

TLR3 and MDA5 signalling, although not expression, is impaired in asthmatic epithelial cells in response to rhinovirus infection.

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Summary

Background Rhinoviruses (RV) are the most common acute triggers of asthma, and airway epithelial cells are the primary site of infection. Asthmatic bronchial epithelial cells (BECs) have been found to have impaired innate immune responses to RV. RV entry and replication is recognized by pathogen recognition receptors (PRRs), specifically toll-like receptor (TLR)3 and the RNA helicases; retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5).

Objective Our aim was to assess the relative importance of these PRRs in primary bronchial epithelial cells (pBEC) from healthy controls and asthmatics following RV infection and determine whether deficient innate immune responses in asthmatic pBECs were due to abnormal signalling via these PRRs.

Methods The expression patterns and roles of TLR3 and MDA5 were investigated using siRNA knock-down, with subsequent RV1B infection in pBECs from each patient group. We also used BX795, a specific inhibitor of TBK1 and IKKi.

Results Asthmatic pBECs had significantly reduced release of IL-6, CXCL-8 and IFN- λ in response to RV1B infection compared with healthy pBECs. In healthy pBECs, siMDA5, siTLR3 and BX795 all reduced release of IL-6, CXCL-10 and IFN- λ to infection. In contrast, in asthmatic pBECs where responses were already reduced, there was no further reduction in IL-6 and IFN- λ , although there was in CXCL-10.

Conclusion and Clinical Relevance Impaired antiviral responses in asthmatic pBECs are not due to deficient expression of PRRs; MDA5 and TLR3, but an inability to later activate types I and III interferon immune responses to RV infection, potentially increasing susceptibility to the effects of RV infection.

Keywords asthma, innate immune response, MDA-5, rhinovirus, TLR-3

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Introduction

Rhinoviruses (RV) are the most frequent trigger for exacerbations of asthma and chronic obstructive pulmonary disease (COPD) [1, 2]. Asthmatics are more likely to have protracted and more severe lower respiratory tract symptoms, which are more severe and last longer [3]. We have previously shown that asthmatic primary bronchial epithelial cells (pBEC) demonstrate an impaired type I (interferon- β) [4] and type III (interferon λ 1/3) response to RV infection [5], although the reasons for this impaired response are not defined, they are crucial to our understanding of virus-induced acute exacerbation of asthma.

Human RV belong to the family of Picornaviridae, and these are non-enveloped, positive-stranded RNA viruses. The viral proteins attach to the receptors on airway epithelium, and this initiates a host innate immune response. The interferons (IFNs) are important regulators of innate and adaptive immune responses as well as regulating cell growth and viability [6]. Induction of IFN- β and IFN- α subtypes (α 2, 4, 5 and 6) occurs early after virus infection and is regulated by phosphorylation of interferon-response factor (IRF)3 [7]. Type I IFNs exert their actions through specific receptors (IFN receptor- α 1/ α 2). Receptor engagement leads to the activation of the IFN-stimulated regulatory factor,

leading to transcription of type I IFNs as well as over a hundred IFN-stimulated genes (ISGs) [8]. These ISGs have specific antiviral effects [6]. IFN- λ s are a recently described group of cytokines (IFN- λ 1-3) that signal through their own receptor comprised of IFNLR1 and the IL10R2 chain (part of the IL-10 receptor) [9]. The IFN- λ s trigger a type I IFN-like gene profile and have been shown to similarly inhibit virus replication [10, 11].

Viral entry into epithelial cells is recognized by pathogen recognition receptors (PRRs) through the recognition of viral nucleic acids. The best characterized of these PRRs are the family of toll-like receptors (TLRs) and the RNA helicases, which include retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). TLR3 is expressed in intracellular endosomes of BECs, macrophages, DCs and lymphocytes [12]. TLR3 responds to the presence of double-stranded (ds)RNA [13], which forms as a product of the replication of RNA viruses, including RV [14]. Signalling through TLR3 involves TIR domain-containing adaptor molecule Toll/IL-1R domain-containing adapter-inducing IFN- β (TRIF), which interacts with TRAF3, Tank-binding kinase 1 (TBK1) and inducible I κ B kinase (IKKi), leading to phosphorylation of IRF3 which forms a dimer and translocates to the nucleus resulting in the expression of type I IFNs. Signalling can also occur that leads to the translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) to the nucleus and results in the release of inflammatory cytokines [15].

The RNA helicases, MDA5 and RIG-I, are present in the cytosol and detect viral dsRNA and single-stranded (ss)RNA [16]. MDA5 detects picornaviruses such as RV, whilst RIG-I detects negative sense ssRNA viruses such as respiratory syncytial virus (RSV) and influenza virus [17]. Once MDA5 detects RV, it is recruited to the mitochondrial surface where it interacts with the mitochondrial antiviral-signalling (MAVS) protein, made up of interferon-beta promoter stimulator protein 1 (IPS-1) and a caspase recruitment domain (CARD). Activation of MAVS is required for the downstream activation of TBK1, IKK, IRF3 and types I and III IFN expression [17].

The initial innate immune response to RV infection is important in determining the subsequent inflammatory response in the airways. An impaired response in asthmatic epithelial cells may account for the increased susceptibility of asthmatics to the effects of RV. Our aim was to assess the relative importance of these PRRs in RV-infected primary bronchial epithelial cells from healthy controls and compare these initial signalling responses in asthmatics to determine whether they are impaired and whether this is responsible for the reduced types I and III IFN antiviral responses.

Methods

Cell culture

Primary bronchial epithelial cells (pBECs) and endobronchial biopsies were obtained from healthy controls (HC) and asthmatic (AS) subjects. The HC were non-smokers, non-atopic, with no history of heart or lung disease and normal lung function. The asthmatics were non-smokers and had a consistent history and evidence of bronchial hyperresponsiveness, with moderate to severe persistent asthma as defined by the GINA guidelines (see Table 1 for demographic subject information). Cells were obtained by bronchoscopy, with brushings taken from third-generation bronchi. These cells were then cultured and passaged as previously described [4]. Endobronchial biopsies were also obtained from second- to third-generation airways. All experiments were conducted in accordance with the Hunter New England Area Health Service Ethics Committee and the University of Newcastle Safety Committee (205/2008).

pBECs were maintained in bronchial epithelial cell growth medium (BEGM; Lonza). Cells were seeded onto placental collagen (Sigma) coated 24-well plates (Nunc) and used at passage 2 once they reached 80% confluency. After exposure to virus, the pBECs were maintained in BEBM minimal media [bronchial epithelial basal medium (no growth factors) (BEBM; Lonza) containing $1 \times$ ITS+1 (insulin, transferring and sodium selenite) liquid media supplement (obtained as a $100 \times$ stock from Sigma)]. All cells were grown at 37°C with 5% CO₂ in air.

Generation of Rhinovirus stocks

Human rhinovirus serotype 1B (RV1B) was isolated from clinical samples in 2005 and sequenced to confirm their identity. The rhinovirus stock was propagated in RD-ICAM-1 cell line in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 1% foetal bovine serum (FBS). Virus was titrated by infecting RD-ICAM-1 with serially diluted RV and assessing the 50% tissue culture infective doses (TCID₅₀) per millilitre [4].

Table 1. Demographic characteristics of subjects

	Healthy control	Asthma
Number (<i>n</i>)	8	12
Age, year (SD)	55.9 (12.3)	54.5 (9.9)
Male, <i>n</i> (%)	4 (50)	3 (25)
ICS, <i>n</i> (%)	NA	12 (100)
Daily ICS dose (BDP equivalent dose), μ g (SD)	NA	2267 (1136)
FEV ₁ , % predicted (SD)	94.6 (11.3)	65.2 (23.0)

BDP, beclomethasone dipropionate; ICS, inhaled corticosteroid; FEV₁, forced expiratory volume in 1 s.

Results are expressed as means (SD) or *n* (%).

Small interfering RNA (siRNA) knock-down of MDA5 and TLR3

Silencer[®] Select Pre-Designed siRNAs, non-specific siRNA (negative control) or GAPDH siRNA (positive control) were reverse transfected into pBECs using siPORT[™] NeoFX Transfection Agent (Applied Biosystems). Reverse transfection (neofection) allows for the cells to be transfected as they adhere to the plate. The Silencer[®] siRNA Transfection II Kit guidelines were followed for specific volumes and concentrations. For MDA5, the pool of sequences used were as follows: 5' GGUGUAAGAGAGCUACUAAAtt-3', 5' GUUCAGGAGUUA UCGAACAtt-3' and 5' GUAACAUUGUUAUCCGUUAtt-3'. For TLR3, the sequence used was as follows: 5' GGAUA GGUGCCUUCGUCAtt-3'. After 24 h of transfection, the appropriate cells were infected with RV. After this, the transfection media were replaced, and RNA lysates harvested 24 h postinfection to confirm knock-down.

Synthetic inhibitors

The TBK1- and IKKi-mediated activation of IRF3 is blocked by BX795 (Axon Medchem). BX795 was dissolved in DMSO and stored as a 10 mM solution at 4°C. A working stock of 100 µM BX795 was prepared by adding 1 µL 10 mM BX795 to 49 µL DMSO and 50 µL PBS. The cells were incubated at 37°C for 1 h in the presence of 1 µM BX795 (10 µL/well of the working stock, with a final DMSO concentration of 0.005%).

Rhinovirus infection

RV were diluted in supplemented BEBM minimal media and added to cells at a multiplicity of infection (MOI) of 1, 5 and 20. Multiple MOIs were tested to characterize an optimal response from the cells (seen at an MOI 20), along with lower doses to determine that host immune responses were not being overwhelmed at high doses. The responses recorded with an MOI 20 have been used to demonstrate the effect. After 1 h of infection on a shaking platform at room temperature, the inoculum was removed and replaced with BEBM minimal media. RNA lysates, cell supernatants and protein lysates were harvested at the times indicated. Supernatants and RNA lysates were stored at -80°C, and protein lysates were stored at -20°C until required.

Real-time quantitative PCR (RT-qPCR)

After treatments, total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen) according to the manu-

facturer's instructions. Extracted RNA (200 ng) was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ABI). First-strand cDNA products were used as the template for qPCR assays. Ribosomal RNA (18S rRNA) was used as the housekeeping gene.

To determine the level of expression by qPCR, premade primers specific for MDA5, RIG-I, TLR3 and GAPDH were purchased from ABI. ABI (Hs00223420_m1) for MDA5, ABI (Hs99999905_m1) for GAPDH, ABI (4319413E) for 18S rRNA and ABI (Hs00152933_m1) for TLR3. Expression levels of mRNA were calculated relative to the housekeeping gene ribosomal RNA (18S rRNA) using the $\Delta\Delta CT$ method. This was further analysed as a fold change relative to media.

Reactions consisted of 12.5 µL Taqman Gene Expression Master Mix (ABI) and 1.25 µL specific primer. One microlitre of cDNA was made up to 25 µL with nuclease-free water (Invitrogen). The reactions were analysed using an ABI7500 real-time PCR system (ABI). The amplification cycle consisted of 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min.

Levels of cytokine/chemokine expression determination

Twenty-four hour postinfection supernatants were analysed for the levels of hIL-29/IFN- λ 1/3 (R&D Systems) by ELISA. Also, CXCL8, IL-6 and CXCL10 were measured using cytometric bead array (CBA) (BD, Becton, Dickinson and Company) by flow cytometry.

Immunoblotting

Cells were lysed in ice-cold lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.1% SDS) containing protease inhibitor cocktail (Roche Diagnostics). Samples were then mixed with a sample buffer (Invitrogen) and heated at 95°C for 10 min. Samples were then electrophoresed on 4–12% Tris-glycine Novex SDS-polyacrylamide gels (Invitrogen), after which the gels were transferred to Hybond polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech). These membranes were washed and incubated with primary or secondary antibodies. Primary antibodies employed in the various studies were rabbit anti-MDA5 (Prosci Inc.), rabbit anti-DDX58 (RIG-I) (Abcam) and anti-pIRF3 (Ser396) (Millipore). Rabbit anti-TRIF (Abcam) was used to detect the immediate downstream measure of TLR3. GAPDH was probed with goat anti-GAPDH antibody (Abcam) and used as a loading control. Proteins were visualized with SuperSignal chemiluminescent substrate (Pierce Biotechnology). Densitometric analysis was performed using ImageJ (NIH, USA) software.

Statistical analysis

Data are expressed as mean $\pm$ standard error of mean (SEM) for eight subjects ($n = 8$) with two replicates for each subject for HC and twelve subjects ($n = 12$) with two replicates for each subject for AS, for all MOI 20 data. Statistical significance was assessed by either paired *t*-test or one-way analysis of variance (ANOVA), and post hoc analysis was performed using the Bonferroni correction. A *P* value of < 0.05 was considered significant. RV1B was compared with media alone, and each condition of RV1B+siMDA5, RV1B+siTLR3 and RV1B+BX795 was compared with RV1B alone.

Results

Baseline expression of MDA5, TLR3 and RIG-I is no different in asthmatic pBECs

To examine baseline expression in untreated primary bronchial cells, total RNA was isolated and mRNA levels were measured at 24 h. MDA5, TLR3 and RIG-I mRNA expression was similar for both HC and asthmatics (Fig. 1a). This finding was confirmed in an *in vivo* setting by measuring the mRNA levels of MDA5, TLR3 and RIG-I mRNA from endobronchial biopsies from the HC and asthmatic subjects (Fig. 1b). Furthermore, there was no difference in baseline levels of protein for MDA5, TRIF and RIG-I in HC and asthmatics (Fig. 1c, d).

Rhinovirus-mediated induction of IFN expression in primary bronchial epithelial cells is virus specific

RV dose and time course were optimized prior to the experiments; pBECs were infected with an MOI 20, 5 and 1. This was to investigate whether the MOI 20 was saturating the responses. The same trend was seen at all MOIs, so the MOI of 20 will be used for these results. To characterize the antiviral response, total RNA was isolated and mRNA levels were measured 24 h post-RV infection and compared with media alone controls. In both HC and asthmatic pBECs, RV increased the PRR gene expression profiles of MDA5, TLR3 and RIG-I (Fig. 2a). There was a greater induction of MDA5 and RIG-I compared with TLR3; however, there was no difference between the two subject groups in terms of PRR induction.

Asthmatic primary bronchial epithelial cells have reduced IFN- λ , IL-6 and CXCL8 release

We next sought to confirm whether HC pBECs had a more effective antiviral response when exposed to RV. After 24 h of infection (with MOI 20 as a representative), asthmatic pBECs had significantly reduced IFN- λ 1/3 (Fig. 2b), IL-6 (Fig. 2c) and CXCL-8 (Fig. 2d) although CXCL-10 (Fig. 2e) release was similar. To confirm that these cytokine inductions were due to the RV infection, a UV-inactivated control was used and this was comparable to untreated control (Figure S1A–C).

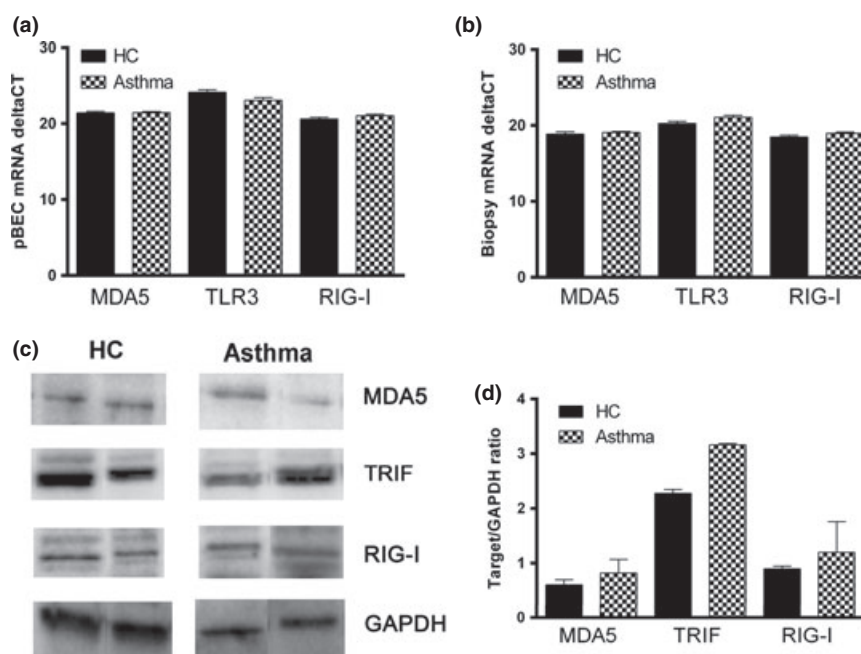


Fig. 1. Healthy control and asthmatic baseline expression of MDA5, TLR3 and RIG-I at the mRNA and protein level. (a) Baseline pBEC mRNA expression of MDA5, TLR3 and RIG-I was assessed by RT-qPCR in HC and asthmatic pBEC. (b) Baseline endobronchial biopsy mRNA expression of MDA5, TLR3 and RIG-I was assessed by RT-qPCR in HC and asthmatic biopsies. (c, d) Baseline expression of MDA5, TRIF and RIG-I from two different subjects (representative) was measured by western blot, and densitometry was measured compared with GAPDH control and presented as a target/GAPDH ratio.

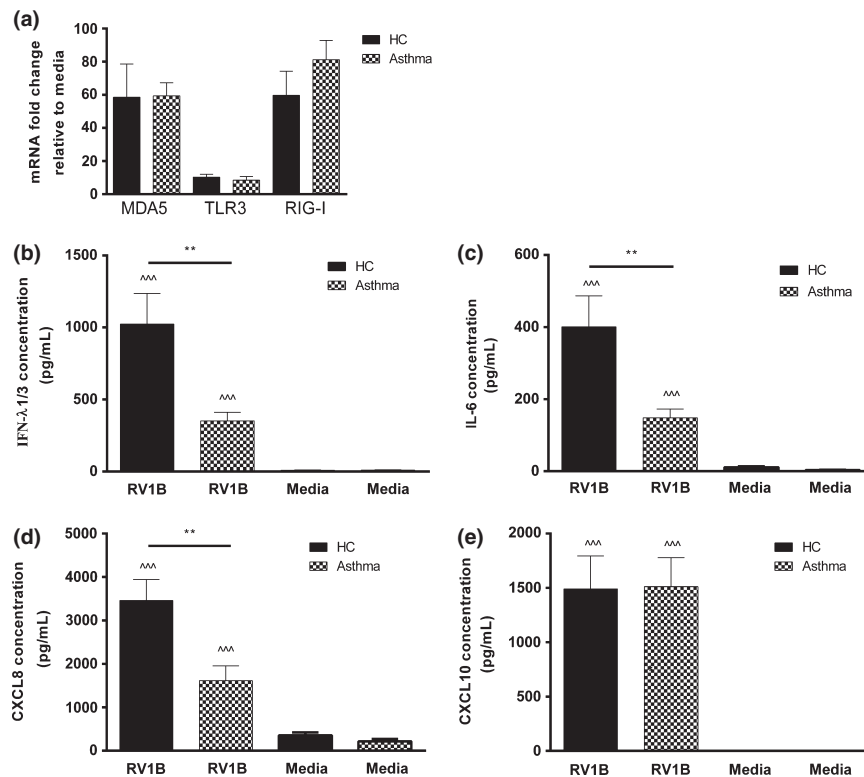


Fig. 2. Induction of PRRs, IFN and ISG expression in pBECs by RV infection. (a) mRNA expression of MDA5, TLR3 and RIG-I was assessed by RT-qPCR after RV1B infection of HC and asthmatic pBEC. Healthy control and asthmatic pBEC supernatant levels of IFN-λ1/3 were measured by ELISA (b); IL-6, CXCL-8 and CXCL-10 were measured by CBA (c-e) $^{***}P < 0.0001$ RV1B expression vs media baseline and $^{**}P < 0.01$ RV1B HC expression vs RV1B asthmatic expression.

Knock-down of MDA5 and TLR3 as well as inhibition of pIRF3 results in down regulation of RV-induced antiviral type I and type III interferon responses

We next investigated the expression patterns and roles of MDA5 and TLR3 using siRNA knock-down. After 24 h of transfection, knock-down of these targets was confirmed by real-time qPCR and western blot. MDA5 and TLR3 expression was knocked down $> 80\%$ through the transfection of specific siRNAs, which occurred to a similar efficiency for each target in both HC and AS pBECs (Fig. 3a–d). The level of expression of negative scrambled siRNA was similar to media controls (Fig. 3a, c). There was no induction of type I or type III IFN responses in cells transfected with a negative scrambled siRNA (data not shown), and when negative scrambled siRNA was combined with RV, there was no effect on replication or cytokine release (data not shown). After confirming the knock-down, transfected pBECs were infected with RV1B. To test whether IRF3 was also required for RV-induced type I IFN and type III IFN responses, we blocked IRF3 activation by blocking the TBK/IKK kinases using a synthetic inhibitor BX795 [18]. Western blot analysis of pIRF3 in cell lysates confirmed that phosphorylation of IRF3 was markedly reduced by BX795 in response to infection with RV

(Fig. 3e). Cells treated with siRNA and BX795 remained viable (data not shown), and the DMSO concentration was too low to have any effect on the cytokine release.

In HC pBEC, RV1B infection induced a significant induction of IFN-λ1/3 release ($P < 0.0001$), although not completely reduced by MDA5 and TLR3 siRNA knock-down. IFN-λ1/3 induction was completely blocked by BX795 ($P < 0.0001$) (Fig. 4a). In stark contrast, in asthmatic pBEC where RV1B-induced IFN-λ1/3 was already decreased, the release following RV1B infection was not further significantly reduced by siMDA5 or siTLR3; in fact, siTLR3 had no effect at all (Fig. 4b). This same effect was seen at an MOI 5 and 1 (Fig. 4e–h). Although release was still completely blocked by BX795 ($P < 0.0001$) (Fig. 4b), again, an effect was seen at all MOIs.

In HC pBEC, at an MOI 20, the release of CXCL10 was reduced by greater than 50% by siMDA5 and siTLR3 ($P < 0.001$), but IFN-λ1/3 was completely eliminated by BX795 ($P < 0.0001$) (Fig. 4c). In asthmatic pBEC, the release of CXCL10 was reduced by siMDA5 ($P < 0.05$) and siTLR3 ($P < 0.001$), although in both cases not to the same extent as in HC (Fig. 4d). CXCL10 release was completely eliminated by BX795 ($P < 0.0001$) (Fig. 4d). This effect was seen at all MOIs tested (Fig. 4e–h).

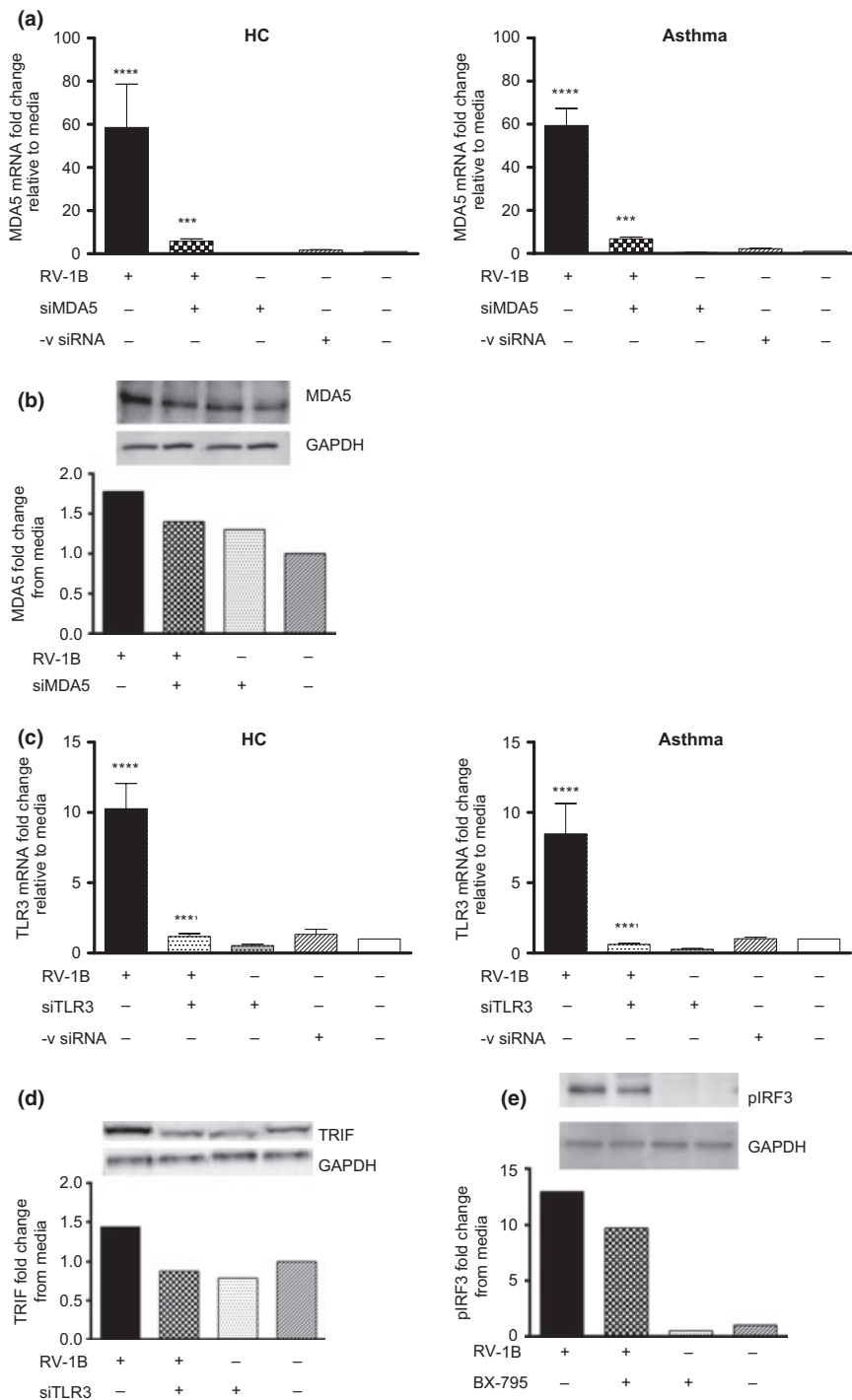


Fig. 3. Successful knock-down of MDA5 and TLR3 using siRNA transfection blocks mRNA and protein expression. (a) 24 h mRNA levels of MDA5 in HC and asthmatic pBECs measured by RT-qPCR represented as a fold change relative to media. (b) 24-h protein lysates were used to determine MDA5 expression by western blot, with the blot being a representative from one HC subject. Densitometry was measured compared with GAPDH control and presented as a fold change relative to media. (c) 24-h mRNA levels of TLR3 in HC and asthmatic pBECs measured by RT-qPCR represented as a fold change relative to media. (d) 24-h protein lysates were used to determine TLR3 knock-down effectiveness by measuring downstream TRIF expression by western blot, with the blot being a representative from one HC subject. Densitometry was measured compared with GAPDH control and presented as a fold change relative to media. (e) Successful blocking of pIRF3 using BX795 pre-treatment inhibits protein expression. Twenty-four-hour protein lysates were used to determine pIRF3 expression by western blot with the blot being a representative from one HC subject. Densitometry was measured compared with GAPDH control and presented as a fold change relative to media. **** $P < 0.001$ RV expression vs. media, *** $P < 0.001$ RV1B vs RV1B + siMDA5/siTLR3

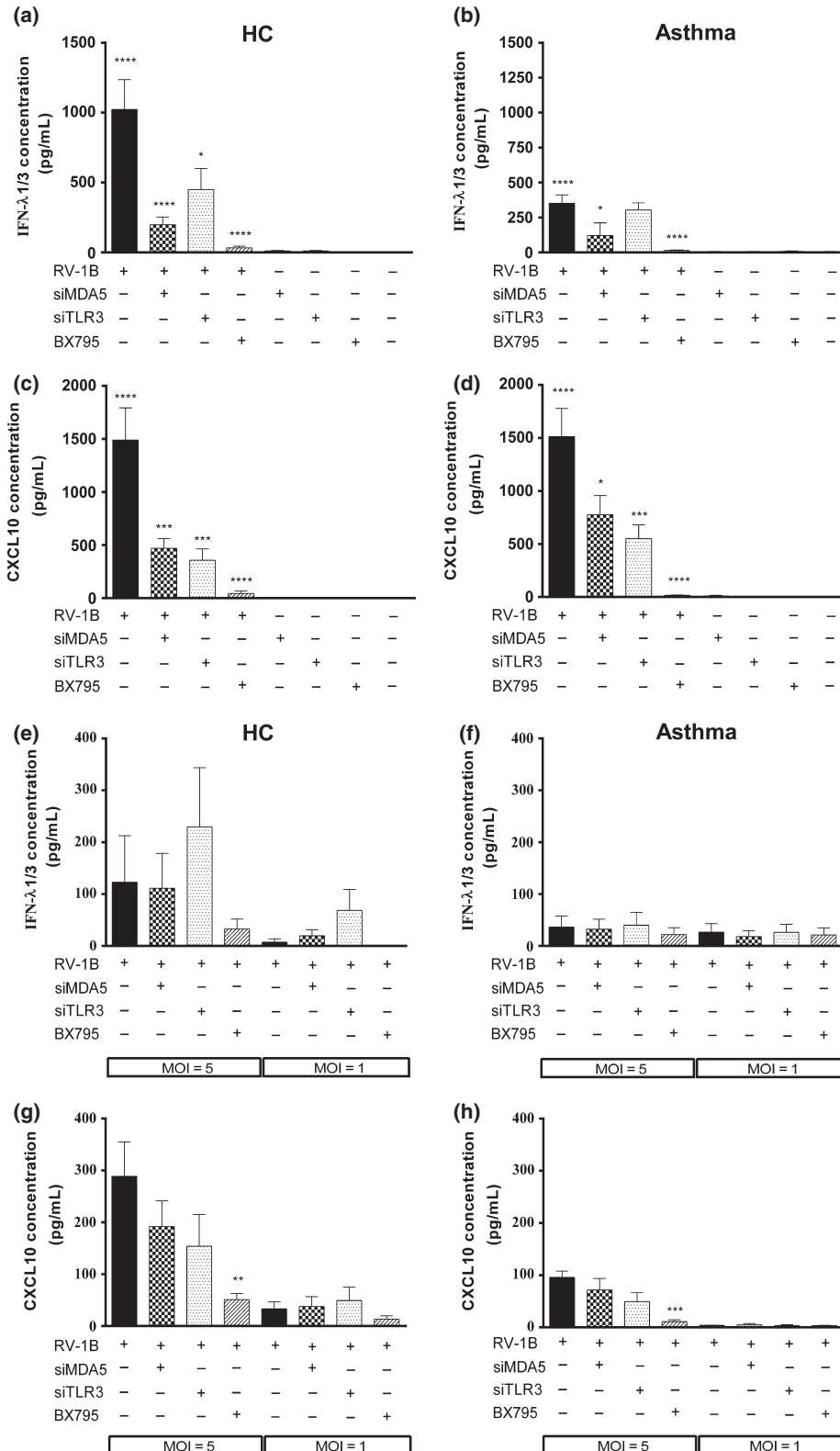


Fig. 4. Knock-down of MDA5, TLR3 or inhibition of pIRF3 results in down regulation of IFN responses in HC. HC and asthmatic pBEC were treated with siMDA5 or siTLR3 or BX795 and then infected with RV1B at an MOI 20. Twenty-four-hour protein secretion of INF-λ1/3 (a–b) and CXCL10 (c–d) in cell supernatants was measured by ELISA/CBA. RV1B infection at an MOI 5 and 1; 24-h protein secretion of INF-λ1/3 (E–F), CXCL10 (G–H) in cell supernatants was measured by ELISA/CBA. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. RV1B expression vs media and RV1B expression vs RV1B + siMDA5/siTLR3 or RV1B + BX795.

Knock-down of MDA5, TLR3 or inhibition of pIRF3 has no effect on RV-induced release of IL-6 and CXCL8

RV infection is known to induce the release of pro-inflammatory mediators that occur through the activation of NF- κ B. In HC pBECs, infection with RV1B led to a significant release of IL-6 (Fig. 5a). HC pBEC knock-down with siMDA5-inhibited IL-6 responses; however, this inhibition was not statistically significant. siTLR3 resulted in significant inhibition of greater than 50% of RV1B response ($P < 0.05$), while BX795 reduced it to that seen at baseline ($P < 0.0001$) (Fig. 5a). In AS pBECs, with inherently reduced RV1B-induced IL-6, siMDA5 or siTLR3 knock-down had no effect at all (Fig. 5b). Furthermore, BX795 caused a small reduction in IL-6 in asthmatic pBECs ($P < 0.05$) (Fig. 5b). In HC pBECs, infection with RV1B led to a significant release of CXCL-8 (Fig. 5c). In HC pBECs, CXCL8 release was unaffected by siMDA5 (Fig. 5c). Similar to IL-6 release in HC, CXCL-8 release was significantly reduced by siTLR3. While in asthmatic pBECs, siMDA5 or siTLR3 had no effect on CXCL-8 RV1B-induced release (Fig. 5d). BX795 essentially caused a pro-inflammatory response from cells of both groups (Fig. 5c–d). These effects were similar at all MOIs tested (Fig. 5e–h).

Discussion

We have demonstrated that expression of the PRRs; MDA5, TLR3 and RIG-I is not different in asthmatic compared with HC subjects, in both their airways and cultured pBECs from these subjects. We have also shown that RV infection of both asthma and HC pBECs leads to an early and similarly robust induction of MDA5 and RIG-I. However, despite this initial response, asthmatic pBECs still demonstrated substantially reduced release of types I and III IFNs to RV infection. In HC cells, signalling through TLR3 and MDA5 results in the release of type I and type III antiviral IFN responses; IL-6 and CXCL-10. Blocking of MDA5 and TLR3 was able to substantially inhibit both the type I and type III IFN responses. While inhibition of both MDA5 and TLR3 substantially reduced the innate immune response to RV infection in HC pBECs, this effect was not noticeable in the asthmatic cells, whose ability to respond was already impaired. These results confirm that asthmatic pBECs have impaired types I and III early immune responses to RV, but demonstrate for the first time, an inability of asthmatic pBECs to effectively signal through MDA5 and TLR3 to initiate this response, despite the normal expression and induction of these PRR in asthmatic pBECs.

The innate immune response in epithelial cells relies on a dual system to recognize viral dsRNA: the helicases MDA5 and RIG-I that are cytoplasmic and TLR3,

which is expressed in cellular endosomes. Activation of either pathway by dsRNA leads to the activation of IRF3 and IRF7 via TBK1 and the inducible IKKi leading to transcription of IFN- β and the induction of interferon stimulated genes [19]. In our experiments, we blocked the activity of these pathways at two separate levels. Initially, we blocked the upstream sensing of RV infection using siRNA to knock down MDA5 and TLR3. Inhibition was greatest with siMDA5, followed by siTLR3. Thus, siMDA5 and siTLR3 reduced expression of IFN- λ s and ISGs (CXCL10). Subsequently, we blocked the activation of IRF3 via TBK1 and the inducible IKKi through the synthetic inhibitor BX795. BX795 reduced expression of IFN- λ and CXCL10, while increasing CXCL8 in both HC and asthmatic cells.

MDA5 is a cytoplasmic sensor of dsRNA that contains a helicase and CARD domain [19]. Infection models with other picornaviruses have shown the importance of the MDA5 pathway in the innate immune response. MDA5 knockout mice when infected with coxsackie B3 virus demonstrate a reduced type I IFN response and increased mortality, although interestingly without an increase in virus replication [20]. Similarly, it was demonstrated that MDA5 was the dominant receptor triggering type I IFN responses to Sendai virus and polyI:polyC [21]. In the case of poliovirus infection, viral proteases cleave MDA5 and this appears crucial in promoting infection [22]. In human epithelial cell models, Wang et al. used siRNA to MDA5, as well as siRNA against the TLR3 adaptor molecule TRIF and siRNA to IRF3 to study innate responses to RV1B and RV39 infection in BEAS-2B cells and primary tracheal epithelial cells [23]. Similar to our own findings, they demonstrated that knock-down of MDA5, TRIF and IRF3 inhibited mRNA induction of IFN- β , IFN- λ , IRF7, RIG-I, MDA5 and CXCL10 but had no effect on CXCL8 and GM-CSF. More recently, Slater et al. [24] demonstrated that this innate immune response relied upon a sequential and coordinated response from the PRRs, with constitutive expression of endosomal TLR3, initiating the early IFN response and leading to subsequent induction of both cytoplasmic MDA5 and RIG-I [24]. They also confirmed that there was minimal baseline expression of MDA5, in endobronchial biopsies from healthy subjects, prior to the experimental infection, but subsequent up-regulation 4 days postinfection. This again is in keeping with our results, where we saw baseline expression of TRIF with minimal induction of TRIF/TLR3 and minimal baseline expression of MDA5 and RIG-I, although marked induction of both to RV infection. In variance with us and Wang et al. [23], Slater et al. [24] found that siRNA to RIG-I did impair subsequent inflammatory responses as well as induction of IFN, although not IFN- λ 1. These differences are difficult to account for, but may relate

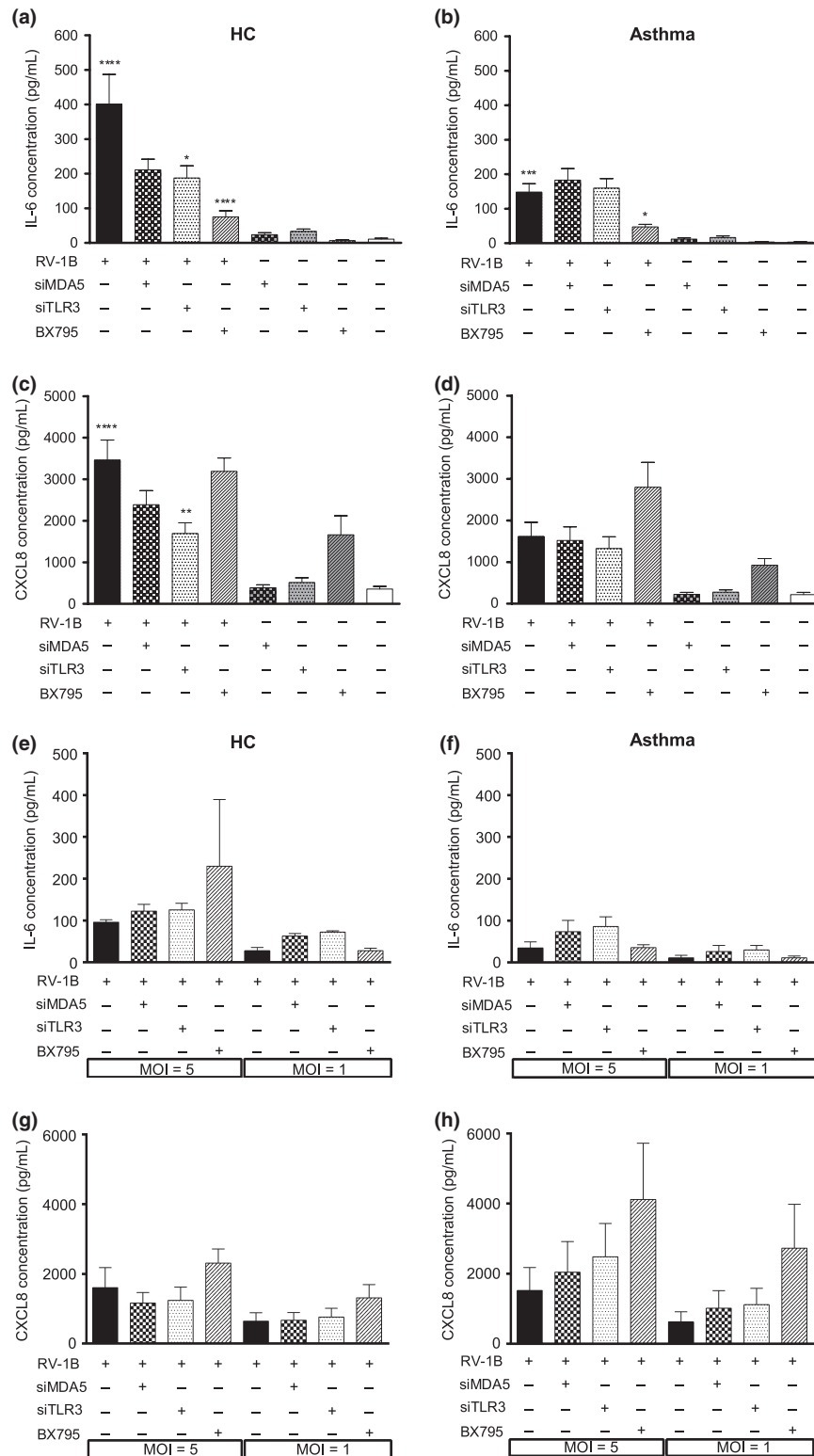


Fig. 5. Knock-down of MDA5, TLR3 or inhibition of pIRF3 has no effect on RV-induced IL-6 and CXCL8. HC and asthmatic pBEC were treated with siMDA5 or siTLR3 or BX795 and then infected with RV1B at an MOI 20. Twenty-four-hour protein secretion of IL-6 (a, b) and CXCL8 (c, d) in cell supernatants was measured by CBA. RV1B infection at an MOI 5 and 1; 24-h protein secretion of IL-6 (E-F) and CXCL8 (G-H) in cell supernatants was measured by CBA. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. RV1B expression vs media and RV1B expression vs RV1B + siMDA5/siTLR3 or RV1B + BX795.

to subtle differences in *in vitro* conditions. As Slater *et al.* did not find RIG-I to be up-regulated in their endobronchial biopsies following RV infection, it may suggest that MDA5 still is the most important of the RNA helicases responding to RV although a degree of redundancy in the ability of MDA5 and RIG-I to be activated may also exist.

We have previously demonstrated that a deficient IFN- β response in pBECs from asthmatics is linked to enhanced RV replication as well as reduced apoptosis following infection [4], while a deficient IFN- λ response is also evident, and these findings are associated with increased lower respiratory tract symptoms in asthmatics [5]. We therefore sought to determine whether this abnormality was the result of impaired activation and signalling at the level of TLR3 and MDA5 PRR expression in asthma pBECs. This, however, was clearly not the case, with similar baseline expression of TLR3 and robust induction of MDA5 seen in asthmatic pBECs and confirmed to be similar in biopsies from asthma and HC airways. This is also consistent with recent findings in subjects who had died from asthma, where airway expression of TLR3 was in fact increased [25]. While *ex vivo* infection of asthmatic bronchoalveolar lavage cells demonstrated similar expression of TLR3, TLR7, MDA5 and RIG-I, these cells still demonstrated an impaired release of IFN- α [26]. Despite the normal levels of PRR expression and up-regulation in response to RV infection, we have demonstrated that asthmatic pBECs still have marked impairment in their antiviral interferon responses. Consistent with this, a recent study by Uller *et al.* [27] showed that induction of IFN- β mRNA is already reduced in pBECs from asthmatic donors just 3 h after challenge with polyI:polyC, and the response to polyI:polyC was inhibited by chloroquine, consistent with an abnormality downstream in the TLR3 pathway. It is also in keeping with the response seen in ovalbumin sensitized MDA5 and TLR3 knockout mice to RV-1B [28]. The null mice had impaired IFN responses, but also had reduced airway inflammation and airway

contractile responses, suggesting that these pathways are important in initiating a response to RV airway inflammation and hyperresponsiveness in this animal model of allergic airways disease. Our results confirm that both MDA5 and TLR3 are expressed but fail to initiate these responses in asthmatic pBECs.

In summary, we have found no difference in the expression of important PRRs; MDA5 and TLR3 that initiate the innate immune response to RV in asthmatic airways. RV infection of healthy pBECs leads to a robust up-regulation of the MDA5 and TLR3, while inhibition of their signalling leads to a marked inhibition of both type I and type III IFN responses. However, we have shown for the first time that asthmatic pBECs while expressing and being able to up-regulate both MDA5 and TLR3, following RV infection, still fail to initiate an effective innate immune response, with impaired type I and type III interferon responses to RV infection. The nature of this abnormality appears to be intrinsic in the response of asthmatic pBECs, at least in those with moderate to severe persistent asthma. However, the exact step(s) of the subsequent IFN response that is impaired in asthma is still to be determined and remains important to define. This will help to better understand asthmatic susceptibility to the effects of RV infection and develop potential therapeutic interventions for reducing the affect of RV on acute asthma exacerbations.

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Conflict of Interest

We have no conflict of interest to declare.

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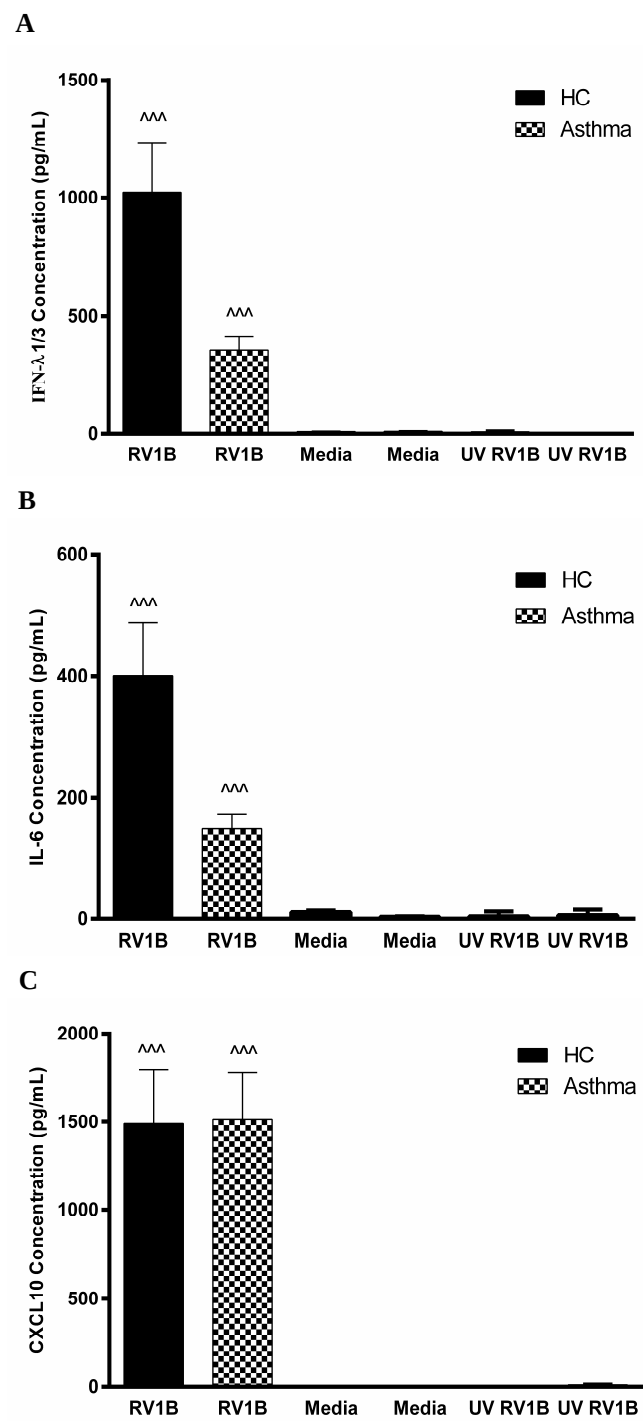
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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Induction of IFN and ISG expression in pBECs by RV infection and not UV-RV. A, Healthy

control and asthmatic pBEC supernatant levels of IFN- λ 1/3 were measured by ELISA (A); IL-6 and CXCL-10 were measured by CBA (B–C) *** $P < .0001$ RV1B expression vs media baseline/UV-RV1B.



Supplement Figure 1