



ORIGINAL ARTICLE

Impaired type I and III interferon response to rhinovirus infection during pregnancy and asthma

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ABSTRACT

Background Acute respiratory tract infections are common ailments to all individuals and the human rhinoviruses (HRVs) cause most of these infections. Pregnant women have increased susceptibility and disease severity to viral infections like influenza and HRVs, as do asthmatics. Successful pregnancy requires immunological modulation to permit fetal tolerance.

Objectives To determine whether pregnant women have reduced innate antiviral interferon (IFN) responses to HRV infection compared with non-pregnant women.

Methods An in vitro culture system was used, where peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of 54 women, including 10 stable asthmatics who were pregnant and 10 who were not pregnant, 10 non-asthmatic women who were pregnant, 10 who were ≥ 6 months post partum and 10 who were not pregnant. Samples were also collected from four exacerbating pregnant asthmatics. PBMCs were cultured with HRV43 and HRV1B. The antiviral proteins IFN α and IFN λ were measured from culture supernatants by ELISA.

Results Compared with healthy non-pregnant women, pregnant women had significantly reduced innate IFN responses to HRV infection ($p < 0.02$), persisting ≥ 6 months post partum ($p \leq 0.02$). Pregnant asthmatics had significantly reduced IFN λ responses compared with healthy non-pregnant women ($p \leq 0.034$), while during current asthma exacerbations a decrease in IFN α ($p \leq 0.023$) and IFN λ ($p = 0.007$) was observed. Induction by a TLR7 agonist induced a similar pattern of decreased innate IFNs during pregnancy as observed when HRV was the inducing agent.

Conclusions Reduced antiviral IFNs during pregnancy and asthma provide an important mechanism for increased susceptibility, morbidity and mortality in pregnant women with respiratory viral infection.

INTRODUCTION

Respiratory virus infections are a major issue during pregnancy, since pregnant women have increased susceptibility to respiratory virus infections as well as subsequent increased disease severity and duration of infection.^{1–2} Pregnancy can be viewed as an ‘immunological balancing act’ where fetal tolerance (via maternal immunosuppression) is essential and yet a degree of maternal protection must be maintained against invading pathogens. Alterations in adaptive immunity have been identified during pregnancy; the most notable being a switch from T-helper-1 immunity towards a T-helper-2 type response.³ The innate immune system is essential

Key messages

What is the key question?

- What are the innate antiviral immune alterations in pregnancy and asthma that contribute to their increased susceptibility and disease severity to respiratory virus infection?

What is the bottom line?

- In pregnancy and asthma, there is a significant reduction in host antiviral type I and III interferons in response to human rhinovirus infections.

Why read on?

- The results of this research are novel and provide us with an important insight as to why pregnant women are more susceptible and have increased disease severity to respiratory virus infections.

in the host's defence during viral infections with the type I (α/β) and type III (λ) interferons (IFNs) known to be a critical component of this innate defence. IFNs are released by virus-infected epithelial cells and peripheral blood mononuclear cells (PBMCs)⁴ and act to facilitate both early innate immunity as well as priming the later antiviral adaptive immune response.^{5–6} Alterations in the innate immune response during pregnancy may have important implications; however, little research has investigated modifications that are likely to arise, and much less the potential effects this may have on immunity during viral infections.

Asthma is a common comorbidity during pregnancy with 18–36% of pregnant asthmatics reported to experience at least one severe asthma exacerbation requiring hospitalisation, emergency department presentation, unscheduled doctor's visit or a course of oral corticosteroids (OCS).⁷ Human rhinoviruses (HRVs) are responsible for 60–80% of all respiratory virus-induced asthma exacerbations.^{8–9} Pregnant asthmatics have increased susceptibility to viral infections compared with pregnant non-asthmatics,¹⁰ as well as increased symptom severity and duration of infections compared with non-asthmatics.^{11–12}

Blood and bronchial epithelial cells from non-pregnant asthmatics exhibit reduced antiviral type I and III IFN responses to respiratory viruses, associated with increased viral replication, airway inflammation and increased asthma symptoms.^{13–15}

While asthmatics exhibit reduced IFN production to respiratory virus infections, it is not known whether pregnant women have a similar reduction or whether asthma in pregnancy further exaggerates this effect, thereby explaining the increased susceptibility observed in pregnant women during respiratory virus infections. We hypothesised that pregnant women would exhibit reduced type I and III IFN responses to in vitro HRV infection, which may be further worsened in asthmatics.

MATERIALS AND METHODS

Subjects

This cross-sectional study of 54 participants included six groups: 10 pregnant women with asthma and 10 without, 10 non-pregnant women with asthma and 10 without, as well as 10 non-asthmatic women in the postpartum period (≥ 6 months after childbirth) and 4 pregnant asthmatics with current asthma exacerbation (table 1). Pregnant women were recruited from the antenatal clinics at the John Hunter Hospital, as part of a larger randomised controlled trial (the 'Managing asthma in pregnancy' study¹⁶), while non-pregnant women were recruited from the Hunter Medical Research Institute Register and John Hunter Hospital respiratory clinics and staff (online supplement). Ethics approval was obtained from the Hunter New England Human Research Ethics Committee and the University of Newcastle Research Ethics Committee and informed consent was obtained from all subjects prior to participation.

Design

Baseline characterisation included height, weight, lung function, smoking status, medication and asthma history. Inclusion criteria for all participants were female gender, aged 18–40 years and currently non-smokers. Pregnant women were recruited between 18 and 28 weeks gestation. Asthmatics had a doctor's diagnosis of asthma, current asthma symptoms and/or asthma medication use. Current and retrospective cold and flu symptoms were assessed using the Common Cold Questionnaire (CCQ) classifying subjects as (1) 'no virus', (2) 'possible' or (3) 'probable', based on cold/flu symptom score (eg, nasal, chest and throat symptoms).¹⁷

For asthmatics, characterisation also included assessment of current therapy and symptoms, OCS use, and asthma control using the Asthma Control Questionnaire (ACQ) and asthma control criteria from the Global Initiative for Asthma (GINA) guidelines.¹⁸ Asthma exacerbations were defined as hospital admissions, unscheduled doctor's visits, % forced expiratory volume in one second (FEV₁) <80% predicted, loss of control (ACQ and GINA criteria) or OCS within the last 2 weeks. Blood

samples were collected within 2 days from the onset of exacerbation. Women were excluded if they had concomitant chronic medical illness or lung disease (besides asthma), drug or alcohol dependence, inability to perform spirometry or cold/flu symptoms within 1 month prior to sample collection.

Viral stocks

HRV43, a major group rhinovirus, was a clinical isolate obtained in 2005 and HRV1B, a minor group rhinovirus, was a gift from the Woolcock Institute of Medical Research in 2005. Viral stocks were propagated in RD-ICAMs (ATCC, Manassas, Virginia, USA), similar to that described previously.¹⁹ Viral concentration was determined by tissue culture infective dose 50%, calculated using the Spearman–Karber formula. UV-inactivated virus was prepared as a negative control (online supplement).

PBMC isolation and culture

Whole blood was collected in EDTA tubes and PBMCs were isolated by density 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 Pty Limited), 5% fetal bovine serum (SAFC Biosciences, Lenexa, Kansas, USA) and plated at a final concentration of 2.0×10^6 cells/ml in 24-well plates (sterile, tissue culture grade, NUNC, Denmark). Virus (live or UV-inactivated) was added at a multiplicity of infection 20, while the TLR3 agonist, polyinosine-polycytidylic acid (Poly (I:C); Sigma-Aldrich, Saint Louis, Missouri, USA), was used at a final concentration of 10 $\mu\text{g}/\text{ml}$ and the TLR7 agonist, imiquimod (Invivogen, San Diego, California, USA), at a final concentration of 5 $\mu\text{g}/\text{ml}$. PBMCs were cultured with virus or TLR agonists for a total of 48 h, at 33°C and 5% CO₂. No media changes were made during this period. Cellular suspensions were centrifuged at $550 \times g$, 10 min and supernatants stored at -80°C for subsequent analyses.

Cell viability

Cell viability was assessed routinely by Trypan-blue exclusion, which discriminates live from dead cells based on cellular necrosis. Cell viability was also tested in optimisation experiments using PE Annexin V Apoptosis Kit I (BD Bioscience California, USA), as per manufacturer's instructions. This method uses annexin and 7AAD to determine apoptotic (annexin⁺ and 7AAD⁺) and necrotic (annexin⁺ and 7AAD⁺) from viable cells (annexin⁺ and 7AAD⁺). PBMCs consistently showed >90% viability using both methods, when cultured with HRV or TLR agonists, as well as in media alone (online supplement).

Table 1 Characteristics of study groups

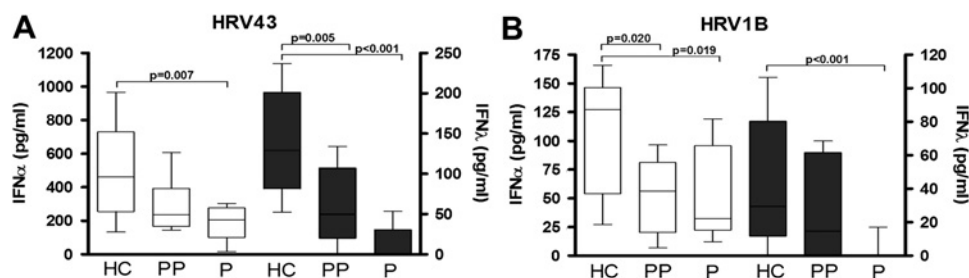
Status	HC	P	PP	A	PA	PA+X
N	10	10	10	10	10	4
Age (years)	29.7 \pm 7.2	29.7 \pm 5	29 \pm 4.3	26.5 \pm 7.9	27.4 \pm 6.1	31.2 \pm 4.9
Gestational age (weeks)	NA	26.2 \pm 2.1	NA	NA	25.1 \pm 1.9	31.3 \pm 4.9
ACQ	NA	NA	NA	0.71 (0.71)	0.35 (0.72)	1.94 (0.95*)
%FEV ₁	109.6 \pm 11.8	101.4 \pm 8.5	98.7 \pm 9.7	92.8 \pm 9.1*	88.2 \pm 11.1*	75.5 \pm 21.9*
Blood IgE (IU/ml)	nd	39.5 (47)	39 (117)	nd	289 (384)*	200.5 (246.5)*
ICS use (n)	NA	NA	NA	2	5	3
ICS daily dose ($\mu\text{g}/\text{day}$ BUD equivalent)	NA	NA	NA	0 (500)	200 (800)	400 (800)

Parametric data represented as mean \pm SD, non-parametric data 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 1 s as per cent predicted; Blood IgE, total allergy score; IU, international units; ICS use, number of women on inhaled corticosteroids at the time of sample collection; ICS daily dose, total $\mu\text{g}/\text{day}$ of budesonide (BUD) equivalents. Asthmatics were currently on budesonide-formoterol (Symbicort), budesonide or fluticasone-salmeterol (Seretide), where 0.5 μg fluticasone propionate=1 μg budesonide; nd, no data were collected; NA, not applicable; HC, healthy control (non-pregnant, non-asthmatic); P, pregnant; PP, post partum; A, asthma; PA, pregnant asthmatic; PA+X, pregnant asthmatic with current asthma exacerbation.

* $p < 0.001$.

Figure 1 Interferon- α (IFN α) and IFN λ responses of peripheral blood mononuclear cells (PBMCs) from pregnant women to in vitro human rhinovirus (HRV) stimulation. Isolated PBMCs from pregnant (P), postpartum (PP) and non-pregnant healthy control (HC) women were stimulated with HRV43 (A) or HRV1B (B). IFN α and IFN λ protein concentrations (pg/ml) are shown on the left and right y-axes, respectively. Values are represented as median (IQR).



ELISAs

IFN α and IFN λ protein concentrations were measured from culture supernatants by ELISA, as per the manufacturer's instructions, and analysed on a Fluorostar Optima microplate reader (BMG Labtech). The assay range for IFN α (PBL Interferon Source, New Jersey, USA) was 12.5–500 pg/ml and for IFN λ (R&D Systems, Minnesota, USA) 15.6–1000 pg/ml. The minimal detectable dose was ≤ 10 pg/ml. The inter- and intra-assay variation was 8%. The %CV between duplicate samples was accepted when $\leq 5\%$.

PCR

RNA collected from nasal and throat swabs was used for detection of respiratory viruses by two-step PCR. Briefly, cDNA was transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsband, California, USA) followed by quantitation of target mRNA with the TaqMan Gene Expression Master Mix (Applied Biosystems) and virus-specific primers (online supplement).

Statistical analysis

Statistical analyses were performed using STATA11 (Stata Corp). For subject statistics, the Kwallis2 test was conducted to determine significant differences in characteristics between groups. For asthmatic groups, correlations between the IFN responses and %FEV or ACQ scores were tested using Kendall's τ . The rank-sum test was used to assess differences in IFN response with inhaled corticosteroid (ICS) use in asthmatics. Simple linear regression was performed on asthmatic groups to determine if ICS dose was a determinant of IFN response. To determine if the study had sufficient power, the PS program (version 2.1.31) was used.²⁰ For experimental statistics, significant differences between the groups were determined using the Kwallis2 test. Rank-sum tests were then conducted, comparing the healthy controls with each of the subsequent five groups (ie, P, A, PA, PP and PA+X). Significance was accepted when $p < 0.05$ (online supplement). All protein data and figures were reported as median (IQR).

RESULTS

The women in this study had a mean age of 28.6 ± 5.6 years, with no significant difference between the groups (table 1). Pregnant women were recruited mid to late third trimester, averaging 26.6 ± 3.2 . Asthmatics (pregnant and non-pregnant) had well-controlled asthma at the time of sample collection (ACQ scores < 1.5), while currently exacerbating pregnant asthmatics showed loss of control with a significantly increased median ACQ score of 1.9 (0.95), ($p < 0.001$). All asthmatic groups showed significantly lower %FEV and significantly higher blood IgE levels compared with the non-asthmatic groups ($p < 0.001$). There was no significant difference in ICS doses between asthmatics. No subjects were taking OCS at the time of sample

collection. No significant difference was seen in virus-induced IFN responses between asthmatics (pregnant and non-pregnant) currently on ICS treatment compared with those who were not and ICS dose was not a determinant of IFN response ($p > 0.10$).

PBMCs from pregnant women have deficient IFN α and IFN λ responses to in vitro HRV infection

PBMCs from pregnant women stimulated with HRV43 (figure 1A) produced significantly less IFN α (median 206.5 (IQR 135.9), $p = 0.007$) and IFN λ (0 (28.8), $p < 0.001$) compared with PBMCs from healthy women (460.8 (193.8) and 136.4 (77.7), respectively). Similarly, in response to the minor group HRV1B (figure 1B), PBMCs from pregnant women produced significantly less IFN α (33.1 (70.5), $p = 0.019$) and IFN λ (0 (5.4) $p < 0.001$) compared with PBMCs from healthy non-pregnant women (127 (90.8) and 33.6 (59.3), respectively). To determine if this reduced IFN response persisted following pregnancy, PBMCs were isolated from women, 6–8 months post partum. HRV43-stimulated PBMCs from postpartum women (figure 1A) produced significantly less IFN λ compared with PBMCs from non-pregnant women ($p = 0.005$). In response to HRV1B, there was significantly less IFN α produced by PBMCs from postpartum women compared with PBMCs from non-pregnant women ($p = 0.020$).

TLR7 stimulation of PBMCs follows the same pattern as HRV-induced IFN α deficiency in pregnancy

Stimulating the TLR7 pathway (figure 2) induced the same pattern of IFN α response observed for live virus with PBMCs from pregnant women producing significantly less IFN α compared with PBMCs from healthy non-pregnant women ($p = 0.015$). Stimulating the TLR3 pathway in PBMCs (online supplement) induced no significant difference in IFN response between the groups ($p > 0.05$).

Asthma during pregnancy leads to reduced IFN λ response to HRV infection

There was no significant difference in HRV43-induced IFN α production in PBMCs obtained from asthmatics (307.3 (183.2)) or pregnant asthmatics (340.6 (326.7)) compared with PBMCs from non-pregnant women (460.8 (193.8)) (figure 3A). No significant difference was observed in IFN λ response from PBMCs of asthmatics (81.2 (95.6)) compared with cells of healthy non-pregnant women (136.4 (77.7)); however, a significant reduction in IFN λ was observed in PBMCs from pregnant asthmatics (46.4 (81.9), $p = 0.012$). In response to HRV1B (figure 3B), a similar pattern was observed, with PBMCs from pregnant asthmatics producing significantly less IFN λ (6.33 (24.6)) compared with PBMCs from healthy non-pregnant women (33.6 (59.3), $p = 0.034$). TLR7 stimulation of PBMCs from pregnant asthmatic women (online supplement) induced a similar IFN response as live virus.

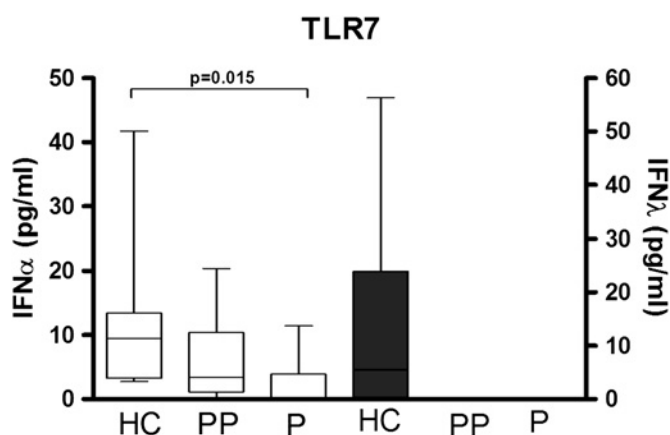


Figure 2 Interferon- α (IFN α) and IFN λ responses of peripheral blood mononuclear cells (PBMCs) to TLR7 agonists. Isolated PBMCs from pregnant (P), postpartum (PP) and non-pregnant healthy control (HC) women were stimulated with the TLR7 agonist imiquimod. IFN α and IFN λ protein concentrations (pg/ml) are shown on the left and right y-axes, respectively. Values are represented as median (IQR).

Pregnant asthmatics with an in vivo exacerbation have deficient type I and III IFN responses to in vitro HRV infection

To see how PBMCs from pregnant women responded to in vitro rhinovirus infection during in vivo respiratory illness, PBMCs were collected from four pregnant asthmatics with cold/flu symptoms (ie, 'probable virus' score according to the CCQ) and current (within 2 days of onset) asthma exacerbations. All four women had ACQ scores >1.5 and showed loss of asthma control by GINA criteria (table 1). None required ED presentation or hospital admission, but all four had an unscheduled visit to their general practitioner or the respiratory research clinician. One woman had a %FEV below 80% and two were prescribed a course of OCS following their doctor's visit (commenced after study sample collection). Exacerbations were considered respiratory virus-induced, as all scored 'probable' to the CCQ. PCR analyses of nose and throat swabs during exacerbation confirmed one positive for human metapneumovirus infection and another for coronavirus.

In response to in vitro HRV43 infection (figure 4A), PBMCs from exacerbating pregnant asthmatics produced significantly less IFN α (135.5 (183), $p=0.023$) and IFN λ (23.9 (38.4), $p=0.007$) compared with PBMC responses from healthy non-pregnant women (460.8 (193.8) and 136.4 (77.7), respectively). HRV1B-induced IFN α (figure 4B) was also significantly lower in PBMCs from exacerbating pregnant asthmatics (19.6 (39.8), $p=0.010$) compared with the response from healthy non-pregnant cells (127.7 (90.8)). No significant correlations were found

between ACQ score or FEV and the IFN responses of PBMCs from all asthmatic groups during in vitro HRV infection.

DISCUSSION

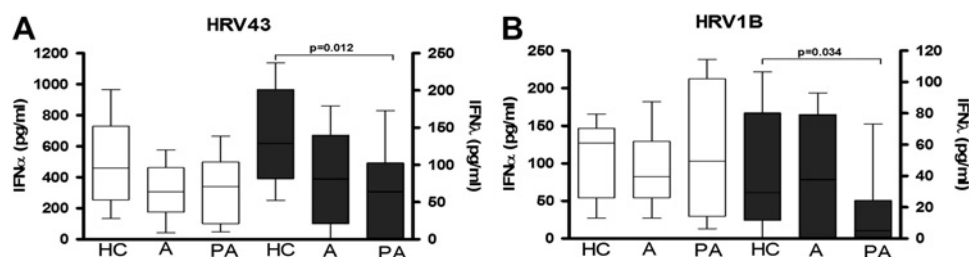
This study shows that pregnant women have deficient type I and III antiviral IFN responses to rhinovirus infection compared with healthy non-pregnant women. A similar pattern of reduced IFNs was observed in PBMCs of pregnant women when stimulated with a TLR7 agonist. A consistent and significant reduction in IFN λ was seen in pregnant asthmatics in response to HRV and both IFN α and IFN λ were significantly reduced in response to HRV in pregnant women during asthma exacerbations. These results indicate innate immune dysfunction as a possible mechanism for the increased susceptibility of pregnant women to respiratory virus infections.

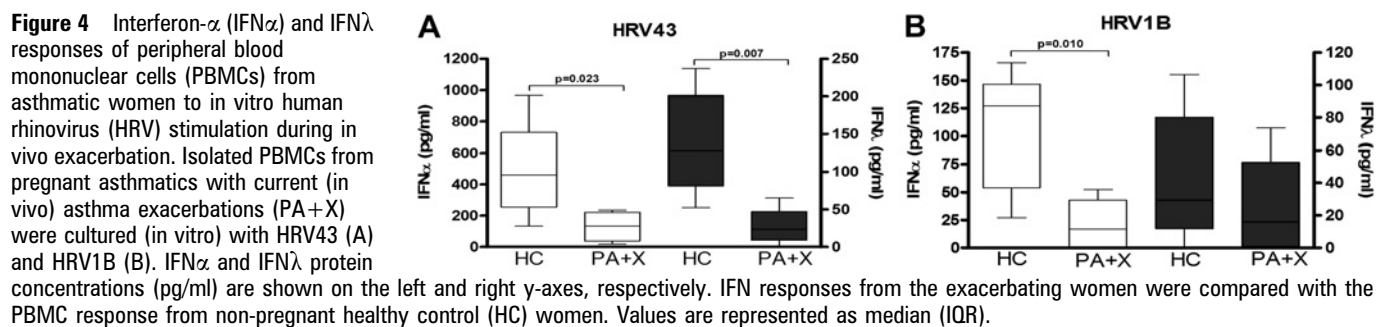
Our data add to current knowledge by demonstrating that PBMCs from pregnant women showed a marked decrease in innate IFNs in response to both HRV43 and HRV1B. The regulation and importance of type I IFNs during pregnancy, especially in response to respiratory virus infection, is still unclear, while the role of type III IFNs has yet to be explored. Epidemiological studies have identified pregnant women as having increased susceptibility to respiratory virus infections^{21 22} and the HRV-induced reduction of IFN α and IFN λ we observed in PBMCs from pregnant compared with non-pregnant women demonstrates an important mechanism explaining why pregnant women are more susceptible.

In response to both strains of HRV, IFN α and IFN λ production from PBMCs of postpartum women was significantly lower than that from PBMCs of non-pregnant women. This suggests a delay in returning to the 'normal' non-pregnant state, confirming heightened risk for respiratory viral infections in the postpartum period.²³

TLR7, an ssRNA pathogen recognition receptor of the innate immune system,²⁴ plays an important role in the type I and III IFN responses observed in this study. IFN α and IFN λ were most markedly reduced in TLR7-stimulated PBMCs from pregnant (asthmatic and non-asthmatic) and postpartum women, indicating pregnancy-associated immune alterations as the most important factor altering TLR7 function. Considering TLR7 is a key receptor for sensing viral RNA, alteration in TLR7 may be an important mechanism for reduced IFNs during respiratory virus infection in pregnancy. Plasmacytoid dendritic cells (pDCs) are key producers of both IFN α and IFN λ and abundantly express TLR7,^{25–29} making them important targets for future studies. TLR7 alterations may be due to reduced numbers of TLR7-expressing cells like pDCs or a change in receptor activity. Oestrogen is known to increase during pregnancy and has been found to alter pDC activity and function,³⁰ providing a plausible link among pregnancy, IFNs and TLR7 activity during respiratory virus infections.

Figure 3 Interferon- α (IFN α) and IFN λ responses of peripheral blood mononuclear cells (PBMCs) from asthmatic women to in vitro human rhinovirus (HRV) stimulation. Isolated PBMCs from non-pregnant healthy control (HC) women and asthmatics who were pregnant (PA) and not pregnant (A) were stimulated with HRV43 (panel A) or HRV1B (panel B). IFN α and IFN λ protein concentrations (pg/ml) are shown on the left and right y-axes, respectively. Values are represented as median (IQR).





This study shows that pregnancy, rather than asthma, appears to be the primary factor for a reduced innate IFN response. Systemic reduction of type I and III IFNs has been previously identified in asthmatic cells when stimulated with respiratory viruses.^{13–15} In our work, we did not observe a significant decrease in IFN production from HRV-stimulated PBMCs of asthmatics. This may be due to methodological differences in cell types used (whole blood, pBECs, BALF macrophages vs PBMCs), viral strains (RSV, HRV16 vs HRV43) and subject selection (male/female vs female only).

Several studies have reported increased susceptibility, symptom severity and duration of respiratory virus infections in pregnant asthmatics compared with pregnant non-asthmatics.^{10–12} We observed that in response to HRV43 and HRV1B, pregnant asthmatics showed significantly less IFN λ production compared with healthy non-pregnant women. This may help explain the increased frequency of exacerbations in pregnant asthmatics that has been observed compared with non-pregnant asthmatics.^{31–32}

In this study, we found that in vitro HRV infection of PBMCs from pregnant asthmatics currently experiencing an in vivo exacerbation resulted in a significantly lower innate IFN response compared with PBMCs from healthy non-asthmatic women. When using an in vitro method to assess antiviral responses from pregnant asthmatics, a more realistic result may be achieved if the PBMCs are isolated from asthmatics during a current asthma exacerbation, rather than when their asthma is well controlled. The exposure of PBMCs in asthmatics to high levels of inflammatory cytokines prior to isolation (as would typically occur during in vivo virus-induced asthma exacerbations³³), and the decreased IFNs in PBMCs of pregnant asthmatics (as we have shown in this in vitro study), lead to a significant reduction of IFNs when pregnant asthmatics are currently exacerbating. This provides useful insight into how a pregnant asthmatic is likely to respond to concurrent respiratory viral infection when the asthma is uncontrolled, further highlighting the importance of well-controlled asthma, especially during pregnancy. The reduction of IFNs seen in pregnant asthmatics was not likely due to steroid use, as no significant association was identified between steroid dose and IFN production in PBMCs, which is similar to previous findings.¹⁴

There are several limitations to our study, including the relatively small sample size, with $n=10$ participants per group. While these numbers are comparable to other studies looking at cellular IFN responses to in vitro virus stimulation,^{13–15} the small numbers may explain lack of significance, especially for the asthma data. This may also prevent us from making generalisable statements about entire populations and may make it difficult to detect a true interaction between steroid use and IFN response. However, the study was adequately powered to detect the difference in IFN response in pregnancy compared with non-pregnant women and provides useful mechanistic

evidence for the epidemiological evidence of increased susceptibility to infection in pregnancy.

CONCLUSION

Pregnant women are classified as a 'high risk' group for respiratory infections. Since IFNs play a critical role in viral defence, reduced levels of type I or III IFNs could render one at increased risk of developing infections and concurrent illness. This study demonstrated that in pregnant women the induction of both IFN α and IFN λ was significantly reduced in response to HRV infection. This effect was observed for at least 6 months following childbirth providing an important insight into one possible mechanism whereby pregnant women are more susceptible to respiratory virus infections. Stimulating the TLR7 pathway in PBMCs induced a similar IFN response from PBMCs of pregnant women as observed by live HRV infection. Reduced type I and III IFNs in pregnant asthmatics, especially during current asthma exacerbations, also provide an explanation as to why pregnant asthmatics experience an increase in severe respiratory symptoms during virus infection. Understanding this alteration in innate immunity will enable the future development of effective target strategies aimed at IFN production, helping to prevent or at least control virus infection during pregnancy, thereby improving health outcomes for both the mother and the baby.

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Correction notice This article has been corrected since it was published Online First. The order of the authors has been updated.

Competing interests None.

Ethics approval Hunter New England Human Research Ethics Committee and the University of Newcastle Research Ethics Committee.

Contributors PGG, PABW and VEM were responsible for the planning and intellectual input/advice for the article. RLF was responsible for the planning, conduct and reporting of the work described in the article.

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Journal club

A possible mechanism of drug tolerance in mycobacteria

In this study, the authors investigated possible mechanisms of drug tolerance in mycobacteria and identified a key mediator to target with drug therapy, with the aim of shortening current treatment and moving towards eradication.

Using an in vivo model, they found that drug tolerant mycobacteria existed in macrophages prior to granuloma formation, indicating that the granuloma is not essential for developing tolerance, but is important for dissemination. By monitoring the movement of individual macrophages from a particular granuloma they observed that these macrophages could leave the granuloma and recruit other macrophages to create new granulomas, often at sites distant from the original. This explained a well-established phenomenon in human tuberculosis infection.

The group confirmed the importance of macrophages in the induction of drug tolerance and showed that efflux pumps are essential for both bacterial growth and development of drug tolerance. They found that bacteria retained intracellularly induced tolerance when they became extracellular. This explained how tolerant extracellular bacteria could arise in the granuloma's necrotic core in human tuberculosis infection.

By challenging current dogma, the researchers have established the important role of macrophages and granulomas in inducing and disseminating tolerance. They have found efflux pumps to be a useful specific target of drug therapy due to their role in tolerance and bacterial growth.

► **Adams KN,** Takaki K, Connolly LE, *et al.* Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 2011;**145**:39–53.

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ONLINE DATA SUPPLEMENT

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METHODOLOGY

1. Study Recruitment Protocol

1.1 Pregnant Participants

Pregnant women (<20 weeks gestation) were recruited for the Managing Asthma in Pregnancy (MAP) study from the John Hunter Hospital antenatal clinic when they presented for routine antenatal care. The midwife or research nurse provided information about the study and consenting participants were included if they were >18 years of age, between 12 and 20 weeks gestation, and for asthmatics, had current asthma symptoms, a doctor's diagnosis of asthma, or had been taking regular inhaled asthma therapy in the past 3 months. The presence of a concomitant chronic medical illness, drug or alcohol dependence, inability to attend study visits, or inability to perform spirometry or exhaled nitric oxide measurement, three or more courses of oral corticosteroids (OCS) in the past year, a hospital admission for an asthma exacerbation in the past 3 months, use of regular oral prednisolone or theophylline, were exclusion criteria. Women attended monthly study visits during the MAP study and for the *in vitro* work done in this study, blood samples were collected from the women at visit 2, which coincided with 20-28 weeks gestation (except for exacerbating pregnant asthmatics where samples were collected at any visit where exacerbation occurred).

1.2 Non-Pregnant Participants

Non-pregnant participants were recruited primarily from the HMRI register volunteer database. The database is a collaborative project between HMRI, the University of Newcastle and the Neuroscience Institute of Schizophrenia and Allied Disorders (NISAD). It consists of volunteers from the community 18 years old and over, recruited through an advertisement campaign under the HMRI banner. No specific gender or ethnicities were targeted and no other specific selection criteria were used for inclusion in the database. An application was made for access to research participants willing to be contacted and individuals that fit the selection criteria for this project were then approached on our behalf. Women recruited from the department of respiratory research were initially contacted by e-mail. After participant consent, appointments were made for baseline characterisation and if suitable for inclusion, sample collection. Identical inclusion and exclusion criteria were required for the non-pregnant asthmatic women as outlined above for the pregnant women.

2. Viral Stock Preparations

2.1 Origin, isolation and characterisation of HRV43

HRV43 was a clinical isolate obtained in 2005 from sputum and nasal/throat swabs collected from subjects over 7 years of age presenting to John Hunter Hospital Emergency Department with an acute asthma exacerbation. Clinical samples confirmed to be HRV positive (as determined by real-time PCR; detailed methodology described below in section 4. “Viral PCR”), were cultured in 70-80% confluent RD-ICAMs (ATCC, Manassas, VA, USA) in T175 tissue culture flasks, at 37°C/5% CO₂ in Dulbecco’s modified eagles medium (DMEM Sigma-Aldrich Co, Castle Hill, NSW, Australia) with 5%FBS (SAFC Biosciences, Lenexa, Kansas,

USA). Media was changed every two days and after 7-10 days cells were freeze-thawed twice to lyse the cells. The cell debris was then pelleted by centrifugation at 250 x g for 10 min and 1 mL of clarified supernatant re-inoculated to fresh RD-ICAM cells for a further 7-10 days. After two passages, or once a cellular cytopathic effect (CPE) was visually evident, clarified cell supernatant was harvested and stored at -80°C for future PCR analyses.

Viral RNA was first extracted from the clarified supernatant that had been stored using the QIAamp viral RNA mini kit (QIAGEN Pty Ltd Doncaster, VIC). A fragment of approximately 549 nucleotides encompassing the VP4/VP2 region and the hypervariable region in the 5'-non-coding region was amplified with one-step RT-PCR (QIAGEN OneStep RT-PCR Kit) as per manufacturer's instructions using HRV specific primers [1]. RT-PCR products were run on a 1.8% agarose gel, visualised using ethidium bromide staining and fragments of expected size were purified using the QIAquick Gel Extraction kit (QIAGEN Pty Ltd Doncaster, VIC). The nucleotide sequences were determined in cycle sequencing reactions using DYEnamic™ ET Dye terminator cycle sequencing kit (MegaBACE™-GE Healthcare Uppsala, Sweden), as per the manufacturer's instructions using the same forward and reverse primers used for RT-PCR. Sequence data of approximately 420 nt (207 nt in VP4 and 213 nt in VP2) [1] was analysed with the Sequencher program (version3.1.1). Nucleotide-nucleotide alignments were performed using NCBI BLAST (Basic Local Alignment Search Tool) to assess percent identity with prototype strains (GenBank).

2.2 Propagation

RD-ICAMs (ATCC, Manassas, VA, USA) were first cultured in T175 tissue culture flasks, at 37°C/5% CO₂ in Dulbecco's modified eagles medium (DMEM Sigma-Aldrich Co, Castle Hill, NSW, Australia) with 5%FBS (SAFC Biosciences, Lenexa, Kansas, USA) until 70-80% confluent. Media was then replaced with 5ml DMEM containing 1%FBS and 1ml of virus stock (either HRV43 or HRV1B). After 1hr of infection at room temperature, 14ml of media was added to each flask and incubated at 33°C until 60-80% cytopathic effects (CPE) was observed (approximately 34-38hrs). Infected RD-ICAMs were then frozen at -80°C for at least 24hrs before freeze-thawing the cells (37°C for 15-20mins and frozen at -80°C). Thawed cell suspensions were transferred to sterile 50ml Falcon tubes, centrifuged at 2000rpm, 10min at room temperature. Cell free supernatant was then transferred into small aliquots and stored at -80°C.

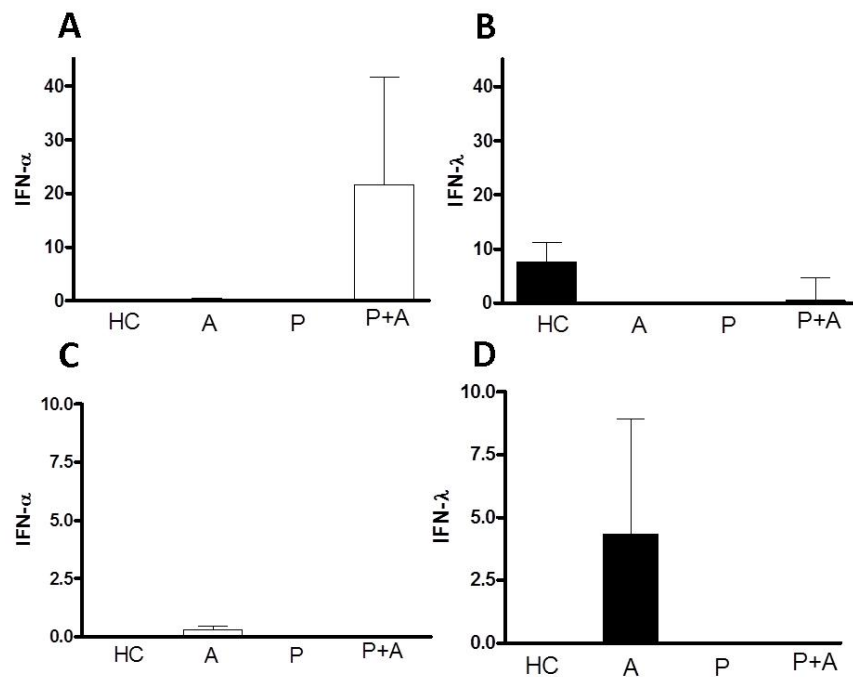
2.3 Viral Concentrations

To calculate the concentration of live virions, the TCID₅₀ (i.e. tissue culture infective dose at which 50% of cells show cytopathic effects; CPE) was performed on viral stocks. RD-ICAMs were first seeded at a 1.0×10^4 cells/well into a 96 well plate using DMEM/5%FBS and cultured overnight 37°C/5%CO₂. Media was removed and replaced with 100µl aliquots of 10-fold serial dilutions (10^{-1} - 10^{-12}) of viral stocks in DMEM/1% FCS; seven replicates were prepared per plate (plus media controls) and three plates were prepared per virus. RD-ICAMs were incubated at 33°C for five days and CPE was scored; any well in which 50% CPE was observed was counted positive for infection. TCID_{50/ml} was then calculated using the Spearman-Kärber formula.

HRV43 stock had a final concentration of 5.34×10^8 /ml and HRV1B stock concentration was 1.88×10^8 /ml.

2.4 UV Inactivation of Virus

UV inactivated virus was prepared by placing 15-20ml aliquots of viral stock supernatant approximately 10cm from a UV lamp for 5-8hrs. To ensure complete inactivation of UV virus, TCID₅₀ was performed similar as for live virus, except with neat stocks only; no cell death indicated successful inactivation of the virions. Inactivated virus was used a negative control to ensure (1) the observed effects were due to live virus only and (2) no significant IFN response was induced from cell lysates in the viral stock. No significant difference in IFN response was observed from UV-inactivated virus compared media only (Supplement Figure 1).



Supplement Figure 1. PBMC Response to UV-inactivated virus. Isolated PBMCs from n=10 non-pregnant healthy controls (HC), non-pregnant asthmatics (A), non-asthmatic pregnant women (P) and pregnant asthmatic women (P+A) were cultured

with UV inactivated HRV43 (panels A and B) and HRV1B (panels C and D). IFN α (panels A and C) and IFN λ (panels B and D) protein concentrations (pg/ml) are represented on the left y axes.

3. PBMC Culture and Cell Viability

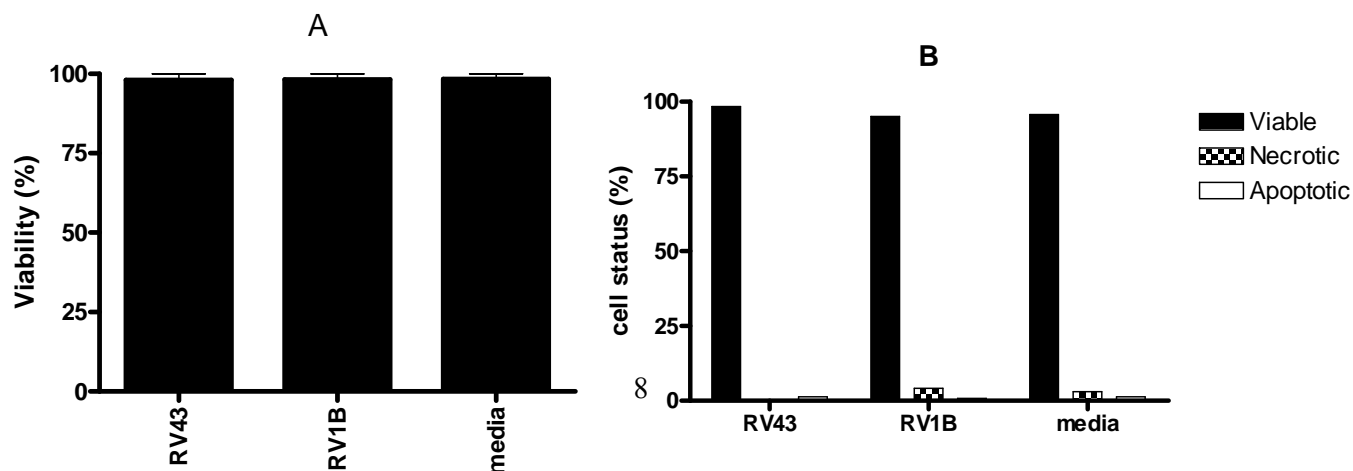
3.1 PBMC culture

Isolated PBMCs were resuspended in 2-4ml of Roswell Park Memorial Institute media, (RPMI; Invitrogen, Australia Pty Limited) 5% foetal bovine serum (FBS;SAFC Biosciences, Lenexa, Kansas, USA) and PBMC suspensions were then seeded at a final concentration of 2.0×10^6 cells/ml into 24 well plates (Nunclon Surface, sterile, tissue culture grade, NUNC, Denmark). HRV43 and HRV1B were added at an MOI 20, while the TLR3 agonist, polyinosine-polycytidylic acid (Poly(I:C); Sigma-Aldrich, Saint Louis, MO, USA) was used at a final concentration of 10 μ g/ml (2 μ l of a 5mg/ml stock) and the TLR7 agonist, Imiquimod (Invivogen, San Diego, CA, USA) at final concentration 5 μ g/ml (5 μ l of a 1mg/ml stock). The virus and TLR agonists were added to the PBMC suspensions directly after seeding the PBMCs and each well was made up to a final volume of 1ml with RPMI/5%FBS (i.e. 2.0×10^6 PBMCs /well). The plates were gently shaken to ensure even distribution of PBMCs over the well surface and cultures were then incubated at 33°C/5% CO₂ for 48 hours. Virus and TLR agonists were left in contact with the PBMCs for the duration of culture and no media changes were made during this period.

Cellular suspensions were centrifuged at 550xg, 10min and supernatants stored at -80°C for subsequent analyses.

3.2 Cell Viability

Cell viability was routinely assessed by Trypan-blue exclusion, directly after PBMC isolation as well as 48 hours after culture with virus or other stimuli. For Trypan-blue exclusion, a 1:20 dilution of PBMC suspension was prepared with Trypan-blue and cells were aliquoted onto an Improved Neubauer haemocytometer (BlauSuperior 0.0025mm²) and analysed using an Olympus IX51 U-RFL-T microscope (Olympus, Australia) at 10x magnification. Viability is measured as percent viable cells and determined by counting the number of live cells divided by the total number of cells (live and dead) divided by 100; dead cells (i.e necrotic cells) appear completely blue whilst live cells are clear. Routine testing showed that cell viability, both directly after PBMC isolation, as well as 48hrs after viral or other stimulation showed >90% viability (Supplement Figure 2A). Apoptosis and necrosis were also tested during optimisation experiments to ensure that neither HRV strain induced more than 10% cell death at MOI20. The method was performed according to manufacturer's instructions. PBMCs stimulated with both strains showed over 90% viability (Supplement Figure 2B).



Supplement Figure 2. Apoptosis and Necrosis of PBMCs stimulated with HRV.

Figures 2A represents routine results obtained with Trypan-blue exclusion, whilst Figure 2B is representative of results obtained during optimisations. Cell status is represented on the y axis as viability (2A) and viable, necrotic or apoptotic (2B). The PBMC responses to HRV43, HRV1B or media alone are indicated on the x axis.

4. Viral PCR

Real time PCR was used to detect human rhinovirus, enterovirus, influenza, respiratory syncytial virus, coronavirus and metapneumovirus from nasal and throat swabs. Prior to RNA extraction, swabs, stored in RNA Later, (RLT QIAGEN Pty Ltd Doncaster, VIC) were homogenised using QIA shredders (QIAGEN Pty Ltd Doncaster, VIC), according to manufacturer's instructions. Total RNA (from homogenised swabs or PBMC lysates in RLT) was then extracted with QIAGEN RNeasy Mini Kit (QIAGEN Pty Ltd Doncaster, VIC), according to manufacturer's instructions. RNA concentration and integrity was assessed using the Nanodrop2000 set at 260nm for RNA analysis (Thermo Scientific, Inc. Waltham, MA). 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 virus detection from swab samples, 5µl of cDNA was added to a virus-specific PCR master mix; consisting of the Eppendorf 2.5X Real Master Mix Probe ROX (Quantum Scientific) and a 20X virus-specific FAM-primer-TAMRA-probe, in 25µl final volume with virus-specific primer-probe sets, adapted from published assays [2-7].

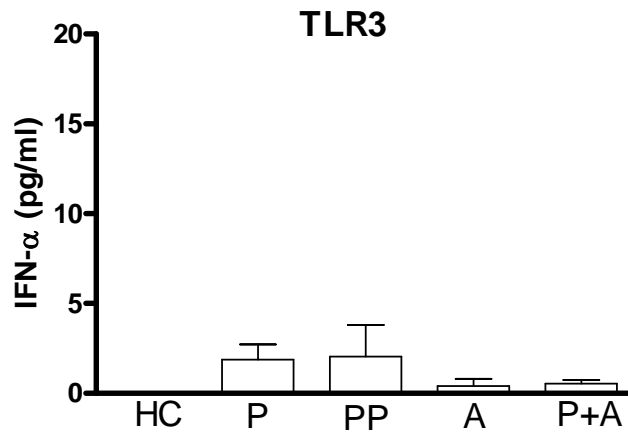
5. Statistical Analyses

Due to small sample size in each group (n=10/group) non-parametric tests were conducted to obtain the final statistics reported. To determine if PBMCs stimulated with virus (live or UV-inactivated) and TLR3/7 agonists showed significantly different IFN responses compared to cells cultured in media alone for 48hrs, the sign-rank test was conducted on each individual group; as differences in positive stimuli versus media within a group represents paired responses, e.g. in the pregnant group, the HRV43 induced IFN concentration versus the IFN concentration measured in the media for this group. For the stimuli that did show a significant difference, the IFN response from the media was first subtracted from the IFN concentration induced by each stimulus, i.e. the change in IFN concentration is used in the final analyses between the groups. This is commonly done in our laboratory for *in vitro* work to ensure that any difference in protein response observed between the groups was purely the result of the stimuli rather than simply 48hrs of *in vitro* culture (which can induce cell stress responses). The Kruskal-wallis test was subsequently used to determine significant differences in IFN response between the groups for each stimulus. Only those stimuli that showed a significant difference between the groups were then analysed by the rank-sum test; where the IFN response of all groups to a given stimulus was compared to the healthy non-pregnant women, as the control. Significance was accepted when $p < 0.05$.

RESULTS

Isolated PBMCs from non-pregnant, healthy controls, as well as pregnant and non-pregnant asthmatics and post-partum non-asthmatics (n=10 subjects for each of the five groups) were also stimulated with the TLR3 agonist Poly(I:C). IFN α but not

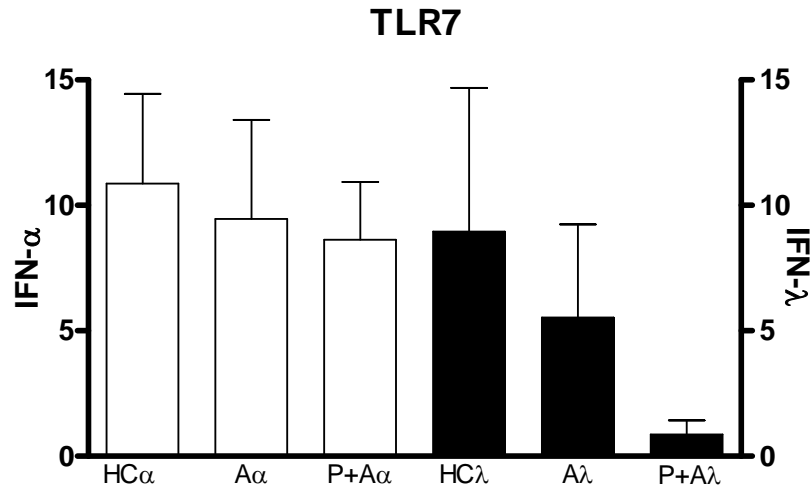
IFN λ was significantly up-regulated in the groups compared to media alone ($p<0.001$), however no significant differences were identified in PBMC response between each group compared to the healthy control PBMCs ($p>0.05$, Supplement Figure 3).



Supplement Figure 3. TLR3 Stimulation of PBMCs. Isolated PBMCs from $n=10$ non-pregnant, healthy controls (HC), pregnant (P), post-partum (PP), asthmatic (A) and pregnant asthmatic women were stimulated with the TLR3 agonist Poly(I:C). IFN- α protein concentration (pg/ml) is represented on the left y axes.

Isolated PBMCs from pregnant and non-pregnant asthmatics (P+A and A respectively; $n=10$ subjects per group) were also stimulated with the TLR7 agonist imiquimod and the IFN α and IFN λ responses compared to non-pregnant healthy control PBMCs. Both IFN α and IFN λ were significantly up-regulated compared to media alone ($p<0.001$). No significant difference was observed in either IFN α or

IFN λ responses from PBMCs of either asthmatic group compared to non-pregnant, healthy control PBMCs.



Supplement Figure 4. TLR7 stimulation of asthmatic PBMCs. Isolated PBMCs from n=10 non-pregnant, healthy controls, (HC) as well asthmatic (A) and pregnant asthmatic (PA) women were stimulated with a synthetic TLR7 agonist, imiquimod. IFN α and IFN λ protein concentrations are represented on the left and right y axes respectively.

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