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Alterations in Inflammatory, Antiviral and Regulatory Cytokine Responses in Peripheral Blood Mononuclear Cells from Pregnant Women with Asthma

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Author Roles
Rebecca Forbes contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data and drafting the article. Vanessa Murphy, Peter Wark and Peter Gibson contributed to the conception and design of the study and revising it critically for important intellectual content and final approval of the version to be submitted. Vanessa Murphy contributed to drafting the article. None of the authors have any conflicts of interest.
Summary at a glance

We measured T helper cytokine patterns of peripheral blood cells from pregnant asthmatic women when stimulated with mitogen and pandemic influenza virus. During pregnancy, inflammatory IL17 activity is enhanced, whilst both antiviral and regulatory responses are suppressed. This provides insight as to why pregnant asthmatics experience severe symptoms during infection.
Abstract

**Background & Objective:** Severe asthma exacerbations during pregnancy are a common complication leading to poor health outcomes for both mother and baby. Asthma exacerbations are caused most frequently by respiratory viruses. A balance between antiviral and inflammatory immune responses is critical during pregnancy, but may be altered by asthma and respiratory virus infection.

**Methods:** Peripheral blood mononuclear cells were isolated from (1) non-pregnant healthy controls, (2) pregnant non-asthmatics, (3) postpartum non-asthmatics, (4) non-pregnant asthmatics (5) pregnant asthmatics, and (6) postpartum asthmatics. Cells were cultured in vitro with the mitogen phytohaemagglutinin or with a strain of the 2009 pandemic swine influenza. Interferon-γ, interleukin-10, and interleukin-17 protein were measured from culture supernatant. Neutrophil counts were obtained in samples from pregnant and postpartum women. **Results:** Following phytohaemagglutinin stimulation of peripheral blood mononuclear cells, pregnant asthmatics had significantly higher IL17 and significantly lower IFNγ responses compared to healthy non-pregnant women. Following infection with influenza, a significant reduction was also observed in interferon-γ and interleukin-10 production from PBMCs of pregnant asthmatics. The interleukin-17 response to phytohaemagglutinin correlated with the neutrophil percentage. Differences in interferon-γ interleukin-10 and interleukin-17 were found to persist for at least 6 months postpartum.

**Conclusions:** A reduction in anti-viral and regulatory immunity with increased inflammation during pregnancy occurs in peripheral blood mononuclear cells from pregnant women with asthma. This novel information may relate to the increased
susceptibility and disease severity to respiratory virus infections observed during pregnancy.

Key words

Asthma, pregnancy, gamma-Interferon, H1N1pdm09, IL-17A human

Short Title

PBMC cytokine responses in healthy and asthmatic pregnancy
Introduction

Asthma is one of the most common diseases to complicate pregnancy. [1] Australian studies have shown that between 36-45% of pregnant women with asthma have exacerbations which require medical intervention during pregnancy. [2, 3] Exacerbations are risk factors for adverse perinatal outcomes such as low birth weight. [4] Major contributors to these exacerbations are respiratory viral infections. [3] Two retrospective studies have demonstrated that pregnant women with asthma have more respiratory virus infections [5] and urinary tract infections [5, 6] compared to pregnant women without asthma. This increased susceptibility may be further influenced by severe asthma. [5] Pregnant women with asthma are also at increased risk of hospitalisation with cardiopulmonary conditions during influenza seasons, [7] suggesting that influenza virus infections in these women have more serious consequences for mothers with asthma and their babies. During influenza pandemics, pregnancy is a risk factor for acute respiratory distress syndrome (ARDS) and pneumonia, as well as increased mortality rates. [8] During the 2009 swine flu pandemic (i.e. H1N1pdm09), pre-existing asthma was found to be the only factor to increase the odds of influenza-like illness in pregnant women (adjusted odds ratio [aOR] 2.0, 95% CI 1.0 to 3.9), whilst in a case-control study conducted in Sydney, Australia, the most prevalent risk factors for hospitalisation during the H1N1pdm09 included pregnancy (odds ratio [OR] 22.4, 95% confidence interval [CI] 9.2-54.5) and asthma requiring regular preventive medication, (OR 4.3, 95% CI 2.7-6.8). [9]

The Th2:Th1 cytokine balance is important for the host response to viral infection. In non-pregnant adults with asthma, a reduction in the interferon (IFN)-γ response to human rhinovirus-16 in a peripheral blood mononuclear cell (PBMC) model was associated with
markers of more severe asthma \cite{10}. In pregnancy, the predominantly Th2 environment may contribute to increased susceptibility and severity of respiratory viral infection in women with asthma. The sensitive balance in T helper cells (Th1, Th2, T\textsubscript{reg} and Th17) during pregnancy \cite{11,12} is an essential adaptation of the maternal immune system required to ensure fetal tolerance and maternal protection against pathogens.\cite{13} Th1 cells are characterised by their release of the anti-viral cytokine, IFN\gamma and are involved in cell-mediated anti-viral immunity,\cite{14} while Th2 cells produce inflammatory cytokines such as interleukin (IL)-4, IL5 and IL13, and are involved in antibody-mediated immunity.\cite{15} Th17 cells are characterised by the release of the pro-inflammatory cytokine IL17.\cite{16,17}

These cells are involved in host defence against extracellular bacterial and fungal pathogens, and more recently, were found to play a role in fetal implantation through angiogenesis and neovascularization in the decidua.\cite{18} T\textsubscript{regs} are key players in maintaining fetal tolerance during pregnancy, mediated partially through their production of IL10, a pleiotropic cytokine with regulatory and immunosuppressive activity.\cite{19-21} IL10 is involved in the maintenance and development of pregnancy,\cite{20-22} and reduced IL10 during pregnancy may be a risk factor for pregnancy failure.\cite{23} During H1N1pdm09, reduced IL10 in infected patients was associated with increased susceptibility and disease severity.\cite{24}

There has been little research into the activity of T-helper cells in pregnant asthmatics in the context of viral infection. Potential alterations in cytokine profiles may provide novel insight into the increased susceptibility and disease severity observed in pregnant women, particularly during influenza pandemics.
We have recently demonstrated using an in vitro PBMC model that pregnant women have alterations in innate antiviral responses to human rhinovirus and H1N1pdm09 infection.\textsuperscript{[25, 26]} In addition, we found that PBMCs obtained from pregnant women with current asthma exacerbations had reduced IFN-α and IFN-λ production following human rhinovirus stimulation compared to non-pregnant healthy controls.\textsuperscript{[25]} In the current study, we hypothesise that PBMCs from pregnant asthmatics would have reduced IFN-γ and IL-10 and increased IL-17 and neutrophils following in vitro stimulation with the mitogen PHA or with H1N1pdm09.

To test these hypotheses, we aimed to: (1) Isolate PBMCs from non-pregnant, pregnant and post-partum women with and without asthma, and to stimulate those PBMCs with PHA or a live strain of H1N1pdm09. (2) Measure and compare the cytokines response of IL-17, IFN-γ, and IL-10 between the groups. (3) Perform neutrophil counts for pregnant and postpartum groups with and without asthma and correlate the neutrophil counts to PHA-stimulated IL-17 production between the groups.
Methods

Subjects

PBMCs from 99 women aged 18-40 years were used in our study: 20 non-pregnant healthy controls, 20 pregnant non-asthmatics, 10 postpartum non-asthmatics, 19 non-pregnant asthmatics, 20 pregnant asthmatics, and 10 postpartum asthmatics. In each experiment, a minimum of 10 samples were used per group. Pregnant and postpartum women were also participating into a larger randomised controlled trial at the John Hunter Hospital, and were recruited from the antenatal clinic as described previously.[27] Non-pregnant women were recruited from the Hunter Medical Research Institute Register and from John Hunter Hospital respiratory staff as described previously.[25] Pregnant women were over 18 weeks gestation and postpartum women were included 6-9 months following delivery. Additional inclusion criteria for asthmatics were current doctor’s diagnosed asthma and asthma medication use. Women were excluded if they had drug or alcohol dependence, chronic medical illness (besides asthma), cold/flu symptoms within the month prior to sample collection, and for asthmatics, any asthma-related hospital admission or unscheduled doctor’s visit, loss of asthma control or oral corticosteroid (OCS) use within the past month. Ethics approval was obtained from the Hunter New England Human Research Ethics Committee and the University of Newcastle Research Ethics Committee. Written informed consent was obtained from all participants prior to subject characterisation and sample collections.
**Study Design**

The women attended a single study visit where baseline characterisation was performed, including height, weight, lung function, smoking status, current medications, and medical history. For asthmatics, characterisation also included assessment of current therapy and symptoms, oral corticosteroid use, and asthma control using the Asthma Control Questionnaire and asthma control criteria from the GINA guidelines. Current and retrospective cold and flu symptoms were assessed using the Common Cold Questionnaire.

**Viral stocks**

H1N1pdm09 (H1N1A/Auckland/3/2009) was obtained from the World Health Organization (WHO, Melbourne). Viral stocks were propagated in Madin-Darby Canine Kidney Cells (ATCC, Manassas, VA, USA) and stock viral concentrations were measured using plaque assays, as described previously.

**Neutrophil Counts**

Whole blood was collected in serum tubes and sent to Hunter Area Pathology Service (Newcastle, NSW, Australia) for standard neutrophil counts.

**PBMC isolation and culture**

Whole blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes and PBMCs were isolated by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare Uppsala, Sweden), as per the manufacturer’s instructions. PBMCs were resuspended in Roswell Park Memorial Institute media, (Invitrogen, Australia) with 5%
fetal bovine serum (SAFC Biosciences, KS, USA), and plated in 24 well plates (NUNC, Denmark) at a final concentration of 2.0x10^6 cells/ml. Phytohaemagglutinin (PHA) (Sigma-Aldrich, MO, USA) was used at a final concentration of 5 μg/ml and H1N1pmd09 stock was added a final concentration of 0.1pfu/ml. PBMCs were cultured for 48 hours at 37°C in 5% CO₂, with no media changes during this period. Cultures were centrifuged at 550xg for 10 minutes and supernatant was stored at -80°C for protein analyses.

Cell viability

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

Cytometric Bead Array (CBA)

IFNγ, IL10, and IL17 protein was measured from culture supernatant as per the manufacturer’s instructions (BD Bioscience CA, USA). Samples were run on a BD FACS Canto II Flow Cytometer and analysed with BD FCAP Array Software (BD Bioscience CA, USA). The minimal limits of detection were 1.8pg/ml (IFNγ), 0.13pg/ml (IL10) and 0.3pg/ml (IL17).

Statistical Analyses

The statistical packages STATA 11 (Stata Corp LP, TX, USA) and Graph Pad Prism4 (La Jolla, CA, USA) were used. Normality of the data was tested using the Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lillie for p value). To determine significant differences between group characteristics, the One-Way ANOVA (or Kruskal-Wallis test) with multiple comparisons was used for continuous data and Fisher’s exact test for categorical data. Group comparisons for the protein data were analysed using the Kruskal-
Wallis test, followed by Dunn’s multiple comparison test. If levels of IL17 were below
detection, they were arbitrarily assigned the value of 0.1pg/ml similar to the method of
analysis previously described by Brooks et al.\[10\] Correlations were made using Kendal’s
tau. Significance was accepted when p<0.05. Parametric data was represented as mean ±
standard deviation and non-parametric data as median (interquartile range).

Results

Subject Characteristics (Table 1)

No significant differences existed between the groups in maternal age, gestational weeks,
months postpartum or smoking status (Table 1). Asthmatics had well-controlled asthma
with median ACQ scores below 1.5, mean forced expiratory volume at one second (FEV\(_1\))
>80% predicted and low inhaled corticosteroid (ICS) use 100(550) µg/day budesonide
equivalents). There was no significant difference in ACQ, %FEV\(_1\), or ICS dosage between
the asthmatic groups, however, pregnant asthmatics had significantly lower lung function
compared to healthy non-pregnant controls (p=0.006) and pregnant non-asthmatics
(p=0.002).

PHA Stimulation of PBMCs (Figure 1)

When PBMCs were isolated and stimulated with the mitogen PHA, which is capable of
inducing non-specific activation of T cells, we observed a significantly higher IL17
response in PBMCs from pregnant women (p=0.002), pregnant women with asthma
(p=0.04), and non-pregnant women with asthma (p=0.001), compared to healthy non-
pregnant women (Figure 1A). Following PHA stimulation, PBMCs from pregnant women
with asthma produced significantly less IFN\(_\gamma\) compared to PBMCs from healthy non-
pregnant women (p=0.002, Figure 1B). IFNγ production was also lower in postpartum
women (asthmatic and non-asthmatic) compared to healthy non-pregnant women (p≤0.02,
Figure 1B). IL10 was induced following PHA stimulation and was found to be
significantly lower in PBMCs isolated from postpartum women compared to healthy non-
pregnant controls (p=0.003, Figure 1C).

Relationship between Blood Neutrophils and IL17 Production from PHA-Stimulated
PBMCs (Figure 2)

Pregnant women, with and without asthma, had a significantly higher percentage of
neutrophils compared to postpartum women (p≤0.01, Figure 2A). When stimulated with
PHA, IL17 production from PBMCs of pregnant women with and without asthma
correlated positively with their neutrophil percentages (r=0.3, p=0.03, Figure 2B).

Respiratory Virus Stimulation of PBMCs (Figure 3)

Following stimulation of PBMCs with H1N1pdm09, IL17 production was detectable in
very low levels in cultures and no significant differences were observed between groups
(Figure 3A). High levels of IFNγ were detected in all groups following stimulation with
H1N1pdm09 (Figure 3B). H1N1pdm09 induced a significantly lower IFNγ response from
PBMCs of pregnant women with and without asthma, compared to the healthy non-
pregnant controls (p≤0.04, Figure 3B). IL10 was significantly induced from all groups of
PBMCs (Figure 3C). Following infection with H1N1pdm09, PBMCs from pregnant non-
asthmatic women and pregnant asthmatic women produced significantly lower levels of
this regulatory cytokine compared to healthy, non-pregnant women (p=0.04).
Discussion

In this *in vitro* study, we demonstrate that PBMCs from pregnant women with asthma have an enhanced IL17 response and a reduced IFNγ response following PHA stimulation compared to PBMCs of non-pregnant women. A higher production of IL17 was also observed in PBMCs from pregnant women, and asthmatic non-pregnant women, while the lower IFNγ response was maintained in postpartum women with asthma. Among pregnant and postpartum women, the production of IL17 following PHA stimulation positively correlated with the percentage of neutrophils. These results suggest that there are immune alterations specific to pregnancy and asthma, which may impair antiviral immunity and enhance inflammatory responses in these women. Whilst previously we have shown that pregnancy seems to be the driving force for an impaired innate IFN response to H1N1pdm09,[25] asthma seems to be the predominant factor that leads to an impaired adaptive immune response during pregnancy.

IL17 plays an important role in early pregnancy, with enhanced placental expression associated with implantation.[18] As pregnancy progresses, Th17 activity decreases as a protective mechanism,[31] since high levels of IL17 in the third trimester are associated with adverse pregnancy outcomes.[32-34] In a study by Toldi et al., it was shown that the percentage of Th17 cells in pregnant women with asthma is higher than pregnant women without asthma.[17] They found that increased Th17 percentages was driven by asthma status, since no difference in Th17 cell proportions were observed between pregnant and non-pregnant women. In the current study, pregnancy and asthma both appear to be driving factors for the increase in IL17 production from PHA-activated PBMCs, since non-
pregnant asthmatic women, and pregnant women with and without asthma women all have
significantly higher production compared to healthy non-pregnant controls.

IL17 is also involved in neutrophil recruitment by inducing the release of the neutrophil
chemoattractant, IL8, from epithelial cells \cite{35} During *in vitro* infection with human
rhinoviruses, IL17 also acts synergistically to enhance IL8 production \cite{36} Neutrophil
counts increase during normal pregnancy \cite{37} however, neutrophil infiltration is also a
characteristic feature of virus-induced asthma exacerbations \cite{38} During pregnancy,
previous research has identified a positive correlation between IL17-producing cells and
blood neutrophils in cases of spontaneous abortions \cite{32} In this study, a significant
correlation was observed between neutrophil percentage and PHA-stimulated IL17
production from PBMCs during pregnancy. This may represent a mechanism of increased
susceptibility and severity of viral infections in pregnant women with asthma.

The inflammatory consequences of H1N1pdm09 infection were investigated in our PBMC
model. Previously we demonstrated that H1N1pdm09 can infect PBMCs from pregnant
women \cite{30} However, when PBMCs were infected with H1N1pdm09, there was no effect
of pregnancy or asthma on IL17 production. This was due to the low levels of IL17
observed from PBMCs following H1N1pdm09 infection. Since the mitogen PHA was able
to induce a significant IL17 response from PBMCs, this demonstrates that these cells were
capable of producing IL17 *in vitro*. Also, the IFNγ and IL10 responses we observed from
PBMCs of all groups following *in vitro* H1N1pdm09 infection indicates that H1N1pdm09
was capable of inducing cytokine responses from PBMCs *in vitro*. Furthermore, it is
known that *in vivo* H1N1pdm09 infection induces a potent IL17 response, especially in
severely ill patients requiring hospitalisation \cite{20, 39} Thus it seems that the lack of IL17
protein response we observed was due to the ‘in vitro’ nature of H1N1pdm09 infection. Indeed others have found that IL17 production following in vitro stimulation is quite varied depending upon the stimulus used.[40]

We did, however, observe significantly lower IFN\(\gamma\) and IL10 responses in PBMCs from pregnant women with and without asthma following H1N1pdm09 infection. During normal pregnancy, the IFN\(\gamma\) response decreases, whilst inflammatory Th2 cytokines are known to increase as gestation progresses.[11] This decreased Th1 response with increased Th2 activity is also observed in asthmatic epithelial cells following in vitro respiratory virus infection. [41] Recently it was also observed that CD4\(^+\)/IFN-\(\gamma\)^+ cells are significantly reduced in pregnant asthmatics (as well as pregnant non-asthmatics and non-pregnant asthmatics).[17] As such, our findings of a significantly reduced IFN\(\gamma\) response from PBMCs following H1N1pdm09 infection (and PHA stimulation) correspond well with these previous findings. These findings also suggest that during pregnancy, there is an impaired early antiviral response which may increase the risk of intense airway inflammation, viral pneumonitis and ARDS as is commonly observed during influenza pandemics.[8] During pregnancy, a reduction in the T\(_{reg}\) cytokine IL10 leads to adverse pregnancy outcomes, including preeclampsia, spontaneous abortion and preterm delivery.[34, 42] IL10 also plays an important role in limiting effector T cell-mediated host tissue damage during viral infections.[43, 44] This was observed during the H1N1pdm09, where reduced IL10 following infection was associated with increased susceptibility and disease severity, potentially inducing lethal pulmonary inflammation.[24, 44] A significant decrease in IL10 following H1N1pdm09 infection, as observed in this study in PBMCs from pregnant women with and without asthma, could be associated with increased risk of
infection, host tissue damage and adverse pregnancy outcomes. A significant reduction in PHA-stimulated IL10 observed in PBMCs from postpartum non-asthmatic women, indicates that a diminished regulatory response may still exist for at least 6 months following delivery.

In conclusion, PBMCs from pregnant asthmatic women have altered inflammatory, antiviral and regulatory cytokine profiles when activated by mitogen or live respiratory virus. An increase in IL17 response following PHA stimulation was common to both pregnancy and asthma, and following PHA stimulation, this IL17 response correlated positively with neutrophil percentages during pregnancy. Reduced IFNγ responses to PHA and H1N1pdm09 stimulation were observed in PBMCs from pregnant women with and without asthma, and may persist postpartum. Whilst previously we have shown that pregnancy seems to be the driving force for an impaired innate IFN response to H1N1pdm09, these findings highlight asthma as the predominant factor that leads to an impaired adaptive immune response during pregnancy. These findings give new information about the T helper cell environment in pregnant women with asthma which may be involved in influencing the susceptibility to and disease severity of respiratory viral infections and exacerbations in pregnancy.

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2. Murphy VE, Clifton VL, Gibson PG. The effect of cigarette smoking on asthma control during exacerbations in pregnant women *Thorax*. 2010; **65**: 739-44.


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Parametric data represented as mean±SD, non-parametric data represented as median(iqr)

ACQ= a measure of asthma control, based on the asthma control questionnaire, where an ACQ score of >1.5 is considered to be uncontrolled asthma. % FEV₁= forced expiratory volume in one second as percent predicted. ICS daily dose = total ug/day of budesonide (BUD) equivalents; asthmatics were currently on budesonide-eformoterol (symbicort), or fluticasone-salmeterol (seretide); where 0.5ug fluticasone propionate = 1ug budesonide. HC = healthy control, A= asthmatic, PP=postpartum, PPA=postpartum asthmatic, P = pregnant, PA=pregnant asthmatic. The one-way ANOVA with Bonferroni’s multiple comparisons test, (or Kruskal-Wallis test for non-parametric data) was used for group comparisons of continuous data, whilst Fisher’s exact test was used to analyse categorical data. a*p=0.006 compared to healthy controls, b*p=0.002 compared to pregnant women.
Figure Legends

Figure 1. Protein production from PBMCs stimulated with PHA

PBMCs were isolated from n=10 healthy non-pregnant controls (HC), n=10 pregnant non-asthmatics (P), n=10 postpartum non-asthmatics (PP), n=10 non-pregnant asthmatics (A), n=10 pregnant asthmatics (PA) and n=10 postpartum asthmatics (PPA). Isolated PBMCs were stimulated in vitro with the mitogen PHA and protein concentrations of IL17, IFNγ and IL10 were measured from culture supernatant. Median protein concentrations in pg/ml. Group comparisons were made using the Kruskal-Wallis test followed by Dunn’s multiple comparison test using non-pregnant non-asthmatics as the control group.*p<0.05, **p≤0.01

Figure 2. Percentage of neutrophils and correlation with IL17 production

(A) Neutrophil counts were performed from whole blood collected from n=10 pregnant non-asthmatics (P), n=10 postpartum non-asthmatics (PP), n=10 pregnant asthmatics (PA) and n=10 postpartum asthmatics (PPA). Group comparisons were made using the Kruskal-Wallis test followed by Dunn’s multiple comparison test. Mean±SD neutrophil counts as percentage of neutrophils from whole blood (% neutrophils). (B) Correlation between IL17 production from the same four groups following PHA stimulation and percentage neutrophils performed using Kendal’s tau.**p≤0.01, ***p≤0.001.

Figure 3. Protein production from PBMCs infected with H1N1pdm09

PBMCs were isolated from n=10 healthy non-pregnant controls (HC), n=10 pregnant non-asthmatics (P), n=9 non-pregnant asthmatics (A) and n=10 pregnant asthmatics (PA).
Isolated PBMCs were infected in vitro with a pandemic strain of 2009 swine influenza (09H1N1pdm) and protein concentrations of IL17, IFNγ and IL10 were measured from culture supernatant. Median protein concentrations in pg/ml. Group comparisons were made using the Kruskal-Wallis test followed by Dunn’s multiple comparison test using non-pregnant non-asthmatics as the control group. *p<0.05, **p≤0.01