# IMPAIRED AIRWAY EPITHELIAL CELL RESPONSES FROM CHILDREN WITH ASTHMA TO RHINOVIRAL INFECTION

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**Keywords:** asthma, human rhinovirus, airway epithelium, wound repair.

**ABSTRACT:**

**Background:** The airway epithelium forms an effective immune and physical barrier that is essential for protecting the lung from potentially harmful inhaled stimuli including viruses. Human rhinovirus (HRV) infection is a known trigger of asthma exacerbations, although the mechanism by which this occurs is not fully understood.

**Objective:** To explore the relationship between apoptotic, innate immune and inflammatory responses to HRV infection in airway epithelial cells (AEC) obtained from children with asthma and non-asthmatic controls. In addition, to test the hypothesis that aberrant repair of epithelium from asthmatics is further dysregulated by HRV infection

**Methods:** Airway epithelial brushings were obtained from 39 asthmatic and 36 non-asthmatic children. Primary cultures were established and exposed to HRV-1b and HRV-14. Virus receptor number, virus replication and progeny release were determined. Epithelial cell apoptosis, IFN-β production, inflammatory cytokine release and epithelial wound repair and proliferation were also measured.

**Results:** Virus proliferation and release was greater in airway epithelial cells from asthmatics but this was not related to the number of virus receptors. In epithelial cells from asthmatic children, virus infection dampened apoptosis, reduced IFN-β production and increased inflammatory cytokine production. HRV-1b infection also inhibited wound repair capacity of epithelial cells isolated from non-asthmatic children and exaggerated the defective repair response seen in epithelial cells from asthmatics. Addition of IFN-β restored apoptosis, suppressed virus replication and improved repair of airway epithelial cells from asthmatics but did not reduce inflammatory cytokine production.

**Conclusions:** Collectively, HRV infection delays repair and inhibits apoptotic processes in epithelial cells from non-asthmatic and asthmatic children. The delayed repair is further

exaggerated in cells from asthmatic children and is only partially reversed by exogenous IFN-β.

## INTRODUCTION

Viral infections are associated with the majority of acute wheezing exacerbations in children and this has led to widespread recognition of the importance of viral respiratory infection in acute asthma [1]. The airway epithelium acts as the first line of defense against injurious insult and several lines of investigation have indicated possible factors that could contribute to the its vulnerability in virus-induced asthma. These include aberrant innate immune responses and inadequate responses to injury. Studies of epithelial cells obtained from asthmatic adults have identified deficient interferon-β [2,3] and interferon-λ responses [4] to HRV infection as well as an inefficient apoptotic response [2]. However, the observation of defective IFN-β production in asthma remains controversial with several studies finding no difference or higher expression in asthmatics [5,6]. Similarly, studies utilizing pediatric subjects have reported either reduced interferon responses [7,8,] or no difference [9] as well. This dichotomy highlights the need to further clarify the innate immune responses of children with asthma to viral infection for whom the asthma burden is greatest [10].

In this study, we explored the relationship between apoptotic, innate immune and inflammatory responses to HRV infection in airway epithelial cells (AEC) obtained from children with asthma and non-asthmatic controls. In addition, we tested the hypothesis that aberrant repair of epithelium from asthmatics is further dysregulated by HRV infection. The combination of deficient innate immune responses to HRV compounded by the inability to repair damaged epithelium effectively could contribute to a state of chronic injury in the asthmatic epithelium following HRV infection.

## METHODS:

### Reagents:

Insulin, bovine serum albumin, fetal bovine serum (FBS), bovine hypothalamus acetone power, hydrocortisone, recombinant human epidermal growth factor, epinephrine hydrochloride, fibronectin, triiodothyronine, transferrin, trans retinoic acid, trypsin, gentamicin, were obtained from Sigma (St. Louis, MO, USA). Bronchial epithelium basal medium (BEBM®) was purchased from LONZA (Clonetics®, Walkersville, MD, USA). Penicillin G, RPMI-1640 media, streptomycin sulfate, amphotericin B and L-glutamine were purchased from Invitrogen (Melbourne, VIC, Australia). Ultroser G was supplied from Ciphergen (Cergy-Saint-Christophe, France). Collagens type I and all tissue culture plastic ware were purchased from BD (Bedford, MA, USA).

### Subjects:

The study was approved by the Princess Margaret Hospital for Children's Human Ethics Committee and written consent is obtained from each participant's legal guardian after being fully informed about the nature and purpose of this study. Here, bronchial brushings were obtained from 34 non asthmatic healthy and 25 asthmatic children undergoing elective surgery for non-respiratory related conditions (Table 1). Children with an existing bacterial or viral chest infection were excluded from the study. Asthma was defined as physician-diagnosed based upon physician documented wheezing episodes in the 12-months preceding their recruitment and confirmed by positive responses on the International Study of Asthma and Allergies in Children (ISAAC) and American Thoracic Society (ATS) respiratory questionnaires [11,12]. All asthmatic children had mild disease, in that although receiving glucocorticosteroids (inhaled or oral) or β-agonists none were taken for at least 1 month prior to surgery. Atopy was defined as a positive radioallergosorbent test (RAST) to a panel of

common allergens including; grass pollens, milk, mould, peanut, egg white and animal hair and measured in all children.

**Sampling procedure and cell types:**

Pediatric derived airway epithelial cells (AECs) were harvested via trans-laryngeal, non-bronchoscopic brushing of the tracheal mucosa of children through an endotracheal tube as previously described [13-15] or via a portable 'bronchoscope directed' sampling methodology [16]. Sampling occurred using a rotational movement of the brush and afterwards the brush tip was withdrawn and cut off into a collection media comprising of cold sterile RPMI-1640 and 20% (v/v) heat inactivated FBS. The process was repeated at least once more before the samples are transported back to the laboratory on ice for immediate processing. Approximately $3 \times 10^6$ AEC cells were obtained at time of brushing and fractionated out as previously described for downstream, RNA, protein and functional experimentation [13,14]. Cultures were grown at 37°C in an atmosphere of 5% $CO_2$/95% air under aseptic conditions, were maintained in BEBM® supplemented with growth additives and 2% (v/v) Ultroser G passaged every 13-16 days and used for experimentation before p3 as previously described [13].

**Epithelial lineage confirmation and purity:**

Prior to experimentation epithelial lineage was confirmed in each established culture via expression of cytokeratin 19 and cultures determined free of contaminant cells including mesenchymal cells, macrophages, dendritic and endothelial cells via immunocytochemistry and reverse transcription PCR (RT-PCR) as previously described [10].

**Cell subculture and culture media collection:**

Briefly, for expansion, cells incubated with 0.25% Trypsin/ 0.05% EDTA in calcium and magnesium free solution for 7 min at 37°C and detached from flasks. The resulting cell suspension was centrifuged at 500g for 7 min at 4°C and re-suspended in culture medium. A viability stain and total cell count were performed on each sample. New culture flasks were pre-coated with fibronectin (10mM), collagen (30mM) and BSA (100mM) and the cells added in fresh BEBM® containing growth supplements, incubation was at 37°C in an atmosphere of 5% $CO_2$ / 95% air in as previously described [13]. Since differentiated AEC have been observed to be resistant to HRV infection [5] and basal cells the primary target of HRV post injury [17] we chose to perform all experiments on undifferentiated cultures grown in monolayer culture.

**Rhinovirus and titrations**

Human rhinovirus (HRV) minor serotype 1B (HRV-1b) and major serotype 14 (HRV-14) were provided by Dr Peter Wark (John Hunter Hospital, Newcastle, New South Wales, Australia) and initially determined viral titres reconfirmed via titration in HF cells (Dr Gerry Harnett, Pathwest, Perth, Western Australia, Australia) (data not shown). In addition, we confirmed all HRV-mediated responses were specific to the active virus, by UV inactivating both serotypes for 120 min and observing a lack of replication in HF cells (data not shown).

**Monolayer wounding, HRV infection and repair experiments**

Our group has developed an in-house wounding device, based on that originally described by Vermeer *et al* [18-20]. This scraping device was developed for the assessment of wound repair capacity of pAEC *in vitro* and produces a circular and consistent wound site (width of 1000μm). Four points of reference were used for all post injury visual analysis, enabling the

construction of a time lapsed visual montage from which we were able to accurately determine the degree of wound closure at designated intervals. Briefly, AECs were grown to confluence, infected with HRV-1b ($5x10^5$TCID$_{50}$/ml) for 24h, wounded and repair assessed as described above.

**Quantification of HRV-1b viral copy number**

Viral copy number of the HRV-1b serotype was determined quantitatively via two-step RT-PCR reactions using a HRV-1b advanced kit (PrimerDesign™ Ltd, UK) in combination with Multiscribe™ Reverse Transcriptase and Taqman® Universal Master Mix (Applied Biosystems, USA) as used previously [21]. Briefly, 200ng total RNA was reverse transcribed in a 20µL total reaction volume containing 1 X RT buffer, 5.5mM $MgCl_2$, 0.5mM of each of the dNTPs, 1µL HRV-1B/ACTB primer mix, 0.4U/µL RNase inhibitor, 0.5U/µL Multiscribe reverse transcriptase and RNase-DNase free water. The reactions were carried out as follow: initial primer incubation step at 25°C for 10 min followed by 1hr incubation at 48°C and ended by heating at 95°C for 5 min. The cDNA was then used in a final PCR reaction volume of 20µL containing 1 X Taqman® Universal Master Mix, 1µL HRV-1B primer/probe mix and 5µL of cDNA which has been diluted 5-fold. The PCR conditions were as described by manufacturer: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. A copy number was determined from a set of standard ranging from 2 copy number/mL to $2x10^7$ copy number/mL that was included in each run.

**Viral release analysis**

The amount of cell lysis following HRV-1b infection was analyzed using the CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI) according to the supplied protocol with slight modification. Briefly, 25µL of the harvested supernatant and the same

volume of a supplied positive control were transferred to appropriate wells of a half-area 96-well plate (Corning, USA). A twenty-five µL volume of reconstituted substrate mix was then added to each well and the plate covered and incubated in the dark at RT for 30 minutes. The reaction was stopped by adding 25µL stop solution and the absorbance read at 490nm. Data was represented as measured lactate dehydrogenase (LDH) readings (arbitrary units) and UV-inactivated HRV were utilized as controls to correlated LDH release with active viral replication.

**Cell viability assay**

To determine the effects of HRV-1b and HRV14 on AEC viability, cells were seeded in 96 well plates and grown to 80% confluence in BEBM containing growth additives. HRV-1b and HRV14 were added to the wells between $1.25 \times 10^5$ to $40 \times 10^5 TCID_{50}$/ml for 12, 24 and 48 h. Following exposure, cell viability was assessed using a 3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium inner salt (MTS) assay (Promega, Madison, WI) [22]. Supernatants were also collected and stored at -80°C for subsequent cytokine assessment.

**Apoptosis Assay**

To ascertain the percentage of cells that underwent apoptosis during HRV-1b infection we utilized a single stranded DNA (ssDNA) Apoptosis ELISA kit (Millipore, Billerica, MA, USA). This procedure is based on the selective denaturation of DNA in apoptotic cells by formamide and detection of denatured DNA with a specific monoclonal antibody for ssDNA. Briefly, cells were seeded at a density of $1 \times 10^4$ cells per well in a 96 well plate and cultured for 24 h in BEGM containing growth additives. Cells were then exposed to HRV-1b at a viral concentration of $10 \times 10^5 TCID_{50}$/ml for 6, 12, 24 and 48 h. The plates were then centrifuged at

200g for 5 min and the media removed and replaced with 200µl of fixative and incubated at room temperature (RT) for 30 min. The fixative was removed from the cell monolayers and the plates dried in a 37°C incubator for 1-2 h to allow for permanent attachment of cells to the plate. Once fully dry, 50µl of formamide solution was added to each well with 10 min incubation at RT. To denature the DNA in apoptotic cells, the plates were heated to 75°C for 10 min in an oven, cooled in a refrigerator for 5 min and the formamide removed. Wells were then rinsed 3 times with PBS and blocked with 200µl of 3% skim milk solution for 1 h at 37°C. The blocking solution was removed and replaced with 100µl of the antibody mixture to each well and incubated for 30 min at RT. The plates were washed a further 3 times with 250µl of wash solution and 100µl of supplied "ABTS" solution added to each well and incubated for 20 min at RT. The reaction was stopped by the addition of 100µl stop solution was added to the wells and the absorbance read at 405 nm.

**Cytokine Assays**

Cytokine concentrations were measured in collected supernatants using commercial ELISA kits. Proteins measured included; IL-1b (Invitrogen, Melbourne, VIC, Australia), IP10 and RANTES (R & D Systems, Minneapolis, MN, USA) and IL-8 (BD Biosciences, San Diego CA). Briefly, each kit was a solid phase sandwich ELISA utilising monoclonal antibodies specific for the target protein. Biotinylated secondary antibodies were used to detect the immobilized capture antibodies and Streptavidin-Peroxidase used at the detection agent. The assays are premised on the fact that the intensity of the coloured product is directly proportional to the concentration of target protein present in the original specimen. IL-6 was measured using an in-house time resolved fluorometry detection system (DELFIA, Wallac, Turku, Finland) based on that described by Taylor *et al.*[23]. Briefly, the DELFIA method was followed by using paired antibodies (Pharmingen, Sydney, NSW, Australia) and the

biotinylated secondary antibody was detected using Europium–labeled streptavidin (Wallac) and fluorescence was quantified using a fluorometer (Wallac VICTOR2; PerkinElmer Life Sciences, Boston, MA, USA). Standard curves were generated using serial dilutions of recombinant human IL-6 (Pharmingen) and were linear between 3 and 30,000pg/ml with a detection limit of 3 pg/ml and sample concentrations determined from triplicate values. Human IFN-α and IFN-β production was detected using an AlphaLISA® IFN-β assay (PerkinElmer Life Sciences, Waltham, MA, USA) and human IFN-λ production measured using relevant DuoSet ELISA Development Systems for IFN-λ (R & D Systems, Minneapolis, MN, USA). All were performed according to manufacturer's instructions and determined cytokines normalized to cell number.

**Statistics:**

Statistical significance was assessed using Mann Whitney non parametric analysis. Experiments were performed 3-6 times using matched samples and all values presented are means ± SE where applicable. All p values less than 0.05 were considered significant.

## RESULTS

### Cohort Characteristics:

The clinical characteristics and demographics for all recruited study participants are provided in Table 1. Of the 75 participants recruited to this study, 36 were non-asthmatic, had no response to the panel of allergens tested and had a group mean total IgE of 20.8 IU/L. A further 39 participants were classified as asthmatic which was previously diagnosed by a physician. Subjects had a history of documented wheeze in the 12 months prior to recruitment and were taking asthma medication although none had taken inhaled/oral corticosteroids for at least a month before surgery. All subjects were considered mild, had one or more positive responses to the panel of allergens tested and had a total group mean IgE of 415.18IU/L.

### Effect of HRV on epithelial cell viability:

Due to the reported various cytopathic effects of HRV on AEC [2], viability assays were performed to determine HRV exposure for downstream experiments. Exposure of AEC from non-asthmatics to a range of HRV-1b titers (2.5-40x$10^5$TCID$_{50}$/ml) caused cytotoxic effects over time, the most significant being an ~50% decrease in the number of viable cells after 48 h when compared to uninfected controls ($p < 0.0001$, Fig. 1a). Cytotoxic effects in AECs from asthmatics were greater at all time points, with viable cell numbers reduced to 20% under the same conditions ($p < 0.0001$: Fig. 1b).

We observed no statistical difference ($p \geq 0.05$) in cell viability between infected UV treated control cells (100% viability) and AEC of non-asthmatics infected to HRV14 irrespective of infection period (Fig. 1c). Even after 72 h of HRV14 infection no decline in cell viability was observed. Furthermore, HRV14 load did not have any effect on cell viability as no significant difference was recorded when comparing cell viability between 1.25x$10^5$TCID$_{50}$/ml and

$40x10^5 TCID_{50}$/ml ($p \geq 0.05$; Fig. 1c). Infection of AEC of asthmatics to HRV14 was observed to have both a time and viral load dependent effect on cell viability (Fig. 1d). When AECS of asthmatics were exposed to all HRV14 loads for up to 12 h, no significant change ($p \geq 0.05$) in cell viability was observed (Fig. 1d). However, these cells demonstrated a significant susceptibility to HRV14 exposure at the higher viral loads after 24 h ($p = 0.0041$) 48 h ($p < 0.0001$) and 72 h ($p < 0.0001$). Since the HRV-1b minor serotype was observed to be more pathogenic than the HRV14 major serotype and that it most closely resembles the pathogenicity of community HRV strains [3,24], we chose to concentrate all subsequent experiments on this serotype only.

**HRV receptor expression, replication and release from airway epithelial cells:**

Basal expression of the Low Density Lipoprotein Receptor (LDLR) was then compared between AEC from non-asthmatics and asthmatics. Results showed overall basal expression of LDLR was significantly higher in AEC from asthmatics ($p< 0.05$; Fig. 2a). Interestingly, receptor expression was not significantly changed by HRV infection in AEC from either non-asthmatics or asthmatics (Fig. 2a). Next, virus replication was assessed by quantifying HRV-1b copy number after infection. There was a significant increase in HRV-1b copy number in AEC from both non-asthmatics and asthmatics post-infection ($p<0.0001$; Fig. 2a), although 24 h after infection AEC from asthmatics contained significantly more HRV-1b copy number compared to non-asthmatics ($p< 0.001$; Fig. 2b). Furthermore, the increase in HRV-1b copy number in both AEC of non-asthmatics and asthmatics was mirrored by an elevation in lactate dehydrogenase (LDH) release at both 24 h and 48h. This was more evident in AEC from asthmatics and correlated with increased cell lysis observed at these time points ($p< 0.01$: Fig. 2c). This release was specific to HRV-1b replication since UV-inactivated virus did not induce LDH release (Fig. 2c).

**Effect of HRV-1b on epithelial cell apoptosis:**

We next investigated whether apoptosis was induced in AEC by HRV-1b infection. The induction of apoptosis in AEC from both non-asthmatics and asthmatics was dependent on the infection period (Fig. 3a) and infection with HRV-1b up to 24 h induced apoptosis in AEC from both cohorts. Unlike cell viability, there were no significant differences between the groups at 24 h. However, at longer infection periods there was a dampened apoptotic response in AEC from asthmatics compared to cells from non-asthmatics (Fig. 3a; $p < 0.05$). Infection with HRV-1b was found to drive the apoptotic response in AECs of non-asthmatics and this was directly associated with reduced viability ($R^2 = 0.84$; $p < 0.05$; Fig. 3b). A similar yet weaker association was also observed in AEC of asthmatics ($R^2 = 0.28$; $p < 0.05$; Fig. 3c).

**Effect of HRV-1b on epithelial cell IFN-α and IFN-β protein production**

The capacity of these cells to produce Type I (IFN-α, IFN-β) and Type III (IFN-λ) interferons in response to HRV infection were also assessed. Human IFN-α was undetectable both in the absence and presence of infection (data not shown). In the absence of infection, IFN-β release was not significantly different between AEC from either phenotype (Fig. 3d). However, following exposure to HRV, AEC from asthmatics produced significantly lower levels of IFN-β compared to AEC from non-asthmatics ($p<0.05$; Fig. 3d). Similarly, there was no difference in the release of any of the IFN-λ subtypes measured between AEC from either phenotype in the absence of infection (Fig. 3e-g). There was however, a significant increase all IFN- λ subtypes in AEC from non-asthmatics following infection ($p<0.05$; Fig. 3e-g). Notably, this not observed in AEC from asthmatics in the presence of infection (Fig. 3e-g). To identify a possible mechanism to explain the impaired IFN response, microarray data from a previous study by our group [19] that compared gene expression in AEC from non-asthmatic

and atopic asthmatic children was reanalyzed, which revealed IRF8 as significantly down regulated (>1.6 fold) in asthmatic AECs. Further assessment using qPCR confirmed this observation ($p<0.05$; Fig. 3h). Post HRV-1b exposure, gene expression of IRF8 was significantly up-regulated in AECs of non-asthmatics ($p< 0.05$; Fig. 3h) but not asthmatics ($p<0.05$; Fig. 3h).

**Effect of HRV-1b on release of pro-inflammatory cytokine release:**

Given these findings, we next measured production of IL-1β, IL-6, IL-8, RANTES, IP10 and TGFβ1 following HRV-1b infection. Infection of AEC from non-asthmatics did not induce IL-1β release at any titer used (Fig. 4), but produced a virus dose-dependent elevation in IL-6, IL-8, RANTES and IP10 release (Fig. 4). Epithelial cells from asthmatics were more sensitive to the effects of infection since they released IL-1β and significantly greater amounts of IL-6, IL-8, RANTES and IP10 (Fig. 4) at lower levels of infection.

**Effect of HRV-1b on epithelial cell proliferation and wound repair:**

Exposure of AEC from non-asthmatics to HRV-1b had a delayed effect on proliferation, significantly decreasing cell number only after 7 days ($p < 0.001$; Fig. 5a). In contrast, infection of AEC from asthmatics inhibited proliferation earlier (day 4) and more severely (Fig. 5b). Subsequent epithelial repair experiments showed that AECs from non-asthmatics were able to fully repair wounds (Fig. 5c), although exposure to HRV-1b significantly delayed repair with only ~70% wound closure observed after 10 days ($p < 0.001$; Fig. 5c). In agreement with our previous findings [18,19], non-infected AEC from asthmatics only partially repaired wounds (Fig. 5d) and infection with HRV-1b further exaggerated this dysregulated response, with only 20% wound closure achieved ($p < 0.01$; Fig. 5d).

**The effect of exogenous IFN-β on epithelial cell apoptosis, viral replication, and wound repair:**

Co-treating or pre-treating AEC with IFN-β could abrogate the effects of HRV-1b exposure and both approaches increased the number of cells undergoing apoptosis in response to virus exposure, although the greatest effect was seen in cells pre-treated with IFN-β (Fig. 6a; $p < 0.05$). In addition, IFN-β-induced apoptosis appeared to positively correlate with viral titer. However, the magnitude of IFN-β-induced apoptosis AEC from asthmatics was still markedly lower than that seen in AEC from non-asthmatics post infection (>6 fold induction: Fig. 6b).

Since the maximal increase in apoptosis in response to HRV-1b exposure was following IFN-β pre-treatment, we then investigated its effect on viral replication. Viral replication was reduced in AECs from asthmatics following IFN-β pre-treatment prior to viral exposure (Fig. 6c). However, pre-treatment with IFN-β had did not reduce production of IL-8 and RANTES following virus infection (Fig. 6d). Finally, the effects of IFN-β pre-treatment on wound repair post infection were investigated. Uninfected and untreated AEC from asthmatics failed to fully repair and worsened following infection with HRV-1b (Fig. 6e). Pre-treatment with IFN-β significantly improved repair of infected cells ($p< 0.05$) to levels no different than uninfected and untreated cells (Fig. 6f).

**DISCUSSION**

Here we have shown that airway epithelium of asthmatic children is more susceptible to HRV-1b infection and that infection has a significant effect on the capacity of asthmatic epithelium to repair. The epithelium from asthmatics was more permissive of viral replication, exhibited reduced apoptotic responses and produced lower levels of type I and III IFN in response to infection than healthy epithelium. Airway epithelial cells of asthmatics also produced greater amounts of IL-1β, IL-6, IL-8, IP10 and RANTES following infection. At viral titers that only minimally affected viability, HRV infection modestly inhibited proliferation of AEC from non-asthmatics, but halted proliferation of AEC from asthmatics. One mechanism for these disparate effects may relate to the greater expression of HRV receptor on AEC of asthmatics compared to AEC of non-asthmatics - suggesting increased viral entry into AEC of asthmatics. Concentrating on type I IFN in this study, we observed lower IFN-ß production in response to HRV from AEC of asthmatics and investigated whether exogenous IFN-ß could reduce the susceptibility of AEC from asthmatics to infection and improve the capacity for repair. Pretreatment of AEC from asthmatics with IFN-β partially restored apoptosis, suppressed HRV replication, improved the repair response to injury, but had little impact on inflammatory cytokine responses to HRV infection, suggesting a potential short-term therapeutic regimen primarily aimed at limiting viral propagation.

Epithelial cells serve as the host for viral replication and initiate both innate and adaptive immune responses. We have showed that the cytotoxic effects of HRV on AEC are dependent on viral load and duration of infection. In addition, AEC from asthmatics facilitate greater HRV replication compared to AEC from non-asthmatics and undergo progressive cell lysis rather than apoptosis. Furthermore, we identified that viral loads detected in our *in vitro* studies were comparable to that identified in asthmatic (3.6 vs 3.08 $\log_{10}$ copies/ml) and non-

asthmatic children (4.35 vs 4.96 $\log_{10}$ copies/ml) [25]. Since expression of LDLR was greater in AEC from asthmatics compared to AEC from non-asthmatic children, our data suggest that the abnormal responses to HRV observed in AEC from asthmatics may be in part due to enhanced viral entry into the cell and consequent downstream effects including innate immune and inflammatory pathways [26].

Apoptosis is an important innate immune response to viral infection that limits virus spread and allows the non-inflammatory removal of infected cells. Our data show that infection with HRV-1b induced apoptosis in AEC and cell death was similar in AEC from both asthmatic and non-asthmatic children early following infection (up to 24 h), but plateaued out in AEC from asthmatics at later time points. The strong association between apoptosis and cell viability observed in non-asthmatics identifies this as the main process to limit viral infection. However, the association was much weaker in asthmatics, suggesting other processes of cell death including necrosis/necroptosis and or autophagy. Since necrosis/necroptosis and autophagy both promote inflammation [27-29], a cycle of infection and inflammation could develop after HRV infection that, in turn, perpetuates prolonged infection and exaggerated inflammation in asthma.

We also observed that AEC from asthmatics, despite similar basal expression, exhibit a dysregulated type I and III IFN response to HRV infection. We were unable to detect IFN-α protein pre and post viral infection which has also been reported by others [30], but were able to detect IFN- β and IFN-λ consistently. IFN-β is a critical component of the innate immune response to viruses and IFN-λ involved in viral containment via the induction of apoptosis in specific target cells including the epithelium [2,31]. Blunted IFN-β and IFN-λ release by epithelial cells from adult asthmatics has been previously reported [2,3,7,8,32] and has also

been observed in other cell types in the asthmatic lung, including macrophages [33]. In our quest to elucidate the underlying mechanism, we revisited a previously generated gene dataset on mild asthmatic children and identified IRF8 as a putative regulator of the damped IFN response. IRF8 is known to directly interact with IRF1 [34,35,36], and both cooperatively regulate IFN-β and IFN-λ induction in particular cellular systems [37,38,39]. However, others have identified additional potential co-regulators including IRF3 [40] and IRF7 [41,42,43] which has been shown to be a major gene network hub driving viral induced exacerbations in asthmatics. Our observation of interferon regulatory factor 8 (IRF8) is unique in these subjects and its failure to upregulate post-infection as seen in the non-asthmatic controls further supports the suggestion that the damped IFN response to HRV represents an intrinsic innate immune defect in asthmatics. Using a genome network approach, we are currently exploring this area further to definitively identify the regulatory factors that could be targeted for therapy.

Furthermore, that apoptotic responses to HRV could be partially normalized in AEC from asthmatics by the addition of exogenous IFN-β reveals a possible pathway defect to explain the consistent observations of damped apoptosis [2,3,29,32]. The increase in HRV induced apoptosis following treatment with IFN-β was accompanied by a reduction in viral replication as reported recently by Cakebread et al., [32]. However, our study sampled cells from children with mild disease and we observed reduced viral replication in response to IFN-β, even at HRV exposures much higher than employed by Cakebread et al., [32].

Another effect of HRV infection of AEC is the initiation of inflammation, resulting in both oxidative stress [44], and inflammatory cytokine release. In agreement with others [45], we found that HRV infection of AEC from asthmatics resulted in a marked elevation of the pro-

inflammatory cytokines IL-1β, IL-6, RANTES and IL-8. These mediators have been shown to induce the accumulation of inflammatory cells in the airways resulting in the release of reactive oxygen species and elastase [46] which cause epithelial damage [47] and their expression is increased in asthma [3,48]. Interestingly, IFN-β pre-treatment had no effect on cytokine release. This is in contrast with work by Cakebread and colleagues [32] who observed a significant reduction in both IP-10 and RANTES with IFN-β pretreatment. However, we used a higher viral titer, which resulted in markedly higher cytokine production than that observed by Cakebread and colleagues [32]. Thus IFN-β pre-treatment at the concentration used in our study may have been ineffective at reducing inflammation against the viral titers used. Although higher titers were used in this study, it is similar to another study performed [6] and appears biological relevant considering the resultant viral load of HRV is similar to that seen in nasal lavage post infection [25]. These data suggest that the beneficial effect of IFN-β treatment against inflammatory cytokine production may occur primarily during lower viral titer infections. Alternatively, our observations were generated using cells from children (mean 8 years) with mild asthma and none were actively taking corticosteroids, whereas Cakebread et al. used cells from asthmatic adults (mean 31 years) most of whom were taking inhaled corticosteroids [32]. Given that the anti-inflammatory activity of corticosteroids has been well reviewed [49], IFN-β pretreatment may act synergistically with these to successfully dampen any inflammation produced following HRV infection. Furthermore, considering HRV infection is more prevalent (>85%) in children hospitalized due to asthma [50] when compared to adults (~30%) [51] data generated here may be more relevant to the pediatric population.

Cells infected with virus utilize virus associated molecular patterns (VAMPS) to trigger the phosphorylation of interferon regulatory factor (IRF) family, which then translocates to the

nucleus to activate interferon responsive genes [52]. We identified IRF-8 as being significantly down regulated in AEC from asthmatics and IRF-8 has recently been found to be expressed by epithelial tumor cells, regulate RANTES production [53] and mediate Fas-mediated apoptosis [54]. Furthermore, epigenetic regulation of the IRF-8 promoter through DNA hypermethylation represses its expression and renders cells resistant to apoptosis [55]. Interestingly, IRF-8 has also been found to directly modulate viral replication as its binding site is homologous with replication specific transcription elements [55-58].

We have previously described dysregulated repair of epithelium from asthmatics [18,19] in response to mechanical wounding. In this study, HRV infection delayed AEC wound repair in both non-asthmatic and asthmatic epithelial cultures but this inhibitory effect was significantly greater in AEC from asthmatics. Viral infection of AEC was also found to have a marked effect on cell proliferation with a delayed inhibitory effect on proliferation of AEC from non-asthmatics by HRV infection and markedly suppressed proliferation of AEC from asthmatics. Work conducted by Tinton and colleagues [59] suggests inhibition may occur in the G2/M phase of the cell cycle. Surprisingly, very few studies have investigated the effects of HRV on epithelial cell proliferation and repair, given that HRV infection have been shown to play a significant role in triggering asthma exacerbations [1]. Infection with HRV significantly delayed wound healing in AEC from non-asthmatics and completely abrogated repair in AEC from asthmatics. Ours are the only data available to functionally demonstrate the differential effect of HRV infection on wound repair in primary AEC from asthmatics and non-asthmatics and our results suggest that impaired IFN-β production by AEC from asthmatics is central to the defective repair response observed. We also observed significantly improved repair of AEC from asthmatics when pre-treated with IFN-β and hypothesize that it is due in part to its suppressive action on HRV replication.

In conclusion, we have identified that HRV infection significantly affects the ability of airway epithelium to repair and this effect is particularly pronounced in the epithelium of asthmatics. We have provided strong evidence that AECs from asthmatic children are more sensitive to the pathogenic effects of HRV than non-asthmatic children, facilitated greater HRV replication as well as viral release. Our data indicate that impaired innate immune responses and dysregulated repair might contribute to a significant susceptibility of the asthmatic epithelium to respiratory virus infections in children that could be amenable to appropriately targeted therapies.

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**FIGURE LEGENDS.**

**Figure 1.** Dose-dependent cytotoxic effects of HRV-1b on AEC viability at $1.5x10^5TCID_{50}/ml$ (Dark Grey), $10x10^5TCID_{50}/ml$ (Light Grey) and $40x10^5TCID_{50}/ml$ (White). The CellTitre 96® $A_{queous}$ Non-Radioactive Cell Proliferation Assay (MTS) was used to assess the number of metabolically active cells post viral exposure. The percentage cell viability in comparison to unexposed cells was assessed at 12, 24 and 48 h. (a) Exposure of from non-asthmatics to HRV-1b produced marked cell death at $40x10^5TCID_{50}/ml$. (b) Exposure of AEC from asthmatics to HRV-1b resulted in marked cell death using all viral concentrations. (c) There was no statistical difference in the viability between uninfected AEC of non-asthmatics and those infected with HRV14 regardless of viral load and infection period. (d) Infection of AEC from asthmatics with HRV14 resulted in marked cell death that was both viral load and infection period dependent. NOTE; * denotes a significant reduction in cell viability from unexposed cells. $p < 0.05$. Experiments were performed on five to eight separate individuals per phenotype and presented as box and whisker plots.

**Figure 2.** HRV-1b receptor (LDLR), replication and release by AEC. Airway epithelial cells from non-asthmatic and asthmatic children were grown to confluence and infected with $10x10^5TCID_{50}/ml$ for 24h and viral receptor, replication and release determined via quantitative PCR and LDH respectively. (a) AEC from asthmatics express greater low-density lipoprotein receptor (LDLR) than their non-asthmatic counterparts however receptor expression did not change in either phenotype once cells were infected with HRV * denotes significant differences observed. $p < 0.05$. (b) AEC from asthmatics were found to facilitate HRV-1b replication far readily than AEC from non-asthmatics with more than twice the viral copy number being observed in these cells. * denotes significant differences observed. $p < 0.05$. (c) Similarly, AEC from asthmatics were also found to release HRV-1b earlier than

their non-asthmatic counterparts with a significantly greater level of LDH released at 24 h compared to their healthy counterparts. Similar levels of LDH were observed when infected for 48h. NOTE; * denotes a significant reduction in cell viability from unexposed cells. # denotes significant difference between non-asthmatic healthy and asthmatic AEC. $p < 0.05$. Experiments were performed on four to eight separate individuals per phenotype and presented as box and whisker plots..

**Figure 3.** Apoptotic responses and IFN-β production in AECs from non-asthmatics (open bars) and asthmatics (shaded bars) to HRV-1b infection. (a) An ssDNA apoptosis ELISA was performed to determine whether AECs underwent apoptosis following HRV infection ($10x10^5TCID_{50}$/ml of HRV-1b). Viral infection induced apoptosis in a time-dependent manner in AEC of both non-asthmatics and asthmatics, however there was an observed damped apoptotic response in AEC of asthmatics over prolonged infection. * denotes significant differences observed. $p < 0.05$; # denotes significant difference between non-asthmatic healthy and asthmatic AEC. data presented as mean ± SD. (b) Using regression analysis, we demonstrated a strong association between apoptosis and cell viability in AEC in non-asthmatics ($R^2 = 0.84$). (c) However, there was a much weaker association between apoptosis and cell viability in AEC of asthmatics ($R^2 = 0.28$). (d) IFN-β protein production was measured from cell supernatants taken from non-HRV-1b infected cultures and no difference was observed in the amount of IFN-β produced by AEC of non-asthmatics and asthmatic children. When IFN-β production was measured in supernatants post HRV-1b infection, AEC of non-asthmatics responded with a >2fold increase in the type 1 interferon, which was not seen in the AEC from asthmatics. * denotes significant differences observed. # denotes significant difference between non-asthmatic healthy and asthmatic AEC. $p < 0.05$. Experiments were performed on at least six separate individuals per phenotype and data

presented as means ± SD. (e) IFN λ1, (f) IFN λ2 and (g) IFN λ3 were also measured from cell supernatants taken from non-HRV-1b infected cultures and no difference were observed in the amount of IFN-λ1-3 produced by AEC of non-asthmatics and asthmatic children. When IFNλ production was measured in supernatants following HRV-1b infection, there was a significant induction of type III interferon protein in AEC of non-asthmatics that was not seen in the AEC from asthmatics. * denotes significant differences observed. # denotes significant difference between non-asthmatic healthy and asthmatic AEC. $p < 0.05$. Experiments were performed on at least six separate individuals per phenotype and data presented as means ± SD. (h) When IRF8 gene expression was assessed prior to and post HRV-1b infection and found to be upregulated in non-asthmatics post infection. However, no increase in gene expression was observed in AEC of asthmatic children. NOTE * denotes significant differences observed. # denotes significant difference between non-asthmatic healthy and asthmatic AEC. $p < 0.05$. Experiments were performed on at eight separate individuals per phenotype and data presented as mean ± SD.

**Figure 4.** Cytokines production in the supernatants AEC of non-asthmatics (open bars) and asthmatics (shaded bars) following HRV-1b exposure. Cytokine release was measured in cell culture supernatants using commercial ELISA kits and an in-house time resolved fluorometry detection system. HRV-1b significantly increased IL-1β production in AEC of asthmatics, and not AEC of non-asthmatics. HRV-1b significantly increased IL-6 levels in both cell phenotypes, though levels were greater in AEC of asthmatics at lower viral doses. IL-8 production was statistically elevated following HRV-1b exposure and levels were significantly higher in AEC of asthmatics compared to their non-asthmatic counterpart. A viral dose dependent increase in RANTES production was evident in both cohorts in response to HRV-1b infection, although this was greater in AEC of asthmatics. A similar pattern was

observed for the cytokine IP10. NOTE; * denotes significant differences observed. $p < 0.05$. # denotes significant difference between non-asthmatic healthy and asthmatic AEC. Experiments were performed on five to eight separate individuals per phenotype and data presented as means ±SD .

**Figure 5.** Effect of HRV infection on AEC proliferation (a & b) and wound repair (c & d). AEC of non-asthmatics (a) and asthmatics (b) were seeded at low density (5000 cells per/well) in a 96 well plate, cultured for 24 hours and exposed to $1.25x10^5 TCID_{50}$/ml of HRV-1b (○ □). The rate of proliferation was measured at 24 hour intervals using the MTS assay and compared to unexposed cells (● ■). Results showed that HRV-1b significantly decreased AEC proliferation in non-asthmatics in comparison to unexposed cells. HRV-1b had a more significant effect on AEC proliferation in asthmatics. In addition, AEC from non-asthmatics (c) and asthmatics (d), were grown to confluence, infected with either HRV-1b, mechanically wounded and repair assessed and compared to non-infected controls. HRV infection in AEC of non-asthmatics (○) resulted in significantly delayed wound repair compared to non-infected controls (●) but full repair was seen by day 15. In contrast, non-infected AEC from asthmatics exhibited a dysregulated repair response (■) which was further abrogated upon HRV infection (□). NOTE; * denotes significant differences observed. $p < 0.05$. Experiments were performed on at least 5 separate individuals per phenotype and data presented as means ±SD.

**Figure 6.** Effect of IFN-β treatment on apoptosis (a & b), viral replication (c), inflammatory cytokine production (d) and wound repair (e & f) in AEC of asthmatics following HRV infection. (a) AEC from asthmatic children were established, and pre-treated with IFN-β for

24 hours prior to HRV-1b infection or co-treated with IFN-β at time of viral infection. There was no significant increase in apoptosis in AEC of asthmatics exposed to IFN-β alone (1.09; data not shown), or UV-inactivated HRV-1b (1.1; data not shown). In cells co-treated with HRV-1b and IFN-β there was only a moderate tendency to increase apoptosis, whereas those pre-treated with IFN-β prior to infection with the higher viral titre there was a significant induction of apoptosis (2.5; $p < 0.05$). Experiments were performed on at 4 separate individuals with data presented as box and whiskers. (b) Effect of HRV-1b infection on apoptosis induction in non-asthmatic airway epithelium. Primary airway epithelial cell cultures from non-asthmatics were established and infected with HRV-1b at various multiplicities of infection (MOI). Apoptosis was then measured via an ssDNA apoptosis ELISA. Results showed a significant induction of apoptosis in AEC of non-asthmatics at both MOI 3 (~4 fold) and MOI 25 (> 6 fold). NOTE; * denotes significant differences observed. $p < 0.05$. Experiments were performed on at 4 separate individuals with data presented as box and whiskers. . (c) The effect of IFN-β pre-treatment on resulting HRV1b replication was also assessed by via two-step RT-PCR reactions using a HRV-1b advanced kit. There was significant reduction (> 2.0 fold) in viral replication in AEC of asthmatics pre-treated with IFN-β irrespective of viral titre infected. Experiments were performed on at 4 separate individuals with data presented as box and whiskers. (d) Effects of IFN-β pre-treatment on inflammatory cytokine production post viral infection in AEC of asthmatics. Production of IL-8 and RANTES protein was measured via ELISA in cells pre-treated with IFN-β prior to HRV-1b infection. No significant difference in the fold change of inflammatory cytokines produced was observed in cells either infected with virus alone (open bars) or those pre-treated with IFN-β prior to infection (shaded bars). Experiments were performed on at 4 separate individuals with data presented as box and whiskers. (e) Effect of IFN-β pre-treatment on wound repair in AEC of asthmatics. Wound closure was assessed between

uninfected AEC of asthmatics (▲), those infected with HRV-1b at MOI 3 (■) and compared to those pre-treated with IFN-β for 24 hours prior to viral infection at MOI 3 (□). Wound repair was significantly improved (□; $p < 0.05$) when cells were pre-treated with IFN-β, however did not restore the reparative capacity of these cells above their normal dysregulated rate when uninfected (▲).Experiments were performed on at least 5 separate individuals and data presented as means ± SD (f) Wound closure was also assessed between uninfected AEC of asthmatics (▲) and those infected with HRV-1b at MOI 25 (●) as well as those pre-treated with IFN-β for 24 hours prior to viral infection at MOI 25 (○). Wound repair was significantly improved (○; $p < 0.05$) when cells were pre-treated with IFN-β, however did not restore the reparative capacity of these cells above their normal dysregulated rate when uninfected (▲). Experiments were performed on at least 5 separate individuals and data presented as means ± SD.

| | Number | Gender (m/f) | Mean Age (range) | mean IgE (IU/L) | Allergies | Hay Fever/Eczema /Both |
|---|---|---|---|---|---|---|
| Healthy | 36 | 18/18 | 8.4 (3.2-15.6) | 20.8 | 0 | 0 |
| Asthmatic | 39 | 25/14 | 8.2 (2.6-14.8) | 415.18 | 39 | 35 |

Table 1: Demographic data for subjects recruited to the study.

**Table 1. Kicic et al., 2015**

| Gene | Primer | Sequence |
|---|---|---|
| *LDLR* | Forward | 5’-GACATGAGCGATGAAGTTGG-3’ |
| | Reverse | 3’-ATTGCAGACGTGGGAACAG-5’ |
| *IRF8* | Forward | 5′-CGTGGAAGACGAGGTTACGCTG-3′ |
| | Reverse | 3’-CGGATACTGTGTGTGGTAAGTCG-5’ |
| *PPIA* | Forward | 5’-TGAGCACTGGAGAGAAAGGA-3’ |
| | Reverse | 3’-CCATTATGGCGTGTAAAGTCA-5’ |

Table 2: Oligonucleotide primer sequences used in the study.

**Table 2. Kicic et al., 2015**

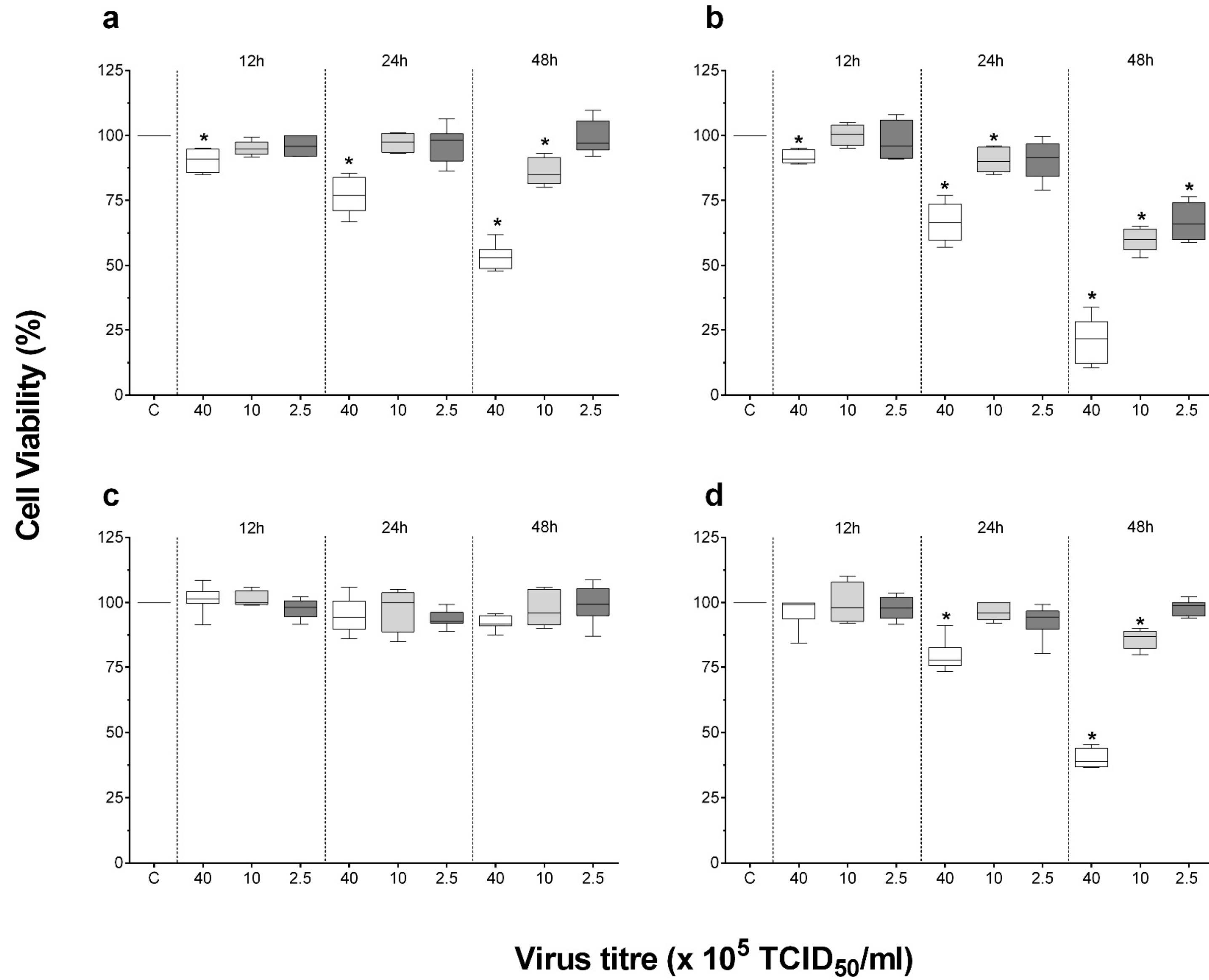



Figure 1. Kicic et al., 2016

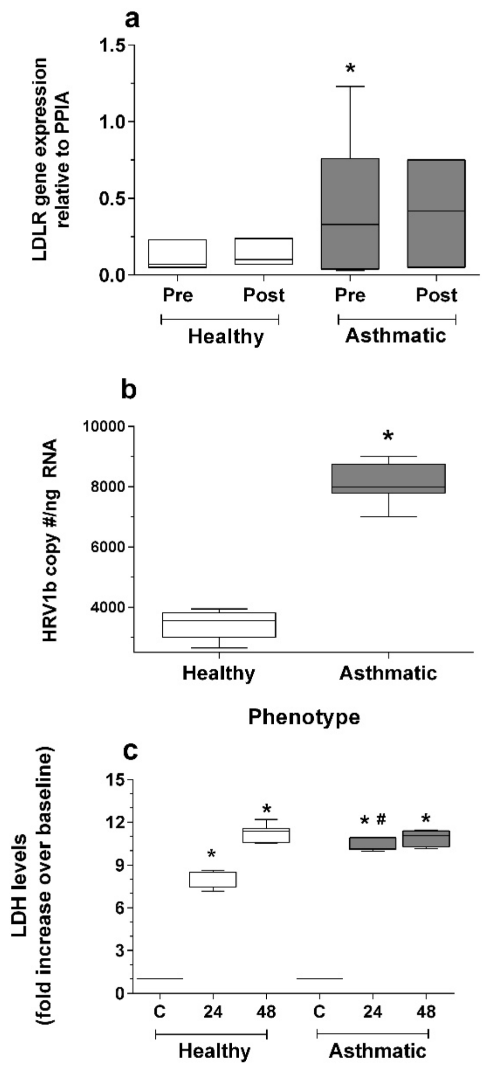



Figure 2. Kicic et al., 2016

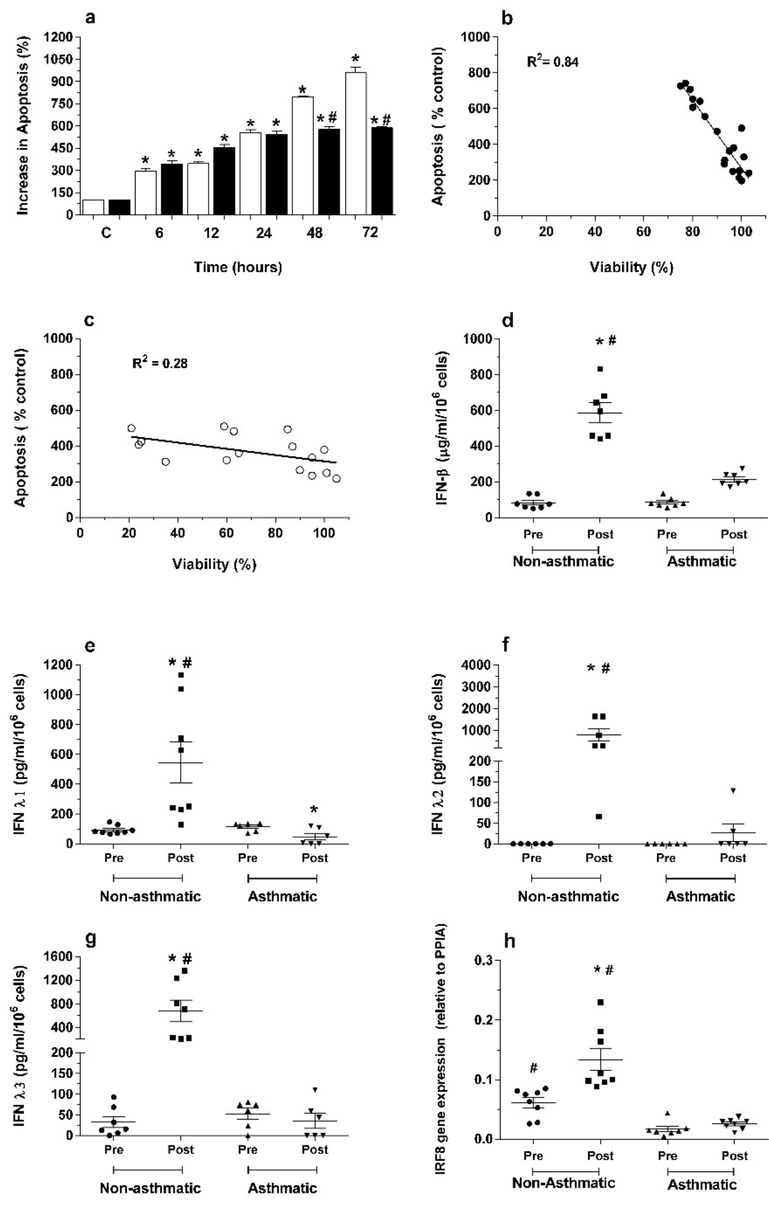



Figure 3. Kicic et al., 2016

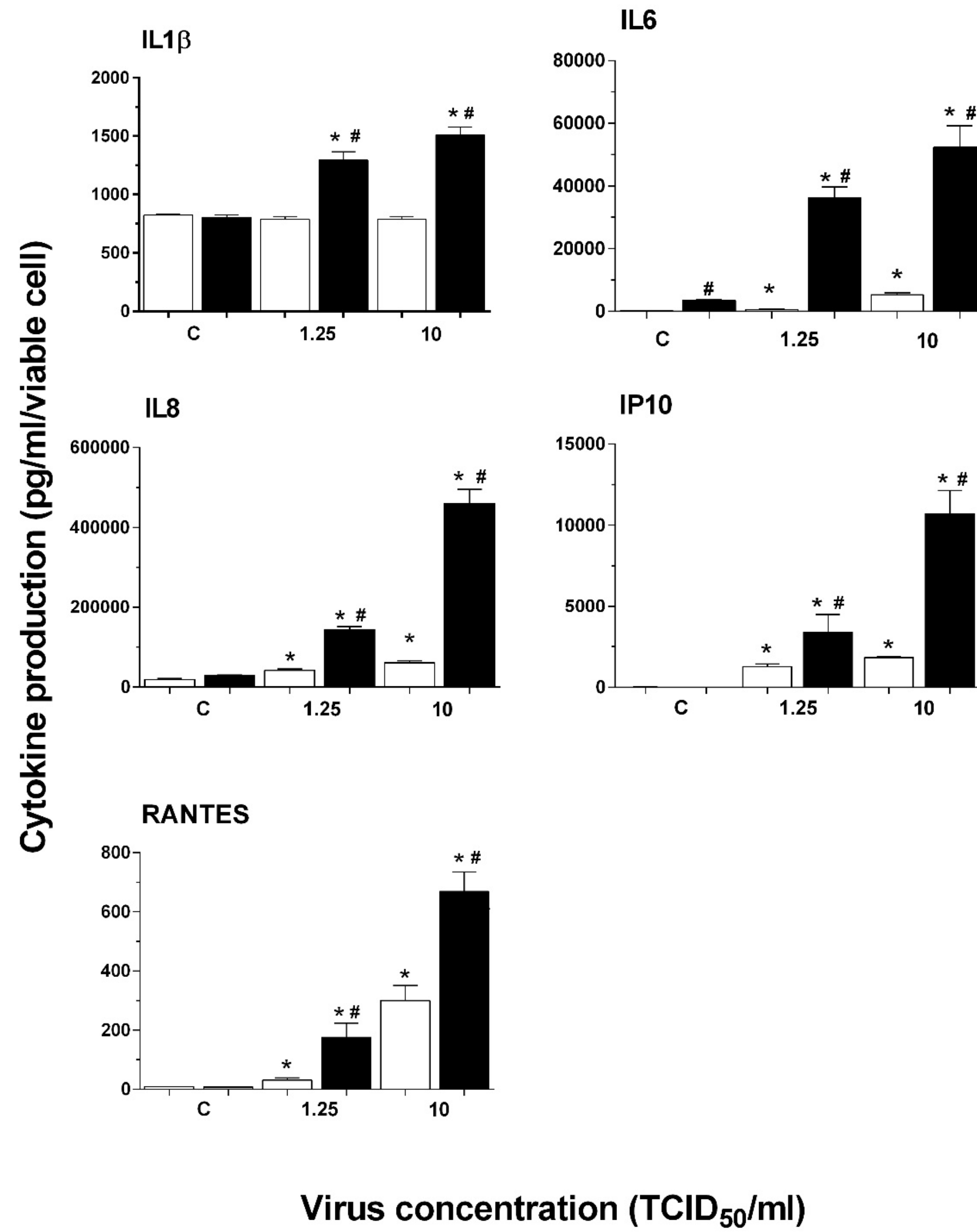
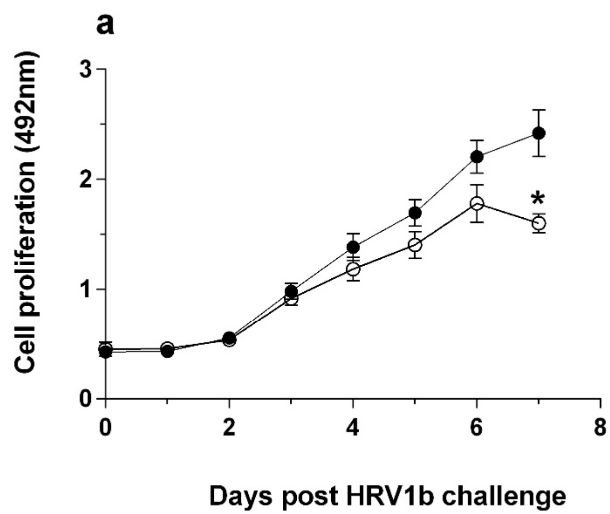
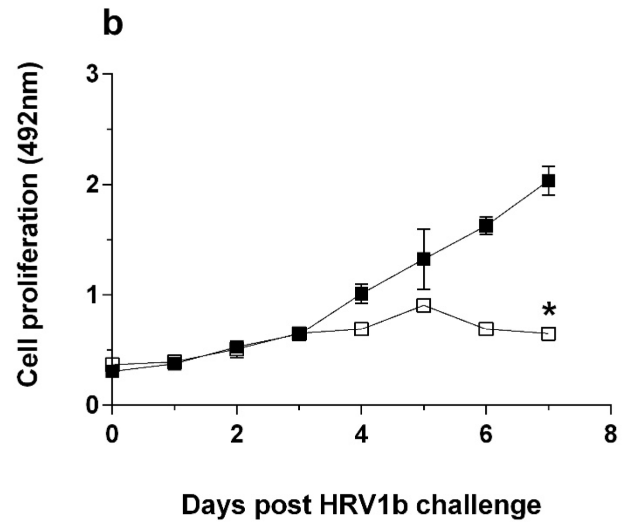
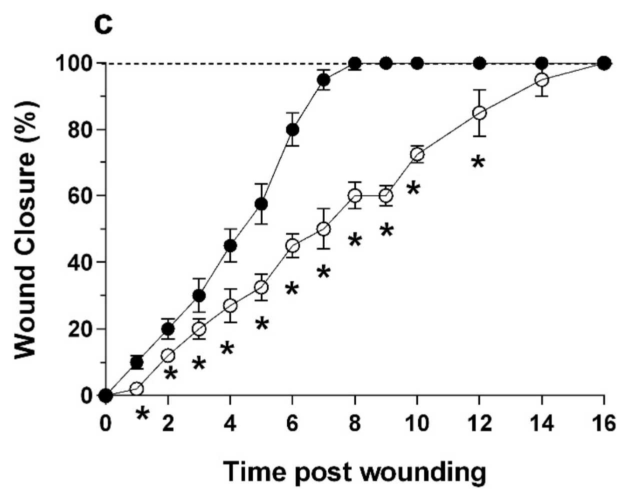



Figure 4. Kicic et al., 2016



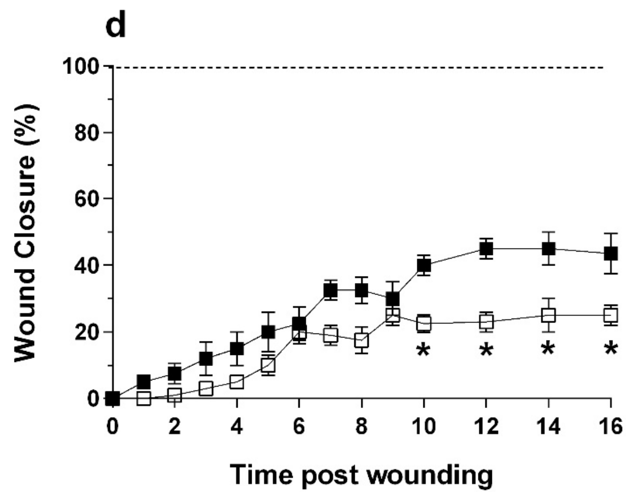




Figure 5. Kicic et al., 2016

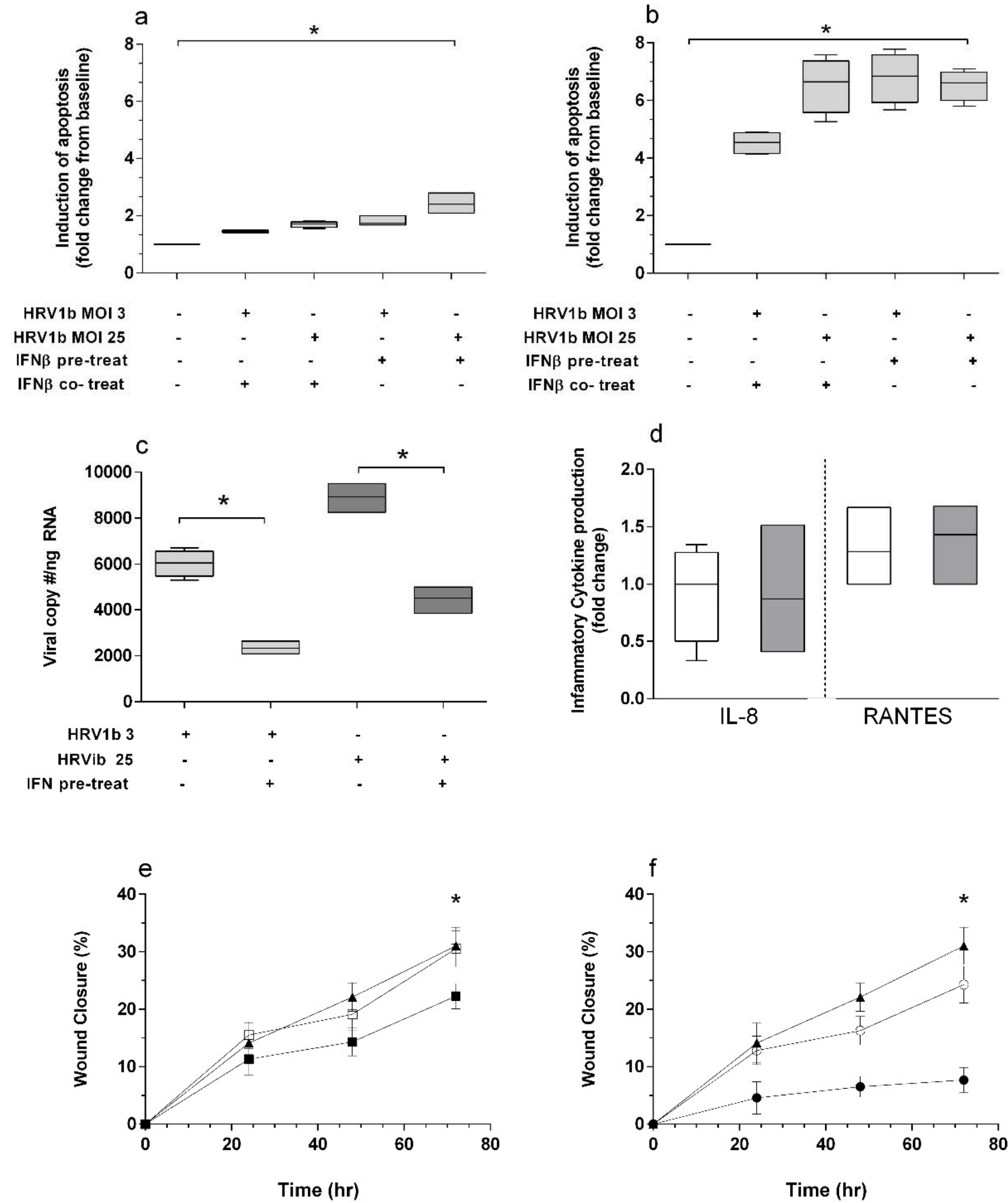



Figure 6. Kicic et al., 2016