolerance may be disrupted by ARV drugs per se. As discussed above, widespread use of ARV drugs has been associated with adverse birth outcomes prevalent among HIV-1-infected women on ART [99, 100]. Whether ARV drugs have teratogenic effects via Hofbauer cells dysfunction remains unknown.

Human Cytomegalovirus

Human CMV (HCMV) is among the leading causes of congenital infections globally [110]. It is a prevalent species-specific herpes virus that causes asymptomatic infections in healthy individuals but leads to sensorineural hearing and neurodevelopmental delay in infants [111] and increased morbidity and mortality in immunocompromised individuals [112]. HCMV can be transmitted from an infected mother to her developing fetus through the placenta [113, 114] and is a major cause of intrauterine growth restriction [115]. The mechanisms involved in transplacental HCMV transmission are poorly understood. Maternal infections with HCMV during pregnancy are associated with high risk of viral

Table 1. Lack of consensus in the literature on the impact of complications of pregnancy and maternal viral infection on HC

Pregnancy complication	Effect on HCs	References
Chorioamnionitis	Decrease in numbers	[62]
Chorioamnionitis	Increase in numbers	[63, 64]
Chorioamnionitis	No effect on polarization status	[60]
Gestational diabetes mellitus	Acquisition of a proinflammatory phenotype	[70]
Gestational diabetes mellitus	Maintain an anti-inflammatory phenotype	[71]
Preeclampsia	Downregulation of CD74 and acquisition of a proinflammatory phenotype	[77]
Preeclampsia	Decrease in numbers	[78]
ZIKV infection	Proliferation and hyperplasia	[90]
ZIKV infection	IFN & antiviral gene induction	[88]
HIV infection	Induction of immune-modulatory cytokines	[48]

transmission to the fetus [111], where primary HCMV transmission from mother to child during gestation is 35–40% [116]. El Costa et al. [117] proposed that transmission of viruses is facilitated by either the hemotogenous spread to the placenta or by cellular transfer from the maternal decidua to the anchoring placental villi in early pregnancy.

A number of studies have reported the ability of HCMV to infect monocytes, macrophages, and their progenitors [118, 119], suggesting that the macrophage lineage provides long-lived reservoirs for HCMV latency [120, 121]. At the maternal–fetal interface, HCMV has been shown to induce a distinct decidual tissue innate immune response and to dysregulate tolerance [122]. A better understanding of innate immune mechanisms at the maternal–fetal interface that modulates transplacental transmission of viruses is important to define, so that adverse birth outcomes that are associated with these infections can be prevented. Figure 2 highlights the lack of consensus and gaps in knowledge (denoted by "?") from studies showing the impact of pregnancy complications and viral diseases on Hofbauer cell quantity, phenotype, and function. The lack of consensus in the literature is summarized in Table 1.

Conclusion

The origin and role of Hofbauer cells at the maternal–fetal interface and in the promotion of tolerance and regulation of pregnancy are not clear, and their function during gestation warrants further investigation. What contributes to the elusive nature of these cells is their apparent ability to adapt to their microenvironment, including the placenta – and likely shape the link between mother and fetus. The selective role of Hofbauer cells to different viruses highlights their pleiotropic nature and a possible gatekeeping role in determining which viral infections are congenital in the newborn infant. How Hofbauer cells appear to be relatively resistant to HIV, as opposed to Zika, remains an evolutionary and developmental enigma. We propose that identifying a biomarker of Hofbauer cell dysfunction associated with various maternal infections during pregnancy will allow for the development of placental interventions to mitigate adverse birth outcomes and improve the health of both mother and her newborn infant.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

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Author Contributions

M.Z.Z. conceived the review; M.Z.Z., F.O.M., S.G., and C.M.G. wrote the review.

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