Plasmacytoid Dendritic Cells and CD8 T Cells From Pregnant Women Show Altered Phenotype and Function Following H1N1/09 Infection

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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 pregnant (n = 26) and nonpregnant (n = 28) women. DC subtypes were enumerated from PBMCs. PBMCs were infected with H1N1/09 and CD86, human leukocyte antigen-DR (HLA-DR), and programmed death ligand 1/2 (PDL1/2) measured on pDCs. PD receptor 1 (PD1) was measured on CD8 T cells. Interferon-alpha (IFN-α), interleukin-2 (IL-2), tumor necrosis factor-alpha (TNFα), and IFN-gamma were measured from culture supernatant.

Results. pDC (ie, CD303+/CD1c− PBMCs) percentages were lower in pregnant compared with nonpregnant women (P < .05). Following H1N1/09 infection, pDCs from pregnant women showed higher expression of CD86 (P < .01), HLA-DR (P < .001), and PDL1 (P < .001) compared with nonpregnant women. Expression of PD1 on CD8 T cells was higher during pregnancy (P < .05). Following H1N1/09 infection, PBMCs from pregnant women displayed reduced IFN-α (P < .01), IL-2 (P < .01), and IFN-γ (P < .01) compared with nonpregnant women. Blocking PDL1 during H1N1/09 infection increased these cytokines during pregnancy (P < .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.
subset and the more recently identified mDC2 [11]. Following influenza infection, pDCs and mDCs undergo activation and maturation, including upregulation of phenotypic markers such as CD83, CD86, human leukocyte antigen-DR (HLA-DR), and chemokine receptor 7 (CCR7) [12], as well as bulk production of the antiviral type I interferon-alpha (IFN-α), 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]. While pDCs appear dispensable for influenza clearance [12], recent studies have shown 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, programmed death ligand 1 (PD1) and PDL2 (ie, CD274/CD273), which interact with the inhibitory receptor, PD1 (ie, CD272), located on numerous cell types, including cytotoxic CD8 T cells [14]. Many viruses can evade host detection by upregulating PD1 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 PD1:PD1 negative costimulatory 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]. It has not yet been explored whether influenza infection during human pregnancy induces alterations in the activity of pDCs. However, 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 prevention of 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,” which is characterized by increased PD1 expression, as well as functional impairment of T-cell activity, including reduced cytokine production (eg, interleukin-2 [IL-2], tumor necrosis factor-alpha [TNF-α], and IFN-γ) 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 defense, 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 hypothesized the following: (1) pregnant women have lower percentages of circulating CD303+/CD1c− pDCs compared with healthy, nonpregnant women; (2) following in vitro H1N1/09 infection of peripheral blood mononuclear cells (PBMCs), pDCs from pregnant women display altered expression of the surface markers CD86, HLA-DR, and PD1/2; (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 IFN-α, IL-2, TNF-α, IL-12, and IFN-γ.

METHODS

Subjects
This was an in vitro study of PBMCs collected from healthy, nonpregnant women and pregnant women. Nonpregnant women (n = 28) were recruited from the Hunter Medical Research Institute database as well as John Hunter Hospital (JHH) staff; pregnant women (n = 26) were recruited from the JHH antenatal clinics as previously described [24]. The Hunter New England Health and University of Newcastle Human Research ethics committees approved this study. 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 4 weeks prior to sample collection. Four of the healthy controls and 5 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 2 papers.

Study Design
Women made a single visit to the JHH; baseline characterization 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]). Trained clinical staff performed venipuncture, and whole blood was collected in 9-mL 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
(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 were performed as described previously [4]. Briefly, isolated PBMCs were cultured in Roswell Park Memorial Institute medium (Invitrogen, Australia Pty Limited, Victoria, Australia) with 5% fetal bovine serum (SAFC Biosciences, Lenexa, KS) at a final concentration of $2 \times 10^{6}$ cells/mL. PBMCs were cultured with H1N1/09 (MOI 0.1) or in media alone. Cells were cultured for 48 hours at 37°C with 5% CO₂.

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, San Jose, CA), according to the manufacturer’s instructions. Previously, we showed that H1N1/09 is capable of low-level replication in PBMCs [4]; however, we confirmed that this does not affect cell viability (Supplementary Data Section 1.7).

Protein Analysis

IFN-α protein was measured from culture supernatants by enzyme-linked immunosorbent assay (assay range, 12.5 pg/mL–500 pg/mL; PBL Interferon Source, Piscataway, NJ) and analyzed on a Fluorostar Optima microplate reader (BMG Labtech, Ortenberg, Germany), according to the manufacturer’s instructions. IL-2, TNF-α, IL-12, and IFN-γ protein was measured from culture supernatant using cytometric bead array (assay range 10–2500 pg/mL; BD Bioscience), according to the manufacturer’s instructions. Samples were assayed on a BD FACs Canto II flow cytometer and analyzed with BD FCS software (BD Bioscience). The theoretical minimal limits of detection were 10 pg/mL (IFN-α), 11.2 pg/mL (IL-2), 1.2 pg/mL (TNF-α), 0.6 pg/mL (IL-12), and 1.8 pg/mL (IFN-γ).

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.); CD14-PECy7, CD19-PECy7, CD3-PeCy7, CD8-PeCy5 (Bio-Scientific Pty. Ltd., Gymea, NSW, Australia); and IFN-α PE, CD86-PECy5, HLA-DR-APC-Cy7, PDL1-PECy7, PDL2-APC, PD1-PE (BD Bioscience). Unstained and isotype controls were used in all experiments. General flow cytometry strategies are described in Supplementary Data Section 1.1.

DC Subtype Enumeration

Freshly isolated PBMCs were resuspended in magnetic activated cell sorting (MACS) buffer (Miltenyi Biotec Australia Pty. Ltd.) at a final concentration $1.5 \times 10^6$ PBMCs. PBMCs were stained with CD303 (BDCA2) for pDCs, CD1c (BDCA1) for mDC1, and CD141(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 analyzed by flow cytometry (Supplementary Data 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 $1.0 \times 10^6$ 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 100 μL in MACS buffer. Surface marker expression was analyzed by flow cytometry, according to the manufacturer’s instructions (Supplementary Data Sections 1.3 and 1.5). To confirm that pDCs were the primary producers of IFN-α within our PBMC cultures, a subset of experiments was performed using intracellular IFN-α staining of pDCs, T cells, monocytes, and B cells (Supplementary Data 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. IFN-α, IL-2, TNF-α, and IFN-γ were measured from culture supernatant (as described above). Cells were also used to visualize blocking by flow cytometry (Supplementary Data Section 1.6).

Statistical Analysis

Analyses were performed using the statistical packages STATA 11 (Stata Corp LP, Texas) and Graph Pad Prism6 (La Jolla, CA). 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 2-sided Student t test was used. For experiments where cells were cultured with H1N1/09, data was transformed by subtracting baseline values (ie, cells cultured in media only). This is a common method when analyzing 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 <.05

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were considered to be significant. Data are represented as mean ± standard deviation (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 = .05; Table 1). 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 nonpregnant women who had been vaccinated or smoked (P ≥ .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). While 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 (ie, CD303+/CD1c−) PBMCs was significantly lower in pregnant compared with nonpregnant women (mean % ± SD, 0.33 ± 0.08 vs 0.41 ± 0.07; P < .05; Figure 1E).

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

PBMCs were infected with H1N1/09. Upregulation of the activation markers CD86 and HLA-DR and of the inhibitory markers PDL1 and PDL2 was measured on CD303+ pDCs (Figure 2A and 2E, respectively). H1N1/09 significantly upregulated the surface markers on pDCs compared with cells in media alone (P < .05; Figure 2B and 2F). Compared with nonpregnant women, H1N1/09 infection of pDCs from pregnant women induced a significantly higher expression of CD86 (mean % ± SD, 8.68 ± 7.5 vs 2.25 ± 4.71; P < .01; Figure 2C), HLA-DR (12.21 ± 7.7 vs 1.15 ± 2.31; P < .01; Figure 2D), and PDL1 (48.48 ± 12.84 vs 19.38 ± 12.63; P < .001; Figure 2G).

Table 1. Demographic Characteristics of Study Groups

<table>
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<th>Characteristic</th>
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<td>26</td>
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<tr>
<td>Age, y</td>
<td>27 ± 4.9</td>
<td>29.2 ± 4.5</td>
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<tr>
<td>Current smoker, N</td>
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Data represented as mean ± standard deviation. Group comparisons of continuous variables analyzed using the 2-sided Student's t-test and categorical variables compared using Fisher exact test.

PD1 Expression on T cells

The percentage of CD3+CD8− and CD3+CD8+ T cells and the upregulation of PD1 on these subsets were 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). H1N1/09 did not significantly alter the percentages of CD3+CD8−, CD3+hi/CD8−, or CD3−lo/CD8− T cells between pregnant and nonpregnant women (Figure 3B–D). Compared with nonpregnant women, H1N1/09 infection of CD8 T cells from pregnant women induced a significantly higher expression of PD1 on CD3+hi/CD8− (mean % ± SD, 2.05 ± 1.0 vs 0.77 ± 0.77; P < .05; Figure 3F) and CD3−lo/CD8+lo (85.68 ± 4.2 vs 73.08 ± 9.3; P < .01; Figure 3G).

Functional Activity of pDCs and CD8 T cells

Protein concentrations of IFN-α, IL-2, TNF-α, IL-12, and IFN-γ were measured from culture supernatant following PBMC infection with H1N1/09 (Figure 4). Compared with nonpregnant women, PBMCs from pregnant women showed significantly reduced levels of IFN-α (mean pg/mL ± SD, 412.1 ± 414.9 vs 985.1 ± 584.6; P < .01; Figure 4A), IL-2 (266.9 ± 162.7 vs 545.8 ± 217.3; P < .01; Figure 4B), and IFN-γ (186.7 ± 130.3 vs 626.5 ± 364.8; P < .01; Figure 4D). TNF-α did not differ significantly between the groups, and IL-12 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; IFN-α, IL-2, TNF-α, and IFN-γ were measured from culture supernatant (Figure 5). Blocking PD1 significantly increased the IFN-α 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 < .05; Figure 5A). Compared to cultures with H1N1/09 alone, blocking PD1 during infection significantly increased the responses of IFN-α (mean pg/mL ± SD, 786.1 ± 315.9 vs 412.1 ± 414.9; P < .05; Figure 5A), IL-2 (643.6 ± 218.3 vs 266.9 ± 162.7; P < .05; Figure 5B), and IFN-γ (1481 ± 832.4 vs 314.2 ± 426.2; P < .05; Figure 5D) from PBMCs of pregnant women. Blocking PDL1 also significantly increased the IL-2 response of PBMCs from nonpregnant women compared with cultures with H1N1/09 alone (mean pg/mL ± SD, 969.4 ± 222.6 vs 545.8 ± 217.3; P < .05; Figure 5B).

DISCUSSION

We show that during human pregnancy there is significant reduction in the percentage of circulating pDCs (ie, CD303+/CD1c−) compared with during the nonpregnant 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 with the nonpregnant state. Blocking PDL1:PD1 during H1N1/09 infection improved maternal immune

Figure 1. Dendritic cell (DC) enumeration. A, Gating strategies to identify DC subtypes. B, Total percentage of DCs obtained by combining myeloid DCs (mDCs) (mDC1 and mDC2) with plasmacytoid DCs (pDCs), C–E, Percentage of DC subtypes. Peripheral blood mononuclear cells isolated from n = 10 nonpregnant (HC) and n = 10 pregnant women (P). Comparisons between pregnant and nonpregnant women were made using the 2-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 ± standard deviation.* P < .05.

Figure 2. Surface marker expression of CD86, HLA-DR, and PDL1/2 on plasmacytoid dendritic cells (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, and H, Surface marker expression on pDCs from pregnant and nonpregnant women following H1N1/09 infection. Peripheral blood mononuclear cells isolated from n = 12 nonpregnant (HC) and n = 10 pregnant women (P). Comparisons between pregnant and nonpregnant women were made using the 2-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 ± standard deviation (SD). * P < .05, ** P < .01, *** P < .001.
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 while the percentage of mDCs is higher in early pregnancy, no difference exists compared with the nonpregnant state by late gestation [19, 31]. Myeloid subtypes display differential immune mechanisms [11, 32]; however, the specific role of these subtypes

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–G, Percentage of CD3+/CD8−, CD3−/CD8+hi, and CD3−/CD8−lo T-cell subsets. E–G, Percentage of PD1 expression on each T-cell subtype. Peripheral blood mononuclear cells isolated from n = 6 non-pregnant (HC) and n = 6 pregnant (P) women. Comparisons between pregnant and non-pregnant women were made using the 2-sided student t test. Experiments were performed in duplicate. Data presented as the percentage of T cells possible for each surface marker and graphs displayed as mean ± standard deviation. *P < .05, **P < .01.

Figure 4. Cytokine production from peripheral blood mononuclear cells (PBMCs) following H1N1/09 infection. Protein concentrations of interferon (IFN)-alpha (A), interleukin-2 (B), tumor necrosis factor-alpha (C), and IFN-gamma (D) from culture supernatant. PBMCs isolated from n = 10 nonpregnant (HC) and n = 10 pregnant women (P). Comparisons between pregnant and nonpregnant women were made using the 2-sided Student t test. Experiments were performed in duplicate. Data presented as protein concentration in pg/mL and graphs displayed as mean ± standard deviation. *P < .05, **P < .01.
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 with nonpregnant women. We also show for the first time that the percentage of pDCs (ie, CD303+/CD1c+ PMBCs) is significantly reduced during pregnancy. Previously, we have shown that PBMCs from pregnant women have significantly reduced IFN-α production following in vitro infection with H1N1/09 [4]. Since pDCs are the primary producers of IFN-α [10], a decreased proportion of circulating pDCs provides a plausible explanation for reduced IFN-α following H1N1pmd09 infection during pregnancy.

Several studies have found that during pregnancy, peripheral and cord blood DCs display “incomplete activation,” characterized by upregulation of activation markers such as CD40, CD83, and CD86, but downregulation of the antigen-presenting molecule HLA-DR [17–19]. This phenotype may result from soluble factors (eg, hormones) and becomes more pronounced as pregnancy progresses [19, 33]. We note 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 with the nonpregnant state. While 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 upregulation 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 costimulatory pathway [20, 35, 36]. During pregnancy, PDL1 is involved in maintaining fetal tolerance by inhibiting effector T-cell activity [20, 35]; however, certain viruses can induce PDL1 expression to evade detection and clearance by the host. For example, hepatitis C virus upregulates 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 that this increase in PDL1 on pDCs following H1N1/09 infection during pregnancy actually suppresses maternal antiviral T-cell responses and contributes 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 with nonpregnant 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 naive CD8<sup>+</sup> T cells by decreasing their responsiveness to antigen stimulation [38]. Since viral infections can induce CD8<sup>+</sup> T-cell exhaustion, which is characterized by increased PD1 expression [23], our finding of influenza-induced expression of PD1 on CD8<sup>+</sup> 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, 37].

As we have observed previously [4], in vitro H1N1/09 infection of PBMCs from pregnant women results in a significant reduction in IFN-α, which we confirmed was produced predominantly by pDCs in PBMC cultures in this study (Online Supplementary Section 1.4). Functional exhaustion of CD8<sup>+</sup> T cells during chronic viral infections is characterized by loss of effector activity, including progressive loss of IL-2, T-cell cytotoxicity, and proliferation, followed by TNF-α and finally IFN-γ [23, 39, 40]. We found that PBMCs from pregnant women showed lower production of IL-2 and IFN-γ compared with nonpregnant women. Collectively, these findings provide evidence that pDCs and CD8<sup>+</sup> 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 [41] and, using PDL1<sup>−/−</sup> or PD1<sup>−/−</sup> knockout mice, reduces disease severity and increases viral clearance and survival following viral infections [36, 42]. In our study, blocking PD1 improved the IFN-α responses from pregnant women, while blocking PDL1 improved IFN-α, IL-2, and IFN-γ production from PBMCs of pregnant women. Thus, PD1/PDL1 blocking may be an effective method for improving pDC and CD8<sup>+</sup> T-cell activity in pregnant women during influenza pandemics. 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 such as 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. Our findings provide a framework for future studies aimed at investigating these cellular pathways and potential therapeutic strategies that could be used to prevent the deleterious effects of influenza infections during pregnancy.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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