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 Schizoprenia 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^{o}C/5% CO_2 in Dulbeco'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^{o}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% CO_2 in Dulbeco'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.

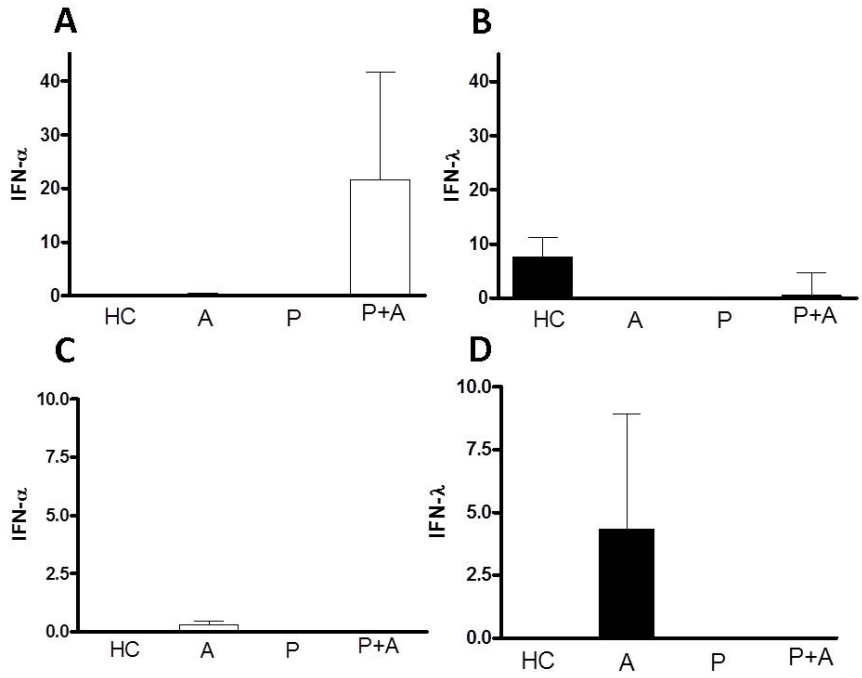
2.3 Viral Concentrations

To calculate the concentration of live virions, the $TCID_{50}$ (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 $1.0x10^4$ cells/well into a 96 well plate using DMEM/5%FBS and cultured overnight 37°C/5%CO_2. 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-Karber formula.

HRV43 stock had a final concentration of $5.34x10^8$/ml and HRV1B stock concentration was $1.88x10^8$/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, $TCID_{50}$ 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). IFNα (panels A and C) and IFNλ (panels B and D) protein concentrations (pg/ml) are represented on the left y axes.

3. PBMC Culture and Cell Viability

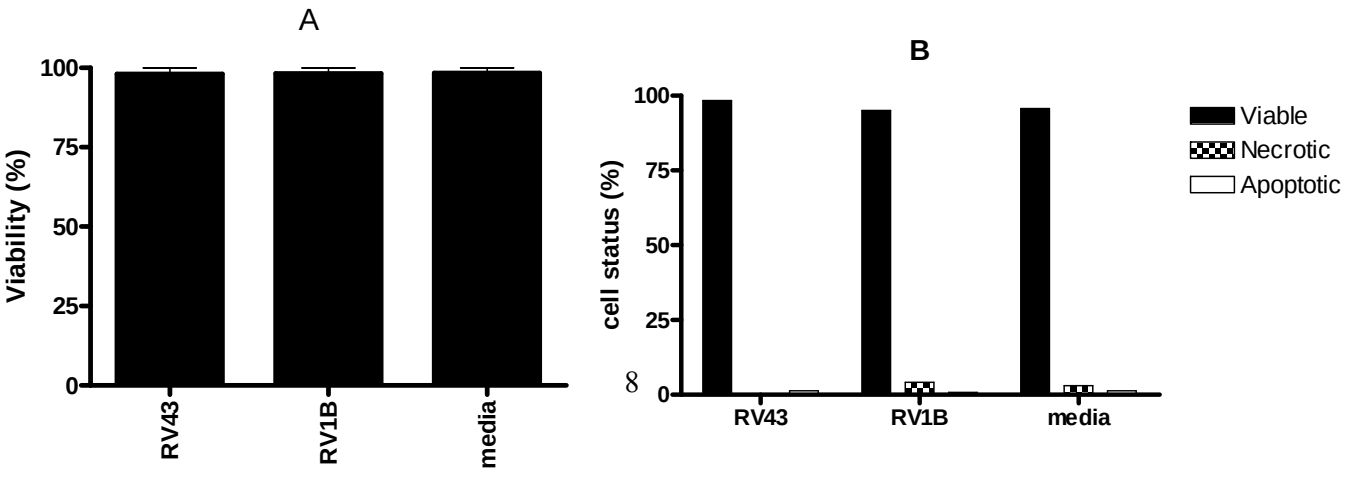
3.1 PBMC culture

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 $2.0x10^6$ 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 μg/ml (2μl of a 5mg/ml stock) and the TLR7 agonist, Imiquimod (Invivogen, San Diego, CA, USA) at final concentration 5μg/ml (5μl 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. $2.0x10^6$ PBMCs /well). The plates were gently shaken to ensure even distribution of PBMCs over the well surface and cultures were then incubated at 33^oC/5% CO_2 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^{o}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 0.0025mm^2) 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 apoptic (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 syncital 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].

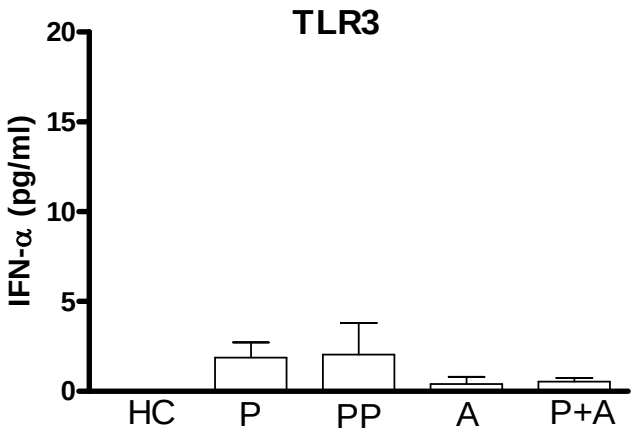
5. Statistical Analyses

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 Kruskall-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). IFNα but not

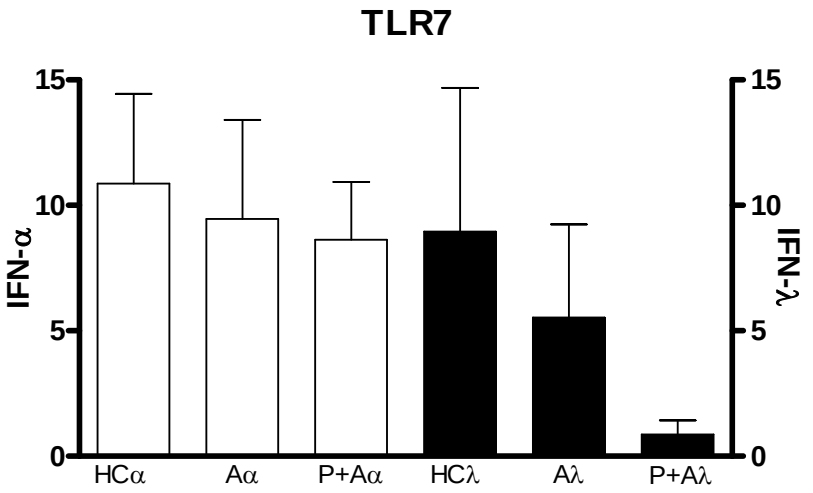
IFNλ was significantly up-regulated in the groups compared to media alone ($p<0.001$), however no significant differences were identified in PBMC response between each group compared to the healthy control PBMCs ($p>0.05$, Supplement Figure 3).



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 IFNα and IFNλ responses compared to non-pregnant healthy control PBMCs. Both IFNα and IFNλ were significantly up-regulated compared to media alone ($p<0.001$). No significant difference was observed in either IFNα or

IFNλ 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. IFNα and IFNλ protein concentrations are represented on the left and right y axes respectively.

Supplement References

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