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Review Article Title

CD8 T cells and Dendritic Cells: Key Players in the Attenuated Maternal Immune Response to Influenza Infection

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Abstract

Pregnancy provides a unique challenge for maternal immunity; requiring the ability to tolerate the presence of a semi-allogeneic fetus, and yet, still being capable of inducing an immune response against invading pathogens. To achieve this, numerous changes must occur in the activity and function of maternal immune cells throughout the course of pregnancy. Respiratory viruses take advantage of these changes, altering the sensitive balance of maternal immunity, rendering the mother with increased susceptibility and disease severity to viral infections. Influenza virus is one of the most common respiratory virus infections during pregnancy, leading to increased risk of ICU hospitalizations, pneumonia, acute respiratory distress syndrome and even death. Whilst much research has been performed to understand the changes that must take place in maternal immunity during pregnancy, considerable work is still needed to fully comprehend this tremendous feat. To date, few studies have focused on the alterations that occur in maternal immunity during respiratory virus infections. This review highlights the role of dendritic cells (DCs) and CD8 T cells during pregnancy and the changes that occur in these antiviral cells following influenza virus infections.

Key words: pregnancy, influenza, H1N1pdm09, dendritic cells (DCs), CD8 T cells, infection

1 The Immunology of Pregnancy

Fetal tolerance is essential for the survival of the semi-allogeneic fetus during pregnancy. Without this tolerance, the maternal immune system would rapidly mount an attack on this “foreign object,” destroying the developing cells of the fetus in the same manner as it would an invading pathogen. To induce this tolerance, changes must take place in the normal function of maternal immune cells. Different viruses use different mechanisms of host evasion and immune cell alteration to increase their chance of survival, and various pathogens, including respiratory viruses, have developed the ability to take advantage of changes that occur in the maternal immune system during pregnancy. The presence of various chronic diseases, such as asthma during pregnancy, may further alter the maternal immune response to the invading pathogen thereby increasing the susceptibility and disease severity that results upon infection during pregnancy

Innate immune cells, such as natural killer (NK) cells, granulocytes and dendritic cells (DCs), as well as CD4 and CD8 T cells from the adaptive immune system, all participate, not only in the regulation of fetal tolerance throughout the course of pregnancy but in maintaining a strong antiviral immune response against invading pathogens. For example, NK cells, which are capable of killing infected host cells through non-specific recognition, are also known to play an active role in maintaining fetal tolerance. One way they do this is through the development of a highly specialized population of cells termed uterine (uNK) or decidual (dNK) NK cells.(Watanabe et al., 1997) These ‘pregnancy-specific’ NK cells alter in number throughout the course of pregnancy, and play an important role in fetal implantation, through interaction with HLA-G molecules on trophoblast cells. Regulatory T cells (T_{regs}) are

one of the most important cell types involved in maintaining fetal tolerance during pregnancy. Once activated, T_{regs} are capable of inhibiting both the proliferation and activity of anti-fetal-specific CD4 and CD8 T cells that would otherwise result in fetal rejection.(Guerin et al., 2009) Dendritic cells and CD8 T cells play a critical role in fetal tolerance and host defence and yet alterations in their activity and their function following influenza infection may provide important understanding as to why pregnant women are increased risk for influenza infections. The remainder of this review outlines the normal role of these two cell types during pregnancy, and highlights novel research that has identified important changes in the phenotype and function of these cells during influenza virus infections, Figure 1.

1.1 Normal Role of DCs during Pregnancy

DCs play a critical role in innate antiviral immunity and in maintaining fetal tolerance during pregnancy. They are the primary antigen presenting cells (APCs) of the immune system, and whilst they account for less than 1% of the leukocyte population, they are key producers of the antiviral type I and type III interferons (IFNs).(Liu, 2005) Following infection, DCs undergo activation and maturation, which includes up-regulation of the antigen presentation molecules, major histocompatibility (MHC) I, (also known as human leukocyte antigen (HLA) A, B and C in humans) which is recognized by CD8 T cells and MHC II, (i.e. HLA-DR, DP, DM etc.) recognized by CD4 T cells. (Sacks et al., 1999) Activated and mature DCs also show increased expression of the phenotypic markers CD80, CD83, CD86 and CD40, as well as the tissue homing marker CCR7(Fonteneau et al., 2003) as well as bulk production of the antiviral type I $\text{IFN}\alpha$, produced especially by pDCs (Liu, 2005).

Mature DCs then accumulate in the lungs and regional lymph nodes, where they present viral antigen to naïve T cells to produce activated virus-specific effector T cells. (GeurtsvanKessel et al., 2008, Fonteneau et al., 2003)

DCs can be subdivided into (1) myeloid (mDCs), which develop *via* the myeloid lineage and migrate to host tissue, and (2) plasmacytoid (pDCs), which are derived from a lymphoid origin and reside primarily within the blood. (Bella et al., 2011) In humans, both are lineage negative (i.e. CD3⁻, CD14⁻, CD19⁻ and CD56⁻), with mDCs commonly classified as positive for CD4, CD11c and HLA-DR, whilst pDCs are positive for CD4, HLA-DR, CD123 and CD45RA. (Dzionek et al., 2000) Blood-derived cellular antigens (BDCAs) are more recently identified factors that can also be used to classify human mDC and pDC subtypes. Using this recent classification, mDCs can be further classified as mDC1 or mDC2 if they are positive for BDCA1 (CD1c) or BDCA3 (CD141), respectively, whilst pDCs are positive for BDCA2 (CD303) and BDCA4 (CD304). (Dzionek et al., 2000)

The total number of DCs, as well as DC subtypes has been shown to alter during pregnancy compared to the non-pregnant state and it appears that these alterations in DC number are largely dependent upon the gestational age of the woman. In the human decidua, an increase in the number of DCs has been identified as pregnancy progresses and this appears to be dominated primarily by mDCs. (Collins et al., 2009)

Conversely the percentage of circulating DCs decreases as gestational age increases (Shin et al., 2009). One recent study in humans identified, that the absolute number of mDCs was significantly higher in the first trimester and decreased as pregnancy progressed compared

to the non-pregnant state (Bella et al., 2011). This study also showed that the percentage of circulating pDCs in pregnant women decreased throughout pregnancy, though not differing significantly compared to non-pregnant women,(Bella et al., 2011). In 2013, we published a study in which peripheral blood DCs from pregnant women primarily in the third trimester (mean $\pm$ SD, 27.5 $\pm$ 6.7 weeks) were enumerated using BDCA5 to specifically identify mDC1, mDC2 and pDC subpopulations. Whilst total DCs and mDC populations did not differ significantly compared to the non-pregnant state, pDCs were significantly reduced at this time point.(Vanders et al., 2013) As a result of these changes in circulating DC populations, there is a higher mDC : pDC ratio that occurs during pregnancy (Shin et al., 2009, Bella et al., 2011)

During embryo implantation “DC entrapment” takes place. These trapped DCs not only have reduced interaction with T cells, but also display a lack of responsiveness towards maternal T cells (Collins et al., 2009). Such unresponsive behaviour is one effective mechanism of controlling T cell reactivity against the fetus. However, the ways in which DCs in the decidua and peripheral blood remain unresponsive, and yet still capable of providing anti-viral protection during pregnancy, is still not clearly understood. It is now evident that this involves marked changes in DC phenotype, which has led to the term coined by Bella et al. “incomplete activation” during pregnancy (Bella et al., 2011) This incomplete activation involves changes in the expression of activation and maturation markers on circulating DCs.(Bella et al., 2011) A number of studies have shown that during human pregnancy, the expression of the activation markers CD40, CD80, CD83 and CD86 increases significantly on mDCs and pDCs while expression of the antigen-presenting

molecule, HLA-DR decreases as gestation progresses (Shin et al., 2009, Bachy et al., 2008, Bella et al., 2011, Cappelletti et al., 2013). It has thus been postulated that this partially activated state, is one mechanism that allows DCs to remain active against pathogen invasion, and yet, tempers their ability to present antigen to naïve T cells, which could otherwise have deleterious effects for the semi-allogeneic fetus. (Bella et al., 2011)

Another mechanism of fetal tolerance involving DCs is the interaction with HLA-G molecules on trophoblast cells. This interaction alters the antigen-presenting activity of DCs to a tolerogenic state. The tolerogenic DCs subsequently induce a state of anergy in effector T cells which suppresses T cell activity. These tolerogenic DCs have been found to express the enzyme, indoleamine 2,3-dioxygenase (IDO),(Grohman et al., 2003) which produces tryptophan metabolites, like kynurenine, that inhibit the activation of effector T cells and NK cells.(Grohman et al., 2003) In humans IDO activity in DCs can also indirectly inhibit T cell activity by activating resting regulatory T cells (T_{regs}), which possess potent immunosuppressive activity.(Guerin et al., 2009). In mice, IDO activity in DCs has been shown to prevent fetal rejection,(Munn et al., 1998).

Whilst there is still much to learn about the specific alterations in DCs during pregnancy, it is clear that these APCs play an important role in maintaining pregnancy, and that an imbalance in their number or function could be fatal to the developing fetus.

1.2 Normal Role of CD8⁺ T cells during Pregnancy

CD8 T cells form part of the adaptive immune response, functioning to effectively clear viruses, aided by the help of the CD4 T cells. CD8 T cells are activated by antigen

presentation, which occurs via interaction of the T cell receptor (TCR) with MHC I molecules on DCs (Ingulli et al., 2009) Upon contact with infected host cells, activated CD8 T cells release $\text{TNF}\alpha$ and $\text{IFN}\gamma$, as well as perforin/granzyme (Pazos et al., 2012b), which create pores within infected host cell membranes resulting in subsequent cellular lysis. CD8 T cells can also utilize a secondary pathway to induce cellular apoptosis, which involves engagement of the Fas ligand (FasL) on T cells, with the Fas receptor (FasR) on infected host cells.(Topham et al., 1997)

Since CD8 T cells must still maintain their function to protect both mother and fetus from infection, their activity during pregnancy must still be tempered to prevent them from targeting foreign cells of fetal origin. Like DCs, this seems to be achieved by modulating the number and activity of CD8 T cells throughout the course of pregnancy, rather than simply by suppressing their activity during gestation. Despite their normal cytotoxic activity, during pregnancy CD8 T cells increase in number, especially in the decidua, where they play a role in fetal implantation; primarily through the release of cytokines such as $\text{IFN}\gamma$, which aid in trophoblastic invasion (Scaife et al., 2006). During pregnancy CD8 T cells can also be induced to play a part in fetal tolerance through the generation of a pregnancy-specific sub-population of CD8 regulatory T cells with immunosuppressive activity.(Shao et al., 2005) These CD8 Tregs are activated through their TCR interaction with the non-classical HLA class I molecule, CD1d expressed by trophoblast cells early in pregnancy.(Shao et al., 2005)

Using mouse models it has been shown that CD8 T cells in the spleen still retain their proliferative capacity during pregnancy, with a significant but transient increase in CD8 T

cells in late gestation, without an apparent induction of apoptosis of these cells.(Norton et al., 2010) In the uterine draining lymph nodes, a significant increase in CD8 T cell proliferation is also observed in later pregnancy, though for a longer period compared to the spleen, and CD8 T cell apoptosis is observed in the lymph nodes in early, mid and late gestation.(Norton et al., 2010) Despite the enhanced proliferative capacity of these cells, fetal demise is not observed, which may be due to alterations in the avidity of the TCR for fetal antigens during pregnancy.(Norton et al., 2010)

These findings suggest that in order to maintain fetal tolerance, the maternal immune system can selectively alter immune cell number and activity throughout the course of pregnancy in a tissue-specific manner, whilst still allowing a strong host immune response. Another study of pregnant mice, showed that paternal antigen-specific CD8 T cells were present, and were activated and capable of proliferating, however, these CD8 T cells also expressed the inhibitory receptor PD1 (CD279), which binds to the ligands PDL1 (CD274) and PDL2) (CD273) located primarily APCs. (Taglauer et al., 2009)These findings indicate that like pDCs, CD 8 T cells are still active during pregnancy but are limited in their response to fetus-specific cells. In addition to the PD1:PD-L1 pathway limiting CD8 T cell activity and preventing accumulation, other mechanisms must also occur by which CD8 T cells maintain fetal tolerance. This is evidenced by PD1 and PD-L1 knockout (-/-) mice, where whilst accumulation of paternal-antigen specific T cells occurred in the spleen, there was no evidence of fetal resorption.(Taglauer et al., 2009)

Further studies are required to resolve the importance of the PD1:PD-L1 pathway in maintaining fetal tolerance, however these findings on CD8 T cells during pregnancy do

provide us with a greater level of insight into the complexity of the mechanisms employed by the maternal immune system to maintain fetal tolerance. In the context of viral infections, these findings also provide us with ‘food for thought’ as to the different changes that may result in these cell types when viral infection occurs in pregnancy..

2 Influenza Infections during Pregnancy

Infection with respiratory viruses such as human rhinoviruses (HRVs) and influenza commonly induce an array of “cold and flu” symptoms, including rhinorrhoea, nasal congestion, sore throat, cough, chest pain, dyspnoea, fever, malaise, headache and myalgia. The severity and extent of these symptoms depends largely upon the specific virus as well as the state of the host.

Influenza viruses are members of the genus *Orthomyxovirus* in the family *Orthomyxoviridae*. They are enveloped, negative sense, single-stranded RNA viruses, containing eight gene segments encoding 11 proteins that constitute viral structure and induce replication, as well as virulence factors that inhibit host antiviral immunity.(Hsu et al., 2011) Influenza viruses are classified into three distinct genera, as influenza A, B, and C. Influenza A viruses are responsible for most human disease and are further classified based on two transmembrane glycoproteins, known as haemagglutinin (HA) and neuraminidase (NA).(Glaser et al., 2007) The HA protein allows the virus to attach and gain entry into host cells through recognition of sialic acid residues located on host cells (α 2,6 in humans and α 2,3 in birds), while the NA is involved in the release of viral particles from infected cells, acting to cleave sialic acid from the HA molecules (Glaser et al., 2007, Hsu et al., 2011).

During influenza pandemics pregnant women are at greater risk of influenza-induced illness leading to increased rates of hospitalisation, ED presentation and influenza-induced mortality rates compared to non-pregnant women. During the 1918 “Spanish flu” influenza outbreak, ~50% of all infected pregnant women developed pneumonia, with an overall 27% case mortality rate.(Harris, 1919) Similarly, in the first outbreak of the 1957 Asian influenza pandemic, pregnant women had a 9-fold increased risk of influenza-induced pneumonia, compared to non-pregnant women,(Widelock et al., 1963) and 50% of all deaths in women of reproductive age occurred in those who were pregnant.(Freeman et al., 1959)

The serious consequences of influenza infections during pregnancy were recently reminisced during the 2009 H1N1 swine influenza pandemic. Here, the rate of hospital admission for pregnant women was substantially higher than in the general population, with 0.32 per 100, 000 [95% CI 0.13, 0.52] versus 0.076 per 100 000 [95% CI 0.07-0.09].(Jamieson et al., 2009) Pregnant women were also more likely to report shortness of breath compared to non-pregnant women of similar age (relative risk (RR) 1.7 [95% CI 1.0, 2.7]) and at least 97% of H1N1pdm09-infected pregnant women presented with febrile illness that developed into pneumonia, leading to acute respiratory distress syndrome.(Jamieson et al., 2009) With such serious adverse outcomes as these, it is not surprising that pregnant women constituted >13% of all fatal cases reported for H1N1pdm09 infections(Jamieson et al., 2009)

The risk of maternal influenza-induced morbidity and mortality increases with gestational age; with the highest mortality rates most often reported in the third trimester. Surveillance data on H1N1pdm09 infection during pregnancy,(Siston et al., 2010) showed that 280

pregnant women were admitted to ICU with influenza-induced illness, and 20% of those women subsequently died. Of those pregnant women who died, 7.1%, 26.8% and 64.3% of these women were in their first, second and third trimester, respectively.(Siston et al., 2010)

Adverse perinatal outcomes are also identified following influenza infections, especially pandemics, including increased congenital deformities, cleft lip, neural-tube defects, cardiovascular malformations, increased risk for behavioural and pharmacological changes and high rates of neonatal mortality are also reported during influenza pandemics.(Shi et al., 2003, Godoy et al., 2011, Acs et al., 2005)

Collectively, these findings highlight the importance of understanding the underlying immune alterations in pregnancy, and the potential imbalance that may result in either antiviral or inflammatory immune responses during respiratory virus infections.

3 Altered Host Immunity to Influenza Infections during Pregnancy

Humans have effective strategies for detecting and clearing virus infections. Viral recognition by host cells is achieved through pathogen-recognition receptors (PRRs), such as toll-like receptors (TLRs), located on the cell's surface, as well as within the cytoplasm. TLR3, TLR7/8 and TLR9, are the virus-specific TLRs located on endosomes within the cytosol, and which recognise viral double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and methylated DNA, respectively.(Arancibia et al., 2007) Recognition of viral RNA can also be achieved by the RNA helicases, retinoic acid inducible gene -1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which also reside within the cytosol.

Influenza infections activate PRRs triggering the rapid release of an array of inflammatory cytokines and chemokines, as well as the release of the innate type I and type III IFNs, which are critical for both early innate antiviral activity, and for priming later adaptive immune responses against the invading viruses (Arancibia et al., 2007).

IFNs are classified into three major types; type I (e.g. $\alpha/\beta/\epsilon$), type II (γ), and type III ($\lambda 1-3$), and whilst type II IFN γ is produced predominantly by T cells of the adaptive immune system, the type I and type III IFNs are produced primarily by the infected epithelial cells and by APCs recruited to the area following viral infection.(Malmgaard, 2004) These antiviral cytokines elicit a broad range of biological responses by inducing the expression of more than 200 interferon-stimulated genes (ISGs) that encode proteins with diverse biological functions including direct antiviral activity, adhesion, apoptosis, anti-proliferation, immunomodulation, chemotaxis, antigen presentation/processing and transcriptional regulation (Malmgaard, 2004).

Host epithelial cells thus act as an important first line of defence during viral invasion, initiating a cascade of antiviral immune responses that are carried out by numerous innate and adaptive immune cells that infiltrate the infected site, like DCs and CD8 T cells.

3.1 DC Response to Influenza Infections during Pregnancy

DCs are heavily equipped with viral recognition receptors, which allow them to respond rapidly to host infection. mDCs are predominantly activated through TLR3, as well as RIG-I and MDA-5 signalling, whilst pDCs highly express the viral sensing receptors, TLR7 and TLR9.(Coccia et al., 2004) Following respiratory virus infection, potent type I and type III

IFN responses are induced from mDCs, and especially pDCs, (Fitzgerald-Bocarsly et al., 2007) triggering an array of antiviral and modulatory ISGs. Once activated, DCs undergo a maturation process, which in humans, includes the up-regulation of phenotypic markers such as CD83, CD86, HLA-DR and CCR7.(GeurtsvanKessel et al., 2008) Mature DCs then accumulate in the lungs and regional lymph nodes, where they present viral antigen to naïve T cells, resulting in the development of activated virus-specific effector T cells. (GeurtsvanKessel et al., 2008) The protective antiviral role of DCs during respiratory virus infections is highlighted by the adverse outcomes that result from their absence. For example, DC depletion in mice has been shown to impair effector T cell activity which leads to increased pulmonary pathology and limited viral clearance following infection.(Wolf et al., 2009, Dolfi et al., 2011)

In 2012, it was shown that *in vitro* infection of PBMCs from pregnant women (during second and third trimesters) with either HRVs or H1N1pdm09 resulted in significant reductions in type I and type III IFN responses compared to those observed in PBMCs from non-pregnant women.(Forbes et al., 2012b, Forbes et al., 2012a) H1N1pdm09 infection of PBMCs from pregnant women, also resulted in significant reduction in mRNA expression of the anti-viral factor, protein kinase receptor (PKR); an ISG which functions to inhibit cellular mRNA expression during viral infections.(Forbes et al., 2012b) Another study in 2012 showed that compared to non-pregnant mice, infection of pregnant mice with influenza virus similarly resulted in a significant reduction in the mRNA expression of IFN β as well as the interferon inducible proteins, retinoic acid inducible protein 1 (RIG-I) and MX-1 (both proteins displaying direct anti-viral capacity).(Pazos et al., 2012b) These findings indicate that

respiratory virus infection during pregnancy leads to altered antiviral activity that not only affects IFN production but which leads to altered activity of key downstream antiviral factors that are important in preventing viral spread.

Alterations in the number or function of DCs in pregnancy during respiratory virus infection are the likely cause for the reduced IFN production during pregnancy and thereby provide a plausible mechanism for the increased disease severity observed in pregnant women. Respiratory viruses are capable of manipulating host anti-viral activity, particularly by inducing specific activation and inhibition markers. For example, in one study examining the effects of influenza A virus infection in mice in the presence and absence of normally non-immunogenic proteins, it was found that influenza infection alone resulted in a significant increase in the maturation markers CD80, CD86 and MHCII (HLA-DR) on CD11c⁺ mDCs in the mediastinal lymph nodes compared to those in non-infected mice (Brimnes et al., 2003). A more recent study used recombinant hemagglutinin (HA) proteins in mice derived from H5N1 and 2009 pandemic H1N1. (Liu et al., 2010) They also showed increased maturation of bone marrow derived mDCs following HA administration, evidenced by enhanced expression of HLA-DR, CD86 and CD40. (Mendola et al., 2014) In 2013 a study was published which identified that influenza infection can also alter DC markers during pregnancy. It was shown that following *in vitro* H1N1pdm09 infection of PBMCs, there was a significant increase in the activation markers CD86 and HLA-DR on pDCs of pregnant compared to non-pregnant women. (Vanders et al., 2013) Not only was the expression of CD86 and HLA-R significantly increased on pDCs from pregnant women compared to non-pregnant women, but following influenza infection these markers were increased on pDCs

from pregnant women compared to pDCs from these same women cultured in media alone. (Vanders et al., 2013) Whilst increased CD86 is normal during pregnancy, (Shin et al., 2009, Bachy et al., 2008, Bella et al., 2011) marked increases in expression following influenza infection, as we observed, may alter antiviral activity. For example, HIV-induced up-regulation of CD86 (and HLA-DR) on DCs, reduces T cell proliferation and cytokine production. (Bjorck, 2004) This potentially occurs through the suppressive activity of CD86 when in contact with the high-affinity inhibitory receptor, CTLA-4. (Petroff et al., 2010) As discussed previously, during normal pregnancy, incomplete activation normally leads to decreased HLA-DR expression on DCs, however, we found that influenza infection during pregnancy induced a significantly higher percentage of pDCs that expressed HLA-DR. Incomplete activation during pregnancy is an important part of fetal tolerance, allowing DCs to retain antiviral activity and yet, preventing presentation and activating effector T cells that could harm the fetus. (Bella et al., 2011) This virus-induced alteration HLA-DR, may therefore provide one mechanism leading to increased maternal and fetal morbidity. Indeed, increased CD86 and HLA-DR has been associated with high abortion rates, which is also a common feature of influenza infections during pregnancy (Arck et al., 2007, Bella et al., 2011).

Interestingly, several other studies have not shown increased CD86 expression on DCs. One study showed that stimulation of PBMCs from pregnant women with a synthetic TLR7 agonist resulted in increased IFN α production and reduced CD86 expression on pDCs (Cordeua et al., 2012). In another study influenza infection was administered during murine pregnancy and resulted in a significantly decreased expression of CD86 on CD11c⁺ mDCs

from mediastinal lymph nodes compared to the non-pregnant state (Pazos et al., 2012b). These differences indicate that it is the live influenza virus infection rather than synthetic agonists that attenuates the antiviral maternal immune responses and that influenza-induced alteration of activation markers is dependent on immune state (e.g. pregnant vs non-pregnant) as well as cell and tissue specific (e.g. mDC vs pDC, mediastinal lymph nodes vs peripheral blood and bone marrow)

Hormones are key pregnancy factors responsible for modulating the changes that occur in maternal immunity during pregnancy. Estrogen and progesterone in particular increase throughout the course of pregnancy and drive many of the changes that occur in immune cell activity during this time. These hormones are responsible for changes in many cytokines, chemokines and growth factors, as well as the Th2 bias that has long been known to exist during pregnancy (Arck et al., 2007, Doria et al., 2006, Kraus et al., 2010). They also appear to be involved in the attenuated immune response that is observed in pregnancy. For example, it has been demonstrated that mDCs express estrogen receptors and upon culture with estrogen, show a significant reduction in antiviral proteins (including IFN α / β and IP-10) following RNA virus infection.(Escribese et al., 2008) Another study demonstrated that in both humans and mice, progesterone can inhibit TLR-mediated IFN α production from pDCs. (Hughes et al., 2008) Pazos et al, (Pazos et al., 2012b) showed that pregnant mice infected with influenza displayed significantly reduced IFN β , MX1 and RIG-I mRNA expression as well as MCP-1, IFN γ , KC and TNF α protein compared to non-pregnant mice. When they treated non-pregnant mice with estrogen (compared to a placebo), they observed the same attenuated immune response as was observed previously in pregnant mice.

In addition to changes in activation markers a number of viruses are known to induce inhibitory markers to suppress T cell activation by APCs. For example, major group HRVs strongly inhibit DC-induced T cell activation through up-regulation of the inhibitory molecule, PD-L1 on DCs. (Kirchberger et al., 2005) Very little is known about the role of PD-L1 expression by DCs particularly during pregnancy following influenza infection. A recently published study examined the expression of PD-L1 on pDCs during pregnancy and the effect of influenza infection. It was shown that following H1N1pdm09 infection of PBMCs from pregnant women, PD-L1 was significantly increased on pDCs compared to cells cultured without influenza.(Vanders et al., 2013) Expression of PD-L1 was also significantly increased on pDCs from pregnant compared to non-pregnant women. Since other studies have found that blocking PD-L1 during viral infection improves host immunity and increases viral clearance,(Channappanavar et al., 2012, Maazi et al., 2013) it is possible that increased PD-L1 expression on pDCs during pregnancy may be an important factor in the attenuation of the maternal host immune response during influenza infections. To explore this, we blocked PD-L1 activity during H1N1pdm09 infection, and found that the maternal type I IFN α responses from PBMCs of pregnant women was significantly improved.(Vanders et al., 2013) The importance of the IFNs produced from DCs during influenza infection is highlighted by a study showing that influenza virus can inhibit the potent antiviral activity of DCs. However, they showed that pre-treating DCs with type I IFNs rescued their anti-viral responses.(Phipps-Yonas et al., 2008) As such, these findings highlight the fact that blocking PD-L1:PD1 activity of DCs could provide a potential therapeutic strategy by improving the innate IFN response has important implications. This

in turn could improve maternal immunity, especially during influenza pandemics where pregnant women are most susceptible.

3.2 CD8⁺ T cell Response to Respiratory Virus Infection during Pregnancy

During influenza infections, antigen presentation to CD8 T cells occurs in the draining lymph nodes, and influenza-specific effector CD8 T cells then cluster primarily within the spleen. The effector CD8 T cells then migrate to the lung, releasing IFN γ and perforin/granzyme, which mediate cytolytic responses against the invading virus.

A 2011 study examined the infiltration of CD8 T cells in the spleen, lymph nodes and BALF following infection of pregnant and non-pregnant mice with H1N1pdm09. They showed that although there was increased pulmonary damage, this was not associated with an increase in the percentage of CD8 T cells in pregnant mice. (Valadares et al., 2013) In 2013 an in vitro model of H1N1pdm09 infection during pregnancy also showed no difference in the percentages of CD8 T cells from pregnant women (primarily in the third trimester) compared to the non-pregnant state.(Vanders et al., 2013) These findings seem to indicate that it may not be a change in the percentage of CD8 T cells, but rather an alteration in their normal function explains the attenuated immune response observed during pregnancy.

As with DCs, influenza viruses have developed mechanisms to suppress the potent antiviral activity of CD8 T cells. One such mechanism is through up-regulation of the inhibitory molecule PD-L1 on DCs, (Kirchberger et al., 2005) which is particularly important in the context of inhibiting CD8 T cell activity. The interaction of PD-L1 on DCs with PD1 on CD8 T cells leads to T cell exhaustion,(Wherry et al., 2007) and promotes respiratory virus-

induced lung damage.(Zdrenghea et al., 2012) In addition, both acute and chronic viral infections, can directly induce PD1 expression on CD8 T cells, resulting in functional impairment as evidenced by a reduction in cytolytic activity (e.g. perforin/granzyme production) and the attenuated release of the key CD8 T cell cytokines, IL2, TNF α and IFN γ .(Wherry et al., 2007) It appears that influenza induced T cell exhaustion also occurs during pregnancy. In a recent study it was shown that CD8 T cells from pregnant women display a significant increase in PD1 expression following *in vitro* infection of PBMCs with H1N1pdm09.(Vanders et al., 2013) In addition, following *in vitro* H1N1pdm09 infection, significantly lower production of IL2 and IFN γ is observed from PBMCs of pregnant compared to non-pregnant women.

As with DC activity, hormones appear to play a key role in mediating the changes that occur in CD8 T cell function during pregnancy. Several studies utilizing murine models have also shown that both oestrogen and progesterone play important roles in modulating the activity of CD8 T cells. One study, examined the role of endocrine activity on the modulation of immune responses during pregnancy (Blois et al., 2004). They found that dydrogesterone (a derivative of progesterone) was essential for protective effects mediated during pregnancy (e.g. prevention of abortion), however these protective effects were dependent on CD8 T cell activity. They showed that depletion of CD8 T cells even in the absence of the progesterone derivative led to increases in inflammatory and Th1 type immune responses. (Blois et al., 2004) Another study looked at the role of oestrogen in pregnancy during influenza infection. (Pazos et al., 2012b) They found that influenza infection during pregnancy led to reduced CD 8 T cell activity (as evidenced by a decrease in granzyme B activity). They also showed

that administration of oestrogen to non-pregnant mice during influenza infection induced a similar decrease in CD8 T cell activity.(Pazos et al., 2012b) Whilst these studies did not directly examine T cell exhaustion, they do highlight the importance of hormone activity in mediating CD8 T cell activity during pregnancy.

Blocking PD-L1:PD1 interactions has been shown to reverse T cell exhaustion (Singh et al., 2010) and following viral infection PD-L1^{-/-} or PD1^{-/-} mice show reduced disease severity and increased viral clearance and survival.(Lafon et al., 2008) In the same study by Vanders et al (Vanders et al., 2013) it was observed that blocking PD-L1 using anti-PDL1 antibodies, significantly improved the IFN α , IL2 and IFN γ responses from PBMCs of pregnant women following *in vitro* H1N1pdm09 infection.(Vanders et al., 2013) These findings indicate that during pregnancy influenza virus can attenuate not only DC activity but also CD8 T cell function. Since influenza induced T cell exhaustion leads to functional impairment, this may provide an important mechanism underlying the increase disease severity that exists following influenza during the pregnant state. However, blocking PD-L1 : PD1 interactions may prove an effective method for improving not only DC, but also CD8 T cell activity in pregnant women, especially during influenza pandemics.

4 Conclusions

Pregnancy is a unique immune state, which requires changes in maternal immunity compared to the non-pregnant state. Pregnant women are known to have increased disease severity to influenza infections, especially during pandemics. This review highlights the role of DCs and CD8 T cells, two important cell types known to undergo changes both in number

and function during the course of pregnancy. These cells are pivotal in providing protection against influenza virus infections. Despite the importance of these cell types in protecting against viral pathogens, little is known about the role of these cells in pregnant women during influenza infections. Research is now uncovering some of the underlying changes that take place in maternal immunity which are responsible for the disease severity observed during influenza pandemics. This review highlights that changes that occur in DC and CD8 T cells activity during pregnancy and following influenza infections. These changes appear to be pregnancy-specific and mediated at least in part by the alterations in key hormones during pregnancy. Whilst vaccination is still considered the major preventative method for influenza infections during pregnancy, few pregnant women follow this advice. As such this review highlights an emerging area of reproductive science, highlighting the potential for targeting key cell types as novel therapeutic strategies to improve health outcomes not only for mothers but also for their babies, especially during influenza pandemics.

Footnotes

Abbreviations

APC – antigen presenting cell

BDCA – blood derived cellular antigen

CD – cellular derived antigen

DC – dendritic cell

dsRNA – double stranded RNA

H1N1pdm09 – 2009 pandemic swine flu

HRVs – human rhinoviruses

IFN - interferon

ISGs - interferon stimulated genes

MDA5 – melanoma differentiation associated gene 5

mDCs – myeloid DCs

NK cells – natural killer cells

PBMCs – peripheral blood mononuclear cells

pDCs – plasmacytoid DCs

PRRs – pathogen recognition receptors

RIG-I – retinoic acid inducible gene -1

Tregs – regulatory T cells

TLRs – toll-like receptors

TCR – T cell receptor

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Figures

Figure 1. Non-Pregnant: **1.** Influenza enters host epithelial cells, via SA receptors. **2.** Replicating viruses are recognized by viral-sensing PRRs (e.g. TLRs7/9), triggering the release of inflammatory and antiviral cytokines and chemokines like IL1 β , IL6, IL8, IL10, TNF α , IP10, IFN β from epithelial cells. DCs are amongst the first to reach the site of infection. **3.** Recruited DCs can be infected with virus and release antiviral type I and III IFNs. IFNs induce ISG and also aid in maturing the DCs. **4.** Mature DCs are characterised by expression of MHC molecules (i.e HLA molecules) as well as activation markers like CD80, CD86, CD40 and the tissue homing marker CCR7. Mature DCs present viral antigens to naïve T cells via direct contact between MHC I/II:TCR. **5.** Activated cytotoxic CD 8 T cells release an array of factors involved in T cell development, recruitment, and especially death of infected host cells. Host cell death prevents the spread of the invading virus to other host tissues.

Pregnant: **1.** During pregnancy respiratory viruses infect host cells in a similar manner. **2.** IFN α and IFN λ are decreased following HRV and influenza infection. The percentage of pDCs is also decreased, especially as gestation increases. **3.** Expression of CD86, HLA-DR and PDL1 is increased on pDCs following influenza infection. **4.** Expression of PD1 is

increased on CD8 T cells following influenza infection. 5. CD8 activity is decreased during, leading to increased influenza virus replication and release from host cells.

α 2,6 SA= α 2,6 sialic acid residue, TLR=toll-like receptor DC=dendritic cell, pDC=plasmacytoid dendritic cell, mDC=myeloid dendritic cell, PDL1=programmed death ligand 1, PD1=programmed death receptor 1, IFN α/λ =interferon α/λ , MHC= major histocompatibility complex, TCR = T cell receptor