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Title Page

Full Title:

Pregnant women have attenuated innate interferon responses to the 2009 pandemic swine flu

Running Title:

Attenuated interferon response to swine flu in pregnancy

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Abstract

Background

Pregnant women are considered a high risk category for influenza infection, though little is known of the biological reasons. Antiviral immunity is critical during influenza infection and understanding the changes that occur during pregnancy and the effect of vaccination is essential for improving health outcomes for mother and baby.

Methods

Peripheral blood mononuclear cells (PBMCs) were isolated from 26 healthy non-pregnant and 28 pregnant women and cultured with H1N1(2009). IFNα, IFNλ and IFNγ protein were measured from culture supernatant. mRNA expression of protein kinase R (PKR) and the Toll-like receptors (TLRs) 3, 7, and 9 was also measured from cell lysates.

Results

PBMCs from pregnant women produced significantly less IFNα (114.06[287.99] pg/ml) and IFNλ (30.65[253.51] pg/ml) compared to non-pregnant PBMCs (800.38[1108.85] pg/ml and 479.87[744.60] pg/ml respectively, p<0.01). PKR expression was also significantly reduced in pregnant PBMCs (p<0.05). Vaccination significantly improved innate and adaptive immunity in pregnancy (p<0.01).

Conclusion

PBMCs from non-vaccinated pregnant women have attenuated antiviral immunity following H1N1 stimulation, however vaccination improves this response. These
novel findings help understand the increased susceptibility and disease severity to influenza infection during pregnancy and the importance of vaccination.

**Key Words**

H1N1, swine flu, pregnancy, innate immunity, IFNα, IFNλ, PKR, TLR, vaccination
Introduction

Pregnancy is a unique immunological challenge; where maternal immunity must accommodate the foetus, whilst endeavouring to maintain maternal protection against pathogens.[1] Such immunological alterations may predispose the mother to increased susceptibility and disease severity to viral infections, especially influenza virus.

Influenza is one of the most common respiratory viruses in humans and is the seventh leading cause of death in the US.[2] Epidemiological studies identify pregnant women as a high risk group; with increased susceptibility and morbidity to influenza infections reported during seasonal influenza epidemics.[3-6] The serious consequences of influenza infection during pregnancy are best highlighted during influenza pandemics; with pregnant women showing significantly increased susceptibility, disease severity and mortality rates compared to non-pregnant women.

In the 1918 H1N1 outbreak, 50% of all infected pregnant women developed pneumonia with an overall 27% case mortality,[7] whilst in the 1957 H2N2 pandemic, 50% of all deaths that occurred in women of reproductive age were in pregnant women.[8]

Despite improvements in healthcare, the gravity of influenza infection during pregnancy remains a problem, as most recently demonstrated during the 2009 swine flu outbreak. US estimates indicated a high incidence of severe disease in pregnant women, with 32-65% being hospitalised.[9, 10] Admission rates were higher in pregnant women, 0.32/100,000 (95% CI 0.13-0.52), compared to the general population, 0.076/100,000 (95% CI 0.07-0.09),[9] with at least 15% of these women admitted to ICU.[10] Within two months of the outbreak in Melbourne, Australia, 25% of all infected females were found to be pregnant or post-partum.[11] Mortality
rates were greatly elevated in pregnant women; accounting for at least 80% of the total H1N1 induced deaths.[10] Most cases resulted from influenza-induced pneumonia, leading to acute respiratory distress syndrome (ARDS),[9, 12] which is similar to that observed in past pandemics.[13]

Increased foetal morbidity and mortality is also reported during both seasonal and pandemic influenza outbreaks. One case-control study reported a higher incidence of congenital malformations in infants whose mothers had influenza infections at some point in their pregnancy compared to those who did not.[14] They found a higher incidence of cleft lip, prevalence odds ratio (POR) 3.2 (95% CI 2.1-5.3), neural-tube defects 1.9 (1.1-3.3) and cardiovascular malformations 1.7 (1.3-2.3). Most effects were indirect, caused by maternal influenza-induced fever.[14] In the 1918 pandemic, approximately 52% pregnancy loss was reported [7] whilst in the 1957 outbreak, 4.1/1,000 infants born to infected mothers were reported to develop leukaemia compared to only 0.8/1,000 infants born to mothers not infected (p<0.001).[15] Infection with seasonal H1N1 influenza during early pregnancy has also been reported to increase the rate of miscarriages.[16]

Despite pregnant women being considered a high risk group to influenza infections, immunization coverage is low. Prior to the 2009 pandemic, US estimates were around 13-15%. By November 2010, just over 50% of pregnant women had received an influenza vaccination;[17] whilst an improvement to past coverage rates, this still accounts for only half of all pregnant women. Other countries still report a low uptake among high risk groups, despite the 2009 pandemic.[18]

Influenza primarily infects epithelial cells of the upper respiratory tract, evoking an array of host inflammatory and anti-viral cytokines and chemokines and the
recruitment of anti-viral immune cells to the site of infection.[19, 20] Type I and III IFNs are amongst the most important early anti-viral cytokines during influenza infection; released not only by epithelial cells but also key immune cells like PBMCs.[21] IFN release, initiated by viral sensors like the TLRs,[22] culminates in transcription of over 200 downstream interferon stimulated genes (ISGs); involved in antiviral, anti-proliferative and immune-modulating activities.[23-26]

Despite the importance of the innate immune system in host defence, little is known about alterations in the type I and type III IFNs during pregnancy, especially in response to influenza infection. Since PBMCs are key producers of IFNα and IFNλ, studying the response of PBMCs from pregnant women, during (in vitro) influenza infection is essential in understanding how immunity may alter in pregnancy during viral infection. We recently showed that PBMCs from pregnant women have an attenuated innate IFN response to human rhinovirus infection (the cause of the ‘common cold’).[27] In this study, we hypothesised that pregnant PBMCs infected with H1N1 would also show attenuated IFNα and IFNλ protein responses, compared to the non-pregnant state and that attenuated IFN responses in pregnancy may lead to an alteration in downstream ISGs. We also hypothesised that vaccination would improve PBMC innate immunity to influenza infection.
Methods

Participants

Twenty-eight pregnant women (17 non-vaccinated and 11 vaccinated) and 26 healthy non-pregnant women (16 non-vaccinated and 10 vaccinated), recruited predominantly in the winter seasons from July 2010-July 2011, participated in this study. Pregnant women were recruited from the John Hunter Hospital (JHH) antenatal clinics and non-pregnant women were recruited from the Hunter Medical Research Institute (HMRI) research database, as well as from JHH respiratory clinics and staff. To study the effect of swine flu vaccination, we recruited women (pregnant and non-pregnant) who had been vaccinated against 09H1N1 swine flu within 12 months prior to sample collection. Inclusion criteria for all participants were females of child-bearing age (18-40 years). Pregnant women were recruited in the second and third trimester; as increased susceptibility and disease severity to influenza infection increases with gestational age.[6, 9] Women were excluded if they had any concomitant chronic medical illness, drug or alcohol dependence, if they had asthma or other respiratory conditions or if they had cold/flu symptoms within the past four weeks prior to sample collection. Informed consent was obtained from all participants prior to sample collection and ethics approval for the study was obtained from the Hunter New England Human Research Ethics Committee and the University of Newcastle Research Ethics Committee.

Study Design

We conducted a cross-sectional study of PBMC immune responses to in vitro influenza infection. Pregnant patients were first contacted to participate in the study either by telephone or direct approach in the antenatal clinics and those women who
consented and fit the selection criteria were then booked in for a study visit at the JHH. Recruitment of the non-pregnant women was primarily through the HMRI database which is a collaborative project between HMRI, the University of Newcastle and the Neuroscience Institute of Schizophrenia and Allied Disorders (NISAD). Access to research participants who were willing to be contacted for this study was made on our behalf by HMRI and those who consented and fit the selection criteria were then booked in for a study visit. Women recruited from the department of respiratory research were samples of convenience as they worked at the hospital and were initially contacted by e-mail or directly approached.

Pregnant and non-pregnant women attended a single study visit at which baseline characterisation was assessed; including height, weight, lung function, smoking status, medication and vaccination history. Current and retrospective cold and flu symptoms were assessed using the Common Cold Questionnaire (CCQ). Venepuncture was performed at the study visit by trained clinical staff and whole blood was collected in 9ml EDTA tubes.

**Virus Preparation**

A strain of 2009 pandemic swine flu (H1N1 A/Auckland/3/2009) was obtained from the World Health Organization (WHO Melbourne) in 2010. Viral stocks were propagated in MDCKs (ATCC, Manassas, VA, USA), similar to the previously described protocol.[28] Stock viral concentrations were measured using plaque assays; which determines live virions based on viral plaque forming units per ml (pfu/ml).[28] (see supplement)
**PBMC Isolation and Culture**

PBMCs were isolated from whole blood by density centrifugation using Ficoll-Paque™ PLUS (GE Healthcare Uppsala, Sweden) and resuspended in Roswell Park Memorial Institute media (RPMI; Invitrogen, Australia Pty Limited) in 5% foetal bovine serum (FBS; SAFC Biosciences, Lenexa, Kansas, USA). PBMCs were cultured in 24 well plates at a final concentration of 2.0x10^6 cells/ml (NUNC, Denmark) stimulated with H1N1 at MOI 0.1 (2.0x10^5 pfu/ml) or cultured in media alone for 48hrs, 37°C and 5% CO₂. These concentrations were based on the ability of the virus to induce maximal IFN production with minimal cell death at 48hrs. After culture, cellular suspensions were centrifuged at 550xg, 10 min. Cell lysates (stored in RLT QIAGEN Pty Ltd Doncaster, VIC) and supernatant were stored at -80°C for subsequent analysis.

**Viral Replication in PBMCs**

To determine if H1N1 could replicate in PBMCs, cultures were prepared as described before and incubated at 37°C for four hours. Cell suspensions were then washed twice (5-10ml RPMI/5% FBS, 550xg) to remove any virus that had not entered the cells and replated with fresh RPMI/5% FBS into new 24well plates. Supernatant was collected at 24, 48, 72, and 96 hrs and plaque assays were performed to determine viral replication.

**Cell Viability**

Cell viability was measured by PE Annexin V Apoptosis Kit I (BD Bioscience CA, USA), as per manufacturer’s instructions.
Protein Analyses

*ELISAs*

IFNα and IFNλ protein was measured from culture supernatants by ELISA and analysed on a Fluorostar Optima microplate reader (BMG Labtech, Ortenberg, Germany). The assay range for IFNα (PBL Interferon Source, NJ, USA) was 12.5pg/ml-500pg/ml and for IFNλ (R&D Systems, MN, USA) 15.6pg/ml-1000pg/ml.

The minimal detectable dose (MDD) was ≤10pg/ml. The inter- and intra-assay variation was 8%. The %CV between duplicates was accepted only when ≤5%.

*Cytometric Bead Array (CBA)*

IFNγ was measured from culture supernatant by CBA (BD Bioscience CA, USA). The samples were run on a BD FACS Canto II Flow Cytometer and analysed with BD FCAP Array Software (BD Bioscience CA, USA), according to the manufacturer’s instructions. The IFNγ assay range is from 2500-10pg/ml with an MDD of 1.8pg/ml.

**mRNA Analyses**

Total RNA was extracted from PBMC lysates using the RNeasy Mini Kit and QIAcube (QIAGEN Pty Ltd Doncaster, VIC), according to manufacturer’s instructions. RNA concentration was assessed using a Nanodrop2000 (Thermo Scientific, Inc. Waltham, MA) and cDNA was transcribed from 200ng of total RNA in 20μl total volume, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems (ABI) Mulgrave, VIC). For realtime-PCR (RT-PCR) the TaqMan gene expression master mix (ABI Mulgrave, VIC) was used together with primer-specific probes for TLR3, TLR7, TLR9, and PKR (ABI Mulgrave, VIC). Primers for the house-keeping gene 18s (ABI Mulgrave, VIC) were run with each sample as an internal calibrator. A positive control, (ALLPOS, Integrated Sciences, Chatswood, NSW) was also run on each plate.
Statistical Analyses

Data were analysed using STATA11.1 (Stata Corp LP, Texax, USA) and Graph Pad Prsim4 (La Jolla, CA, USA) and significance was accepted when \( p<0.05 \). To determine the sample size required, sample size calculations were performed based upon several previous studies looking at the cellular IFN response to in vitro respiratory virus infections [27, 29, 30]. Using a Type I error set at \( \alpha = 0.05 \) and a Type II error of \( \beta =0.20 \) to give us 80% power it was estimated that an average of \( n=8 \) women would be required in each group. To compare subject characteristics between groups, age and gestational age were analysed by one-way ANOVA and smoking status by Fisher’s exact test. For experimental statistics comparing protein, mRNA expression and viral replication, the Kruskal Wallis test was performed with Duns post hoc test comparing all groups to the non-pregnant, non-vaccinated, healthy controls. To compare viral replication within each group at different time points, the paired sign-rank test was used. In text parametric data was reported as mean±SD and non-parametric data as median[iqr]. All graphs are represented as median[iqr]. Protein data was reported in pg/ml, mRNA expression as fold change from media \( (2^{-\Delta\Delta ct}) \) and viral replication in pfu/ml.
Results

Women in this study had an age range of 25-29 years and pregnant women were primarily within their third trimester (Table 1). There were no significant differences in age (p=0.32), gestational age (p=0.87), or smoking status (p=0.4) between the groups.

Table 1. Characteristics of Study Groups

<table>
<thead>
<tr>
<th>Status</th>
<th>aHC</th>
<th>bHC (v)</th>
<th>cP</th>
<th>dP(v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>16</td>
<td>10</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>Age (years)</td>
<td>25.8±3.2</td>
<td>28.2±7</td>
<td>28.9±5.3</td>
<td>27.6±4.7</td>
</tr>
<tr>
<td>Gestage (weeks)</td>
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<td>n/a</td>
<td>26.7±6.8</td>
<td>26.4±5</td>
</tr>
<tr>
<td>Smoke (N)</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

NOTE. All data represented as mean±SD.

ahealthy control, bvaccinated healthy control, cpregnant, dvaccinated pregnant.

PBMCs from pregnant women have significantly reduced type I and type III IFN responses to 09 H1N1 infection

PBMCs from pregnant and non-pregnant women were cultured with a strain of influenza isolated during the 2009 swine flu pandemic (herein referred to as 09H1N1) or cultured in media only. As no IFN protein was produced in cultures without the presence of H1N1 (i.e. media only), all subsequent results reflect the PBMC
responses to H1N1 stimulation only. PBMCs from pregnant women produced significantly less IFNα (median 114.06 iqr [287.99] pg/ml) compared to non-pregnant PBMCs (800.38[1108.85] pg/ml), p<0.01 (Figure 1A). Similarly, pregnant PBMCs showed an attenuated IFN λ response following 09H1N1 stimulation (30.65[253.51] pg/ml) compared to cells from healthy non-pregnant women (479.87[744.60] pg/ml), p<0.01 (Figure 1B).

Expression of the interferon stimulated gene, PKR, is significantly reduced in pregnant PBMCs after 09H1N1 infection

To see whether downstream ISGs would be affected by reduced IFNs, expression of PKR (an early antiviral ISG directly activated by type I and III IFNs), was tested (Figure 2). The median mRNA expression of PKR from pregnant PBMCs infected with 09H1N1, was more than 50% reduced (4.5 fold change[2.76]) compared to PBMCs from non-pregnant women (10.09 fold change[10.14]), p<0.05.

Expression of the viral sensing TLRs is no different in pregnant compared to non-pregnant PBMCs after 09H1N1 infection

To determine if the anti-viral TLRs were altered in pregnancy by influenza infection, mRNA expression of TLR3, TLR7 and TLR9 was measured in PBMCs (Figure 3). In response to 09H1N1, TLR3 showed the highest expression, with a median fold change of 7.30[15.12] in pregnant PBMCs and 4.2 fold change[7.0] from non-pregnant PBMCs (Figure 3A). However, there was no significant difference in TLR3
09H1N1 replicates in pregnant PBMCs but shows no significant difference compared to non-pregnant PBMCs

Although PBMCs are not the primary host cells of influenza infection, 09H1N1 was capable of low-level replication in PBMCs from pregnant and non-pregnant women (Figure 4). Viral replication was highest at 24hrs post-infection, decreasing slightly but not significantly in either group (p≤0.08) by 48hrs, with only minimal replication observed beyond this time-point. There was no significant difference in viral replication in pregnant compared to non-pregnant PBMCs at either 24hrs (160[416] pfu/ml vs 226[425.5] pfu/ml, p=0.47) or 48hrs (67[126.5] pfu/ml vs 120[200] pfu/ml, p=0.17). Whilst H1N1 can replicate in PBMCs, it did not affect cell viability, which was consistently ≥90% after culture.

Expression of the viral sensing TLRs is no different in pregnant compared to non-pregnant PBMCs after 09H1N1 infection

To determine if the anti-viral TLRs were altered in pregnancy by influenza infection, mRNA expression of TLR3, TLR7 and TLR9 was measured in PBMCs (Figure 3). In response to 09H1N1, TLR3 showed the highest expression, with a median fold change of 7.30[15.12] in pregnant PBMCs and 4.2 fold change[7.0] from non-pregnant PBMCs (Figure 3A). However, there was no significant difference in TLR3
(p=0.22), TLR7 (p=0.62) or TLR9 (p=0.31) mRNA expression between pregnant and non-pregnant PBMCs

PBMCs from vaccinated pregnant women do not have a significantly reduced type I, II or III IFN response to 09H1N1 infection

To determine the effect of vaccination, PBMCs were collected from pregnant and non-pregnant women who had received the 09H1N1 vaccination within the past 12 months. Following in vitro influenza infection, the IFN responses of the PBMCs from the vaccinated women were compared to those responses of the PBMCs from non-vaccinated healthy women (Figure 5). Whilst PBMCs from pregnant vaccinated women still showed a reduced IFNα and IFNλ response compared to the non-vaccinated non-pregnant PBMCs (Figure 5A and 5B respectively), this was not significantly reduced as observed previously from PBMCs of pregnant women who had not been vaccinated. To see if vaccination affected adaptive anti-viral immunity, IFNγ (the type II IFN), was measured. IFNγ production followed the same pattern as observed by the innate IFNs (Figure5C). [Figure 5 goes here]
**Discussion**

Pregnant women are known to be a high risk group for influenza infections [6] and imbalance in anti-viral immunity during infection is a likely cause. In this study, we found that PBMCs from pregnant women have an attenuated innate anti-viral immune response; showing a significant reduction in IFNα and IFNλ following in vitro stimulation with 09H1N1 influenza. However, this impaired response is improved in pregnant women by influenza vaccination.

IFNα and IFNλ are critical for host defence against influenza infection; mediating their anti-viral activity by activating hundreds of downstream ISGs. In this study we also found decreased expression of the antiviral ISG, PKR; indicating that an impairment in the IFN response in pregnancy, also affects expression of downstream antiviral genes. Diminished type I and type III IFNs, therefore provides key insight at the molecular level into why pregnant women have increased susceptibility and disease severity to influenza infection.
Type I and III IFNs are rapidly released upon host recognition of virus by the viral sensing TLRs.[22] We did not find any significant difference in the expression of TLR3, TLR7 or TLR9 from pregnant compared to non-pregnant PBMCs after culture with 09H1N1. This suggests that the reduced IFN response we observed in pregnancy is not mediated by a change in TLR expression. Interestingly, we found that after influenza infection, TLR3 showed the highest expression in PBMCs from pregnant and non-pregnant women. Since TLR3 recognizes dsRNA, and influenza is a ssRNA virus, this implies that viral replication takes place not only in epithelial cells,[31] but also in PBMCs. Indeed, when we tested viral replication from supernatant collected after PBMC culture with 09H1N1, we found low level replication at 24 and 48hrs post infection. Influenza A (H3N2 and H1N1) have been shown to infect macrophages and dendritic cells (DCs); inducing IFNα and IFNλ, as well as downstream expression of ISGs and up-regulation of TLR3.[32, 33] It is therefore reasonable to assume that it is these antigen-presenting cells which are being infected and producing the IFN responses observed in our PBMC cultures in this study.

Whilst 09H1N1 could infect PBMCs, we did not find any significant difference in viral replication between pregnant and non-pregnant women; possibly since viral replication was quite low in PBMCs. Since DCs are key IFN producers and 09H1N1 is highly sensitive to the anti-viral actions of IFNs[33] viral replication would be kept to a minimum in PBMCs, as we observed. Therefore, altered viral replication in pregnant PBMCs is not likely to contribute to the increased susceptibility and disease severity in pregnancy. Since there is no difference in viral replication or TLR expression in PBMCs from pregnant compared to non-pregnant women, it is possible that the attenuated IFN responses we observed resulted from altered function of DCs present within the PBMC population.
Vaccination not only successfully decreases influenza-induced illness in adults[34, 35] and therefore a reduction in the risk of influenza-induced infant morbidities,[14] but also directly improves health outcomes for the infant. Several studies have shown increased influenza-specific antibodies with delayed onset and severity of infection in infants whose mothers were vaccinated during pregnancy.[36] Interestingly, we found that PBMCs taken from pregnant women who had been vaccinated against 09H1N1 did not show the same attenuation in IFN response as observed in the PBMCs from non-vaccinated pregnant women. Whilst the median IFNα and IFNλ responses in these vaccinated pregnant women were still lower when compared with the non-pregnant controls, it was not significantly different compared to the response from healthy non-pregnant women. This effect seemed specific to pregnancy, as there was no difference in IFN response in non-pregnant healthy women regardless of vaccination; possibly since their IFN response is already maximal.

To determine if adaptive immunity was also altered by vaccination, we measured the type II IFN; typically released by activated Th1 cells and cytotoxic T lymphocytes, as part of the adaptive immune response.[19, 37] As expected, we found that the IFN-γ response was significantly reduced in PBMCs from non-vaccinated pregnant women, which is required for successful foetal implantation and tolerance.[38, 39] However, we found that the IFNγ response to 09H1N1 was significantly higher in PBMCs from vaccinated pregnant women; to the best of our knowledge this effect of vaccination on IFNγ in pregnancy has not been previously observed. Since type I and III IFNs are responsible for priming the adaptive immune system[40] and the success of vaccination in pregnancy appears independent of IFNγ,[41] these findings indicate that the vaccination effect we observed in pregnancy stems from innate alterations, subsequently manifested in the adaptive immune system.
Vaccination in pregnancy not only provides protection through antibody mediated immunity, but this study shows that it acts by boosting the mother’s early innate anti-viral immune response and subsequent downstream adaptive anti-viral immunity. This may be especially important given the recent evidence for trans-placental transmission of influenza.[16]

**Future Directions**

The work herein provides novel insights into the underlying mechanisms that are important in our understanding of how the immune system responds to influenza infection during pregnancy and why these women are at high risk. These findings may prove useful in developing new approaches to boost the host immune responses to influenza infection throughout pregnancy, especially during pandemics.

**Conclusion**

Pregnant women are a high risk group for influenza infections resulting in serious consequences for both mother and baby. We found an attenuated anti-viral innate immune response from PBMCs of pregnant women following stimulation with 09H1N1 pandemic influenza. Whilst 09H1N1 can infect PBMCs, there is no difference in viral replication nor viral TLR expression in PBMCs from pregnant compared to non-pregnant women. This study confirms the importance of vaccination in pregnancy; revealing a novel feature whereby vaccination appears to improve the innate antiviral immune response in pregnant women and subsequently enhances anti-viral adaptive immunity. Collectively, these findings provide important insight at the molecular level into why pregnant women and their babies have increased risk of influenza-induced morbidity, especially during influenza pandemics, and how vaccination works to improve this response.
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References


Footnotes

Conflict of interest statement

The authors of this manuscript do not have any associations that may pose a conflict of interest for the work herein with any pharmaceutical stock ownership, consultancy, advisory board member, relevant patent or research funding.

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Figure Legends

**Figure 1.** Isolated PBMCs from n=10 non-pregnant (HC) and n=12 pregnant (P) women were cultured with 09H1N1. IFNα (A) and IFNλ (B) protein were measured from culture supernatant by ELISA. Protein concentrations represented as pg/ml and graphed as median[iqr]. **p<0.01

**Figure 2.** Isolated PBMCs from n=5 non-pregnant (HC) and n=5 pregnant (P) women were cultured with 09H1N1. PKR mRNA expression was measured from cell lysates by RT-PCR. mRNA expression represented as fold change from media (2^-ΔΔct) and graphed as median[iqr]. *p<0.05

**Figure 3.** Isolated PBMCs from n=5 non-pregnant (HC) and n=5 pregnant (P) women were cultured with 09H1N1. TLR3, TLR7 and TLR9 mRNA expression was measured from cell lysates by RT-PCR. mRNA expression represented as fold change from media (2^-ΔΔct) and graphed as median[iqr].

**Figure 4.** Isolated PBMCs from n=6 non-pregnant (HC) and n=5 pregnant (P) women were cultured with 09H1N1. Viral replication was calculated from supernatant collected at 24hr, 48hr, 72hr, and 96hr time points. Viral replication represented as plaque forming units (pfu)/ml and graphed as median[iqr].

**Figure 5.** Isolated PBMCs from n=10 non-vaccinated non-pregnant (HC) and n=12 non-vaccinated pregnant (P) women as well as n=10 vaccinated non-pregnant (HC(v)) and n=11 vaccinated pregnant (P(v)) women were cultured with 09H1N1. IFNα and IFNλ were measured from culture supernatants by ELISA and IFNγ was measured by
cytometric bead array. Protein concentrations represented as pg/ml and graphed as median[iqr]. ** p<0.01