Title Page

MAJOR ARTICLE

Title: Plasmacytoid Dendritic Cells and CD8 T cells from Pregnant Women Show Altered Phenotype and Function Following H1N1/09 Infection

Running Title: H1N1/09 Alters Maternal Immunity

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Abstract

Background: Pregnant women are a high risk group during influenza pandemics. In this study we determined whether plasmacytoid dendritic cells (pDCs) and CD8 T cells from pregnant women display altered activity following *in vitro* infection with 2009 pandemic swine influenza (H1N1/09). **Methods:** Peripheral blood mononuclear cells (PBMCs) were isolated from n=26 pregnant and n=28 non-pregnant women. DC subtypes were enumerated from PBMCs. PBMCs were infected with H1N1/09 and CD86, HLA-DR, and Programmed Death Ligand 1/2 (PDL1/2) measured on pDCs. Programmed Death receptor 1 (PD1) was measured on CD8 T cells. IFNα, IL2, TNFα and IFNγ were measured from culture supernatant. **Results:** pDC (i.e.$CD303^{+}/CD1c^{-}$ PBMCs) percentages were lower in pregnant compared to non-pregnant women ($p<0.05$). Following H1N1/09 infection, pDCs from pregnant women showed higher expression of CD86 ($p<0.01$), HLA-DR ($p<0.001$) and PDL1 ($p<0.001$), compared to non-pregnant women. Expression of PD1 on CD8 T cells was also higher during pregnancy ($p<0.05$). Following H1N1/09 infection, PBMCs from pregnant women displayed reduced IFNα ($p<0.01$), IL2 ($p<0.01$) and IFNγ ($p<0.01$) compared to non-pregnant women, however blocking PDL1 during H1N1/09 infection increased these cytokines from PBMCs of pregnant women ($p<0.05$). **Conclusion:** Altered maternal cellular antiviral activity is implicated in the increased morbidity during pregnancy following influenza pandemics.

Keywords

CD8 T cell, H1N1/09, pDCs, pregnancy, PDL1, PDL2, CD86, HLA-DR, PD1, IFN

Introduction

Pregnancy induces a unique challenge for the maternal immune system, which must tolerate the presence of a semi-allogeneic fetus and yet still maintain a strong immune response against invading pathogens. Influenza viruses take advantage of alterations in maternal immunity, as evidenced during influenza pandemics, where pregnant women have increased rates of influenza-induced hospitalisation and intensive care admissions, as well as increased mortality risk.[1-4] Adverse neonatal outcomes caused by maternal infection are also identified during influenza outbreaks including congenital malformations, leukaemia and fetal death.[5-7] Numerous changes are known to take place in the activity and function of innate and adaptive immune cells during pregnancy,[8, 9] but exactly how these maternal immune changes may contribute to increased disease severity during influenza infections is not clearly understood. Identifying changes in the activity of specific antiviral immune cells during influenza infections is of great importance in developing therapeutic strategies to improve the health outcomes for mothers and their baby.

Dendritic cells (DCs) are antigen presenting cells which play a critical role in antiviral immunity.[10] DCs are divided into two major types known as plasmacytoid (pDCs) and myeloid (mDCs).[11] mDCs can be further subdivided into the prototypic mDC1 subset

and the more recently identified, mDC2.[11] Following influenza infection, pDCs and mDCs undergo activation and maturation, including up-regulation of phenotypic markers like CD83, CD86, HLA-DR and CCR7,[12] as well as bulk production of the antiviral type I IFNα, produced especially by pDCs.[10] Mature DCs accumulate in the lungs and regional lymph nodes, where they present viral antigen to naïve T cells leading to activated virus-specific effector T cells.[12] Whilst pDCs appear dispensable for influenza clearance,[12] recent studies show that depletion of pDCs during influenza infection results in reduced antiviral antibody production following viral clearance and impaired host resistance to infection.[13] DCs also possess a negative regulatory role through expression of the ligands, PDL1 and PDL2 (i.e.CD274/CD273), which interact with the inhibitory receptor, PD1 (i.e.CD272), located on numerous cells types, including cytotoxic CD8 T cells.[14] Many viruses can evade host detection by up-regulating PDL1 on DCs[15] and epithelial cells,[16] which consequently limits virus-specific CD8 T cell activity and potentially worsens virus-induced tissue damage.[15, 16]

DCs also play an important role in mediating fetal tolerance during pregnancy, with alterations observed in their number and location in the decidua and peripheral blood,[17] as well as in their activation/maturation status.[17-19] This allows DCs to remain active and yet maintain fetal tolerance. The PDL1:PD1 negative co-stimulatory pathway is also utilized by regulatory T cells to maintain fetal tolerance through suppression of paternal-antigen specific T cell activity against the fetus.[20] Whether influenza infection during human pregnancy induces alterations in the activity of pDCs has not yet been explored, but given the important role that these cells play both during viral infection

and in pregnancy, such changes may lead to increased risk for influenza infection and disease progression.

CD8 T cells are an essential component of the adaptive immune system. They display potent cytolytic activity against pathogen-infected host cells, but are also involved during pregnancy where they aid in fetal implantation and preventing fetal abortion.[21, 22]. Activated CD8 T cells express PD1 as part of the host regulatory response to control T cell activity.[14] However, both chronic and acute viral infections can induce CD8 T cell 'exhaustion,' characterised by increased PD1 expression, as well as functional impairment of T cell activity, including reduced cytokine production (e.g. IL2, TNFα and IFNγ) and cytolytic ability.[14, 23] If influenza infection during pregnancy increased expression of PD1 on CD8 T cells, this could suppress their antiviral activity, and subsequently increase influenza-induced disease severity.

Given the importance of pDCs and CD8 T cells for fetal tolerance and in viral defence, alteration in their number or activity during pregnancy following influenza infection would provide a plausible mechanism for the increased susceptibility and disease severity commonly reported during pregnancy. We hypothesised that (1) pregnant women have lower percentages of circulating $CD303^{+}/CD1c^{-}$ pDCs compared to healthy, non-pregnant women, (2) following in vitro H1N1/09 infection of PBMCs, pDCs from pregnant women display altered expression of the surface markers CD86, HLA-DR, and PDL1/2, (3) CD8 T cells from pregnant women display higher PD1 expression and (4) pDCs and CD8 T cells display reduced function, evidenced by a decrease in production of the cytokines IFNα, IL2, TNFα, IL12 and IFNγ .

Methods

Subjects

This was an in vitro study of PBMCs collected from healthy, non-pregnant women and pregnant women. Non-pregnant women (n=28) were recruited from the Hunter Medical Research Institute database as well as John Hunter Hospital (JHH) staff, while pregnant women (n=26) were recruited from the JHH antenatal clinics as previously described.[24] This study was approved by the Hunter New England Health and University of Newcastle Human Research Ethics Committees. All patients gave written informed consent prior to sample collection. Inclusion criteria for all participants were females aged 18-40 years. Pregnant women were recruited after 18 weeks gestation. Women were excluded if they had any concomitant chronic medical illness, drug or alcohol dependence, respiratory or other medical conditions requiring immunosuppressive therapies or if they had "cold/flu" symptoms within the past four weeks prior to sample collection. Four of the healthy controls and five of the pregnant women who participated in this study also participated in another study conducted by our group.[4] However, there is no overlap in the patient samples collected or the results presented in these two papers.

Study Design

Women attended a single visit at the JHH and baseline characterisations included height, weight, lung function, smoking status, medication and vaccination history, as

well as current and retrospective cold and flu symptoms (assessed using the Common Cold Questionnaire[25]). Venepuncture was performed by trained clinical staff and whole blood was collected in 9ml EDTA tubes. Subsequent experiments were conducted with n=3-12 women per group.

H1N1/09 Stock

A strain of 2009 pandemic swine flu (H1N1 A/Auckland/3/2009) was obtained in 2010 from the World Health Organization (WHO, Melbourne, Australia).Viral stock propagation and concentrations of H1N1/09 were performed as described previously.[4]

Isolation and in vitro Culture of PBMCs with H1N1/09

PBMC isolation and culture was performed as described previously.[4] Briefly, isolated PBMCs were cultured in RPMI (Invitrogen, Australia Pty Limited) with 5% fetal bovine serum (SAFC Biosciences, Lenexa, Kansas, USA) at a final concentration of $2.0x10^6$ cells/ml. PBMCs were cultured with H1N1/09 (MOI 0.1) or in media alone. Cells were cultured for 48hrs at 37^oC with 5% CO_2.

Cell Viability

Cell viability for freshly isolated PBMCs was measured using a dead cell discrimination kit (Miltenyi Biotec Australia Pty. Ltd. North Ryde, NSW, Australia). Viability of H1N1/09-infected PBMCs was measured using the PE Annexin V Apoptosis Kit I (BD Bioscience CA, USA), according to the manufacturer's instructions. Previously, we

have shown that H1N1/09 is capable of low level replication in PBMCs,[4] however we confirmed that this does not affect cell viability (Online Supplement, section 1.7).

Protein Analysis

IFNα protein was measured from culture supernatants by ELISA (assay range, 12.5pg/ml-500pg/ml, PBL Interferon Source, NJ, USA) and analysed on a Fluorostar Optima microplate reader (BMG Labtech, Ortenberg, Germany) according to the manufacturer's instructions. IL2, TNFα, IL12 and IFNγ protein was measured from culture supernatant using cyotometric bead array (assay range 10-2500pg/ml BD Bioscience, CA, USA), according to the manufacturer's instructions. Samples were assayed on a BD FACS Canto II Flow Cytometer and analysed with BD FCAP Array Software (BD Bioscience CA, USA). The theoretical minimal limits of detection were 10pg/ml (IFNα), 11.2pg/ml (IL2), 1.2pg/ml (TNFα) 0.6pg/ml (IL12) and 1.8pg/ml (IFNγ).

Antibodies for Flow Cytometry

The following fluorochrome-conjugated antibodies were used in these experiments: CD303-FITC, CD1c-PE, CD141-APC (Miltenyi Biotec Australia Pty. Ltd. North Ryde, NSW, Australia), CD14-PeCy7, CD19-PeCy7, CD3-Pe-Cy7,CD8-Pe-Cy5 (Bio-Scientific Pty. Ltd. Gymea, NSW, Australia), IFNα-PE, CD86-PeCy5, HLADR-APC-Cy7, PDL1-Pe-Cy7, PDL2-APC and PD1-PE (BD Bioscience, CA, USA). Unstained and isotype controls were used in all experiments. General flow cytometry strategies described in Online Supplement, Section 1.1.

DC Subtype Enumeration

Freshly isolated PBMCs were resuspended in MACs buffer (Miltenyi Biotec Australia Pty. Ltd. North Ryde, NSW, Australia) at a final concentration $1.5x10^6$ PBMCs. PBMCs were stained with CD303 (i.e. BDCA2) for pDCs, CD1c (i.e. BDCA1) for mDC1 and CD141(i.e. BDCA3) for mDC2. PBMCs were also stained with CD14 and CD19 to exclude monocytes and B cells, respectively. Dead cell discrimination and surface marker staining was performed according to the manufacturer's instructions and samples were analysed by flow cytometry (Online Supplement, section 1.2).

Expression of CD86, HLA-DR and PDL1/2 from pDCs and PD1 on T cells

Isolated PBMCs were cultured with H1N1/09 and resuspended in MACs buffer at $1.0x10^6$ cells. To measure surface marker expression on pDCs, PBMCs were stained with antibodies for CD303, CD86, HLA-DR, PDL1 and PDL2. To measure PD1 expression on T cells, PBMCs were stained with antibodies for CD3, CD8 and PD1. Following incubation for 30 minutes at room temperature, PBMCs were washed twice with MACS buffer, centrifuged and resuspended at a final volume of 100µl in MACS buffer. Surface marker expression was analysed by flow cytometry according to the manufacturer's instructions (Online Supplement, sections 1.3 and 1.5). To confirm that pDCs were the primary producers of IFNα within our PBMC cultures, a subset of experiments were performed using intracellular IFNα staining of pDCs, T cells, monocytes and B cells (Online Supplement, sections 1.3-1.5).

Blocking PD1 and PDL1

Isolated PBMCs were cultured with H1N1/09 alone or with H1N1/09 in combination with a fluorochrome-conjugated antibody for PD1 or PDL1. IFNα, IL2, TNFα and IFNγ were measured from culture supernatant (as described above). Cells were also used to visualise blocking by flow cytometry (Online Supplement, section 1.6).

Statistical Analysis

Analyses were performed using the statistical packages STATA 11 (Stata Corp LP, Texas, USA) and Graph Pad Prism6 (La Jolla, CA, USA). Normality of the data was tested using the Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lillie for P value). To compare differences in subject characteristics, the two-sided student t test was used for continuous data or Fisher's exact test for categorical data. For experimental statistics, comparison was made between virus stimulation to media alone within each group using the two-sided paired t test. For comparison of DC enumeration, surface marker expression, and protein concentrations between pregnant and non-pregnant groups, the two-sided student t test was used. For experiments where cells were cultured with H1N1/09, data was transformed by subtracting baseline values (i.e cells cultured in media only). This is a common method when analysing in vitro cultures to ensure that the results are the direct effect of viral stimulation and not caused simply by culture conditions.[4, 24, 26] P values <0.05 were considered to be significant. Data are represented as mean±SD, protein concentrations in pg/ml and surface marker expression as percentage (%) of positive cells.

Results

The average age of the women in this study was 28±5 years, with no significant difference between groups, ($p=0.05$, Table I.). Pregnant women were recruited between the second and third trimester (20-34 weeks). Four women in each group currently smoked, however no significant difference was observed between the proportion of pregnant and non-pregnant women who had been vaccinated or smoked ($p \geq 0.51$).

DC Enumeration

To determine if there was a difference in the percentage of mDCs or pDCs during pregnancy, freshly isolated PBMCs were stained with the surface markers CD1c, CD141 or CD303 (Figure 1A). Whilst no significant difference was observed in the percentage of total DCs (Figure 1B) or in myeloid subtypes, (Figure 1C and 1D), the percentage of pDCs(i.e.$CD303^+/CD1c^-$ PBMCs) was significantly lower in pregnant compared to non-pregnant women (mean%±SD, 0.33±0.08 versus 0.41±0.07, $p<0.05$, Figure 1E).

CD86, HLA-DR and PDL1/2 Expression on pDCs

PBMCs were infected with H1N1/09 and up-regulation of the activation markers CD86 and HLA-DR, and the inhibitory markers PDL1 and PDL2, was measured on $CD303^+$ pDCs (Figure 2A and 2E respectively). H1N1/09 significantly up-regulated the surface markers on pDCs compared to cells in media alone. ($p<0.05$ Figure 2B and 2F). Compared to non-pregnant women, H1N1/09 infection of pDCs from pregnant women

induced a significantly higher expression of CD86 (mean %±SD, 8.68±7.5 versus 2.25±4.71, $p<0.01$, Figure 2C), HLA-DR (12.21±7.7 versus 1.15±2.31, $p<0.001$, Figure 2D) and PDL1 (48.48±12.84 versus 19.38±12.63, $p<0.001$, Figure 2G).

PD1 Expression on T cells

The percentage of $CD3^{+}/CD8^{-}$ and $CD3^{+}/CD8^{+}$ T cells and the up-regulation of PD1 on these subsets was measured following infection with H1N1/09 (Figure 3A). $CD3^{+}/CD8^{+}$ T cells were classified as 'hi' or 'lo' depending on the position of the population in the scatter plot (Figure 3A). H1N1pmd09 did not significantly alter the percentages of $CD3^{+}/CD8^{-}$, $CD3^{+hi}/CD8^{+hi}$ or $CD3^{+lo}/CD8^{+lo}$ T cells between pregnant and non-pregnant women (Figure 3B-D). Compared to non-pregnant women, H1N1/09 infection of CD8 T cells from pregnant women induced a significantly higher expression of PD1 on $CD3^{+hi}/CD8^{+hi}$ (mean%±SD, 2.05±1.0 versus 0.77±0.77, $p<0.05$, Figure 3F) and $CD3^{+lo}/CD8^{+lo}$ (85.68±4.2 versus 73.08±9.3, $p<0.01$, Figure 3G).

Functional Activity of pDCs and CD8 T cells

Protein concentrations of IFNα, IL2, TNFα, IL12 and IFNγ were measured from culture supernatant following PBMC infection with H1N1/09 (Figure 4). Compared to non-pregnant women, PBMCs from pregnant women showed significantly reduced levels of IFNα (mean pg/ml±SD, 412.1±414.9 vs 985.1±584.6, $p<0.01$, Figure 4A), IL2 (266.9±162.7 vs 545.8±217.3, $p<0.01$, Figure 4B) and IFNγ (186.7±130.3 vs 626.5±364.8, $p<0.01$, Figure 4D) w. TNFα did not differ significantly between the groups and IL12 levels were below detection, and thus not included.

Blocking PD1 and PDL1

To determine the effect of blocking PDL1 and PD1 during H1N1/09 infection, PBMCs were cultured with H1N1/09 alone, or in combination with a blocking antibody against PD1 or PDL1, and IFNα, IL2, TNFα and IFNγ were measured from culture supernatant (Figure 5). Blocking PD1 significantly increased the IFNα response of PBMCs from pregnant women compared to cultures with H1N1/09 alone (mean pg/ml ±SD, 632.3±297.7 vs 412.1±414.9, $p<0.05$, Figure 5A). Compared to cultures with H1N1/09 alone, blocking PDL1 during infection, significantly increased the responses of IFNα (mean pg/ml ±SD, 786.1±315.9 vs 412.1±414.9, $p<0.05$ Figure 5A), IL2 (643.6±218.3 vs 266.9±162.7, $p<0.05$, Figure 5B) and IFNγ (1481±832.4 vs 314.2±426.2, $p<0.05$, Figure 5D) from PBMCs of pregnant women. Blocking PDL1 also significantly increased the IL2 response of PBMCs from non-pregnant women compared to cultures with H1N1/09 alone (mean pg/ml ±SD, 969.4±222.6 vs 545.8±217.3, $p<0.05$, Figure 5B).

Discussion

We show that during human pregnancy there is significant reduction in the percentage of circulating pDCs (i.e.$CD303^{+}/CD1c^{-}$) compared to the non-pregnant state. We also demonstrate that during pregnancy, H1N1/09 infection alters pDC phenotype by increasing expression of activation and antigen-presenting molecules, as well inhibitory ligands. In addition, CD8 T cells from pregnant women demonstrate phenotypic and functional exhaustion following H1N1/09 infection compared to the non-pregnant state. Blocking PDL1:PD1 during H1N1/09 infection improved maternal immune responses during pregnancy. These findings may provide novel insight into pregnancy-related alterations at the cell-specific level induced by H1N1/09 infection, increasing our understanding of why pregnant women are considered a high risk group during influenza pandemics.

In this study, we used blood-derived DC antigens (BDCAs), a recently identified group of ligands with increased sensitivity and specificity in detecting DC subtypes from peripheral blood.[11, 27] Blood samples were collected from women primarily in the third trimester (27.5±6.7 weeks), since the risk of influenza-induced morbidity and mortality increases with gestation.[28-30] Previously, it has been shown that whilst the percentage of mDCs is higher in early pregnancy, by late gestation, no difference exists compared to the non-pregnant state.[19, 31] Myeloid subtypes display differential immune mechanisms,[11, 32] however, the specific role of these subtypes during pregnancy has yet to be explored. We extend current knowledge by showing that no difference exists in

either mDC1 or mDC2 subtypes in late-stage pregnancy compared to non-pregnant women. We also show for the first time that the percentage of pDCs (i.e.$CD303^{+}/CD1c^{-}$ PBMCs) is significantly reduced during pregnancy. Previously, we have shown that PBMCs from pregnant women have significantly reduced IFNα production following *in vitro* infection with H1N1/09.[4] Since pDCs are the primary producers of IFNα, [10] a decreased proportion of circulating pDCs provides a plausible explanation for reduced IFNα following H1N1pmd09 infection during pregnancy.

Several studies have found that during pregnancy, peripheral and cord blood DCs display 'incomplete activation'; characterised by up-regulation of activation markers like CD40, CD83 and CD86, but, down-regulation of the antigen presenting molecule HLA-DR.[17-19] This phenotype may result from soluble factors (e.g. hormones) and becomes more pronounced as pregnancy progresses.[19, 33] We identify for the first time that following H1N1/09 infection, there is a significantly higher percentage of pDCs from pregnant women that express these activation markers compared to the non-pregnant state. Whilst increased CD86 is normal during pregnancy, [17-19] 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.[34] This potentially occurs through the suppressive activity of CD86 when in contact with the high-affinity inhibitory receptor, CTLA-4.[35] During normal pregnancy, incomplete activation 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.[19] Together these novel findings identify an important mechanism whereby influenza infection during pregnancy may increase the adverse outcomes not only for the mother, but also for the unborn fetus.

We also found that H1N1/09 infection induces a significantly higher percentage of pDCs from pregnant women that express the inhibitory ligand PDL1. Through interaction with the PD1 receptor, PDL1 forms a potent negative co-stimulatory pathway.[20, 35, 36] During pregnancy, PDL1 is involved in maintaining fetal tolerance through inhibiting effector T cell activity [20, 35] but certain viruses can induce PDL1 expression to evade detection and clearance by the host. For example, hepatitis C virus, up-regulates CD86 and PDL1 on DCs, leading to T cell suppression and impaired adaptive antiviral immune responses.[37] Similarly, human rhinoviruses can induce PDL1 expression on DCs, which inhibits effector CD4 and CD8 T cell function.[15] It is possible then, that this increase in PDL1 on pDCs following H1N1/09 infection during pregnancy, may actually suppress maternal antiviral T cell responses and contribute to increased influenza-induced illness during pregnancy.

Since PDL1 was increased on pDCs, we wanted to determine whether PD1 is also increased on T cells from pregnant women during influenza infection. We found a significantly higher percentage of $CD8^+$ T cells from pregnant women that expressed PD1 following H1N1/09 infection compared to non-pregnant women. Interestingly, we observed increased PD1 on $CD8^{+lo}$ T cells only after influenza infection. Others have

also identified the existence of peripheral $CD8^{+lo}$ T cells, which displayed reduced proliferation and cytokine production and which function to regulate naïve $CD8^{+hi}$ T cells by decreasing their responsiveness to antigen stimulation.[38] Since viral infections can induce CD8 T cell exhaustion, characterised by increased PD1 expression,[23] our finding of influenza-induced expression of PD1 on CD8 T cells during pregnancy could pose a serious problem given the central role of these killer cells during viral infection and in maintaining fetal tolerance.[21, 22, 39]

As we have observed previously,[4] *in vitro* H1N1/09 infection of PBMCs from pregnant women show a significant reduction in IFNα, which we confirmed in this study was produced predominantly by pDCs in PBMC cultures (online supplement, section 1.4). Functional exhaustion of CD8 T cells during chronic viral infections is characterised by loss of effector activity, including progressive loss of IL2, T cell cytotoxicity and proliferation, followed by TNFα, and finally IFNγ. [23, 40, 41] We found that PBMCs from pregnant women showed lower production of IL2 and IFNγ compared to non-pregnant women. Collectively, these findings provide evidence that pDCs and CD8 T cells from pregnant women exhibit not only phenotypic but also functional alterations in their antiviral activity following H1N1/09 infection.

Blocking PDL1:PD1 interactions can reverse T cell exhaustion [42] and using $PDL1^{-/-}$ or $PD1^{-/-}$ knockout mice, reduces disease severity and increases viral clearance and survival following viral infections.[36, 43] In our study, blocking PD1 improved the IFNα responses from pregnant women, whilst blocking PDL1 improved IFNα, IL2 and IFNγ production from PBMCs of pregnant women. Thus PD1/PDL1 blocking may be an

effective method for improving pDC and CD8 T cell activity in pregnant women during influenza pandemics. Performing blocking studies using animal models would be an important step to understand the full extent for the efficacy of such a therapeutic strategy in improving the antiviral response during influenza infection in pregnancy, without posing a risk for either mother or baby.

This study identifies significant changes that occur in maternal antiviral cellular immunity that may explain increased disease severity observed in pregnant women during influenza pandemics like H1N1/09. Understanding the cellular mechanisms that lead to increased morbidity and mortality during influenza infection in pregnancy is of great importance in the development of effective strategies to improve health outcomes for both mother and baby. The findings herein provide a framework for future studies aimed at investigating these cellular pathways and potential therapeutic strategies that could be employed to prevent the deleterious effects of influenza infections during pregnancy.

Footnotes

Conflict of Interest Statement: The authors do not have a commercial or any other association that might pose a conflict of interest.

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Tables

Table I. Demographic Characteristics of Study Groups

	Healthy Controls	Pregnant
N	28	26
Age (years)	27±4.9	29.2±4.5
Gestational Age (weeks)	n/a	27.5±6.7
Current Smoker (N)	4	4

Data represented as mean±SD, Group comparisons of continuous variables analysed using the two-sided student t test and categorical variables compared using Fisher's exact test.

Figure Legends

Figure 1.DC Enumeration

(A) Gating strategies to identify DC subtypes **(B)** Total percentage of DCs obtained by combining mDCs (mDC1 and mDC2) with pDCs. **(C-E)** Percentage of DC subtypes. PBMCs isolated from n=10 non-pregnant (HC) and n=10 pregnant women (P). Comparisons between pregnant and non-pregnant women were made using the two-sided student t test. Experiments were performed in duplicate. Data presented as the percentage of cells positive for each surface marker and graphs displayed as mean±SD.*$p<0.05$

Figure 2.Surface Marker Expression of CD86, HLA and PDL1/2 on pDCs following H1N1/09 Infection

(A and E) Gating strategy to identify surface markers expressed on $CD303^{+}$ pDCs. **(B and F)** Surface marker expression on pDCs from pregnant women following culture with H1N1/09 or in media alone. **(C, D, G, H)** Surface marker expression on pDCs from pregnant and non-pregnant women following H1N1/09 infection. PBMCs isolated from n=12 non-pregnant (HC) and n=10 pregnant women (P). Comparisons between pregnant and non-pregnant women were made using the two-sided student t test. Experiments were performed in triplicate. Data presented as the percentage of pDCs positive for each surface marker and graphs displayed as mean±SD and graphs displayed as mean±SD. Negative values indicate that H1N1 induced down-regulation of

the marker compared to media alone (i.e. H1N1/09 values minus media alone). *p<0.05, **p<0.01, ***p<0.001

Figure 3.Percentage of CD4 and CD8 T cells and their Expression of PD1 following H1N1/09 Infection

(A) Scatter plots used to identify $CD3^{+/-}$ and $CD8^{+/-}$ T cell subsets. **(B-D)** Percentage of $CD3^{+}/CD8^{-}$, $CD3^{+hi}/CD8^{+hi}$ and $CD3^{+lo}/CD8^{+lo}$ T cell subsets. **(E-G)** Percentage of PD1 expression on each T cell subtype. PBMCs isolated from n=6 non-pregnant (HC) and n=6 pregnant (P) women. Comparisons between pregnant and non-pregnant women were made using the two-sided student t test. Experiments were performed in duplicate. Data presented as the percentage of T cells positive for each surface marker and graphs displayed as mean±SD. *p<0.05, **p<0.01

Figure 4.Cytokine Production from PBMCs following H1N1/09 Infection

Protein concentrations of **(A)** IFNα **(B)** IL2 **(C)** TNFα and **(D)** IFNγ from culture supernatant. PBMCs isolated from n=10 non-pregnant and n=10 pregnant women.

Comparisons between pregnant and non-pregnant women were made using the two-sided student t test. Experiments were performed in duplicate. Data presented as protein concentration in pg/ml and graphs displayed as mean±SD. *p<0.05, **p<0.01

Figure 5.Effect of PD1 and PDL1 Blocking on Cytokine Production following H1N1/09 Infection

Protein concentrations of **(A)** IFNα **(B)** IL2 **(C)** TNFα and **(D)** IFNγ from culture supernatant following infection with H1N1/09 only, or with H1N1/09 in combination with blocking antibodies for PD1 (aPD1) or PDL1 (aPDL1). For H1N1/09 only, PBMCs isolated from n=10 non-pregnant women and n=10 pregnant women, for H1N1/09+PD1 and H1N1/09+PDL1, n=5 non-pregnant and n=5 pregnant women for IFNα and n=3 non-pregnant and n=3 pregnant women for IL2, TNFα and IFNγ. Comparisons were made within pregnant and non-pregnant groups for the TNFα response induced by H1N1, H1N1+aPD1 and H1N1+aPDL1, using the two-sided paired t-test. Individual experiments were performed. Data presented as protein concentration in pg/ml and graphs displayed as mean±SD. *$p<0.05$, **$p<0.01$