

Original article

Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells

Background: Respiratory viruses, predominantly rhinoviruses are the major cause of asthma exacerbations. Impaired production of interferon- β in rhinovirus infected bronchial epithelial cells (BECs) and of the newly discovered interferon- λ s in both BECs and bronchoalveolar lavage cells, is implicated in asthma exacerbation pathogenesis. Thus replacement of deficient interferon is a candidate new therapy for asthma exacerbations. Rhinoviruses and other respiratory viruses infect both BECs and macrophages, but their relative capacities for α -, β - and λ -interferon production are unknown.

Methods: To provide guidance regarding which interferon type is the best candidate for development for treatment/prevention of asthma exacerbations we investigated respiratory virus induction of α -, β - and λ -interferons in BECs and peripheral blood mononuclear cells (PBMCs) by reverse transferase-polymerase chain reaction and enzyme-linked immunosorbent assay.

Results: Rhinovirus infection of BEAS-2B BECs induced interferon- α mRNA expression transiently at 8 h and interferon- β later at 24 h while induction of interferon- λ was strongly induced at both time points. At 24 h, interferon- α protein was not detected, interferon- β was weakly induced while interferon- λ was strongly induced. Similar patterns of mRNA induction were observed in primary BECs, in response to both rhinovirus and influenza A virus infection, though protein levels were below assay detection limits. In PBMCs interferon- α , interferon- β and interferon- λ mRNAs were all strongly induced by rhinovirus at both 8 and 24 h and proteins were induced: interferon- α > β > λ . Thus respiratory viruses induced expression of α -, β - and λ -interferons in BECs and PBMCs. In PBMCs interferon- α > β > λ while in BECs, interferon- λ > β > α .

Conclusions: We conclude that interferon- λ s are likely the principal interferons produced during innate responses to respiratory viruses in BECs and interferon- α s in PBMCs, while interferon- β is produced by both cell types.

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Respiratory virus infections are major triggers of acute exacerbations of asthma in both adults and children, implicated in around 80% of paediatric (1) and 75% of adult (2) asthma attacks. They are therefore major causes of asthma morbidity and mortality (3). Of viruses

detected in asthma exacerbations, two thirds are rhinoviruses (1). Influenza viruses are also implicated in asthma exacerbations during annual influenza epidemics (2, 4, 5).

Current therapy of asthma exacerbations is of limited efficacy (6–8), new approaches are therefore required.

Asthmatic individuals are more susceptible to naturally occurring rhinovirus (RV) infection than normal individuals (9) and we have recently reported that primary bronchial epithelial cells (BECs) from asthmatic subjects exposed to RV *in vitro* had profoundly impaired production of interferon (IFN)- β compared to normal bronchial epithelial cells (10). In these studies, normal BECs were almost completely resistant to RV infection, and impaired IFN- β production in asthmatic cells was strongly correlated with increased RV replication. Restoring IFN- β responses with exogenous IFN- β in asthmatic cells restored antiviral activity and rendered asthmatic cells as resistant to infection as normal ones (10). These studies suggest IFN- β could be an effective therapy for RV-induced asthma exacerbations.

The major human type I IFNs are IFN- α and IFN- β . There is only one human IFN- β , but there are 14 human genes that comprise the IFN- α family, excluding the pseudogene IFNAP22, and 13 proteins are expressed from these genes (11, 12). In response to viral infections IFN- α 4 and IFN- β are induced first and then enhance virus-mediated expression of themselves, as well as all the other IFN- α s acting through autocrine and paracrine mechanisms involving signalling via the type I IFN receptor (13, 14). These combined type I IFNs then induce multiple IFN-inducible genes with antiviral properties as well as promoting apoptosis in virally infected cells (10, 15).

It has recently been reported that peripheral blood mononuclear cells (PBMCs) from asthmatic subjects produce less IFN- α 2 than PBMCs from normal subjects in response to stimulation with respiratory syncytial virus (RSV) or Newcastle disease virus, implicating IFN- α deficiency in the pathogenesis of asthma (16).

A further family of human IFNs has recently been described, consisting of IFN- λ 1 (also known as IL-29), IFN- λ 2 (IL-28A) and IFN- λ 3 (IL-28B), these are now known as the type III IFN family (17, 18). They are distinct from type I IFNs in that they are located on a different chromosome (nine for type I IFNs and 19 for type III), have only ~20% amino acid homology with type I IFNs and signal via a distinct novel receptor (17, 18). They are a family as their genes are located together on chromosome 19 and - λ 1 has 80% homology with - λ 2/3, which have 96% homology with each other (5, 18). Like type I IFNs, type III IFNs are produced by human cells on infection with viruses and signal via STAT1 and STAT2 to stimulate IFN-inducible genes (17), however, their role in respiratory viral infections is poorly understood.

We have recently demonstrated that these novel type III IFNs are also produced by human BECs on infection with RV and are antiviral against RVs *in vitro* (19). Further, they are also produced by RV-infected bronchoalveolar lavage (BAL) cells (~90% macrophages) and production by both BAL cells and BECs was deficient in asthmatic compared to normal subjects (19). Importantly, IFN- λ production was strongly inversely correlated with severity of clinical illness, and with virus load and airway inflam-

mation, when subjects were experimentally infected with RV *in vivo* (19). These data strongly implicate type III IFN deficiency in the pathogenesis of asthma exacerbations.

These studies combined suggest that administration of type I or type III IFNs, or augmentation of BEC and/or macrophage type I or type III IFN production is likely to be an attractive approach to prevention and/or therapy of virus induced asthma exacerbations. However, little is known about the types of IFNs produced by virus infected BECs, nor about the relative contributions of the different types of IFNs in response to rhinoviruses.

We have recently shown that macrophages are major producers of type I IFNs on RV infection (20) and that RV infection of BECs induces both IFN- β (10) and IFN- λ (19, 21).

We have therefore investigated respiratory virus induction of type I and type III IFNs in the BEAS-2B BEC line, in primary BECs, and PBMCs. We elected to study RVs as the most common virus type implicated in asthma exacerbations, and influenza virus type A as a respiratory virus known to suppress type I IFN responses, as the patterns of induction could be different for this virus type.

Materials and methods

Primary BECs and BEAS-2B tissue culture

All cells were cultured at 37°C in 5% CO₂. Primary BECs obtained from three different donors were purchased from Cambrex BioScience (Walkersville, MD, USA). Primary cultures were established by seeding bronchial epithelial cells into supplemented bronchial epithelial growth medium (BEBM) according to the manufacturer's instructions (Cambrex). Cells were seeded onto 12 well trays and cultured until 80% confluent (22) before exposure to virus.

The human bronchial epithelial cell line BEAS-2B (ECACC) was cultured in RPMI-1640 supplemented with 10% FCS (Invitrogen, Paisley, UK). BEAS-2B cells were cultured in 12-well tissue culture plates (Nalge Nunc, Rochester, NY, USA) for 24-h before being placed into 2% FCS RPMI medium for a further 24-h prior to infection.

Viral stocks

Rhinovirus serotypes 16 and 1B obtained from the Medical Research Council Common Cold Unit were grown in Ohio HeLa cells and prepared as previously described (23). Viral stocks were used at 1×10^7 TCID₅₀/ml (5). The identities of all RVs were confirmed by titration on HeLa cells and neutralization using serotype-specific antibodies (ATCC). Ultraviolet (UV) inactivation was performed as previously described (24).

Influenza A Victoria 75/3 (gift from Peter Morley, GSK, Stevenage) was propagated in MDCK cell cultures (gift from Peter Morley). At 80% confluence cell cultures were washed twice with sterile PBS and then growth medium replaced with serum free eagle minimum essential medium (Invitrogen). 0.5 ml of influenza stock was added to each flask and incubated for 1 h. Cells were then washed to remove any non-adherent virus and resuspended in serum free medium for culture for 48 hours. Two days after infection, supernatants were collected from flasks, aliquotted and stored at -80°C

for future use. Viruses were titrated on MDCK cells to determine TCID₅₀/ml. Stock was assessed as being at 10^{7.5} TCID₅₀/ml.

Infection of cells with RV and influenza virus

BEAS-2B cells were seeded in 12 well plates (Nunc, Roskilde, Denmark) at 1.7×10^5 cells/ml, allowed to attach and grow for 24 h. BEAS-2B were then placed in RPMI 1640 + 2% FCS (infection media) overnight. Cells were then treated with RV16 or RV1B [multiplicity of infection (MOI) of 1] for 1 h at room temperature with shaking. Cells supernatants and RNA lysates were harvested at the times indicated. Supernatants and lysates were stored at -80°C until required.

The same protocol was used for infecting cells with Influenza A Victoria 75/3 virus. Virus was used at a MOI of 1.

PBMC separation and rhinovirus 16 infection

Peripheral blood mononuclear cells were separated from whole blood from three healthy donors using gradient density centrifugations (Sigma-Aldrich, UK). A total of 4×10^6 cells/2 ml were exposed to rhinovirus 16 for 1 h. At the end of exposure time cells were centrifuged at 300 *g* for 10 min at 4°C once, supernatant was removed and medium containing RPMI 1640 and 5% FCS was added to infected and mock-infected cells. Peripheral blood mononuclear cells were cultured for 4, 8 and 24 h after the infection. Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. This study was approved by St Mary's NHS Trust Ethics Committee and all subjects gave informed consent.

RNA extraction, reverse transcription and TaqMan® real-time PCR

RNA was extracted from cells using the RNeasy method (RNeasy Mini Kit; Qiagen) following the manufacturer's instructions, including the optional DNaseI digestion of contaminating DNA (Dnase (Rnase free Dnase); Qiagen). cDNA was synthesized using Omniscript RT and components as directed by the manufacturer (Qiagen).

Primers specific for IFN- α s, IFN- β , IFN- λ 1, IFN- λ 2/3 were purchased from Invitrogen and probes from Qiagen (Table 1). TaqMan® analysis of - α , - β and - λ interferon mRNA was normalized with respect to 18S rRNA and presented as log₁₀-fold induction relative to medium control. For detection of IFN- α s it was not possible to design a single primer and probe set to detect all subtypes, therefore subtypes 1, 6 and 13 were detected with one set of primers and probe (IFN- α .1); and subtypes 2, 4, 5, 8, 10, 14, 17 and 21 by another (IFN- α .2) (25). Reactions consisted of 12.5 μ l 2 $\times$ QuantiTect Probe PCR Master Mix (Qiagen) and 300 nM of forward primer and 900 nM of reverse primer for IFN- α .1, 300 nM of each primer for IFN- α .2, 300 nM and 900 nM for IFN- β , IFN- λ 1 and IFN- λ 2/3, 300 nM of each primer for 18S; 175 nM of each specified probe was used. Two microliters of cDNA (18S diluted 1/100) was made up to 25 μ l with nuclease-free water (Promega, Southampton, UK). The reactions were analysed using an ABI7000 Automated TaqMan (Applied Biosystems, Warrington, UK). The amplification cycle consisted of 50°C for 2 min, 94°C for 10 min and 40 cycles of 94°C for 15 s, 60°C for 15 s.

Enzyme-linked immunosorbent assay to evaluate IFN- α , IFN- β and IFN- λ and CCL5/RANTES release

Interferon- α , IFN- β and CCL5/RANTES proteins were quantified by enzyme-linked immunosorbent assay (ELISA) in supernatants from untreated and infected cell cultures collected and stored at -80°C using commercially available paired antibodies and standards, following the manufacturer's instructions using a high sensitivity IFN- α human Biotrak ELISA (GE Healthcare, Amersham Biosciences, Amersham, UK), a human IFN- β ELISA kit and human RANTES Cytoset (both BioSource, Biosource International, Nivelles, Belgium). All the measurements were done according to manufacturer's instructions. The detection limits for described assays are 0.63 pg/ml for IFN- α , 2.5 pg/ml for IFN- β , 0.169 pg/ml for RANTES.

Amersham Biosciences (now part of GE Healthcare) technical support group report that the antibodies used in the IFN- α human Biotrak ELISA (Amersham Biosciences) were designed to recognize an IFN-alpha epitope common for all IFN-alpha subtypes.

Human IFN- β ELISA kit (Biosource) selectively detects only IFN-beta protein.

Table 1. Sequences of primers and probes used for detection of IFNA, IFNB, IL-29, IL-28A/B and 18S

Primer and probe set	Subtypes detected	Sequence of primers and probes	References
IFN- α .1	1,6,13	Forward – 5'-CAG AGT CAC CCA TCT CAG CA-3' Reverse – 5'-CAC CAC CAG GAC CAT CAG TA-3' Probe – 5'-FAM ATC TGC AAT ATC TAC GAT GGC CTC GCC TAMRA-3'	25
IFN- α .2	2, 4, 5, 8, 10, 14, 17, 21	Forward – 5'-CTG GCA CAA ATG GGA AGA AT-3' Reverse – 5'-CTT GAG CCT TCT GGA ACT GG-3' Probe – 5'-FAM TTT CTC CTG CCT GAA GGA CAG ACA TGA TAMRA-3'	25
IFN- β	IFN- β	Forward – 5'-CGC CGC ATT GAC CAT CTA-3' Reverse – 5'-GAC ATT AGC CAG GAG GTT CTC A-3' Probe – 5'-FAM TCA GAC AAG ATT CAT CTA GCA CTG GCT GGA TAMRA -3'	14
IFN- λ 1	IL-29	Forward – 5'-GGA CGC CTT GGA AGA GTC ACT'3' Reverse – 5'-AGA AGC CTC AGG TCC CAA TTC'3' Probe – 5'-FAM AGT TGC AGC TCT CCT GTC TTC CCC G TAMRA-3.	19
IFN- λ 2/3	IL-28A/B	Forward – 5'-CTG CCA CAT AGCCCA GTT CA-3' Reverse – 5'-AGA AGC GAC TCT TCT AAG GCA TCT T-3' Probe – 5'-FAM TCT CCA CAG GAG CTG CAG GCC TTT A TAMRA-3'	19
18S	18S	Forward – 5'-CGC CGC TAG AGG TGA AAT TCT-3' Reverse – 5'-CAT TCT TGG CAA ATG CTT TCG-3' Probe – 5'-FAM ACC GGC GCA AGA CGG ACC AGA TAMRA-3'	37

To measure interferon- λ s we developed an assay using a monoclonal anti-human IL-29/IFN- λ 1 antibody as capture, a polyclonal anti-IL-29 antibody as secondary and biotin conjugated donkey anti-goat IgG as third antibody (all R&D Systems) followed by streptavidin conjugated HRP (Biosource). Recombinant human IL-29 (Peprotech, Pepro Tech EC Ltd, London, UK) was used as standard. The sensitivity of the assay was 25 pg/ml. The assay for IFN- λ detection detects IL-29 protein but also detects some IL-28 as there is 25% cross-reactivity with IL-28.

Statistical analysis

Data are presented as mean (SEM). For time course experiments data were analysed using one-way ANOVA for repeated measures followed by paired *t*-tests between baseline and individual time points where appropriate. For other comparisons paired *t*-tests and Bonferroni's multiple comparison *post hoc* test were used. Data were accepted as significantly different when $P < 0.05$.

Results

Rhinovirus induction of type I and type III interferons in BEAS-2B BECs

The time course of expression of type I and type III IFN mRNAs was studied during infection of BEAS-2B cells with RV-16, a RV serotype implicated in asthma exacerbations (1, 10, 19, 23). There was no significant

induction by RV-16 of IFN- α subtypes 1, 6 or 13 detected by IFN- α .1 at either 4, 8 or 24 h, however, subtypes 2, 4, 5, 8, 10, 14, 17 and/or 21, detected by IFN- α .2, were significantly induced in comparison to medium at 8 h [2.74 (0.3) log₁₀-fold induction compared to medium, $P < 0.001$], but not at 4 or 24 h (Fig. 1A).

IFN- β mRNA expression was induced by RV-16 only at 24 h [3.22 (0.33), $P < 0.001$], while IFN- λ 1 mRNA expression was statistically significantly increased by RV-16 at 8 h [3.1 (0.75), $P < 0.01$] and further increased at 24 h [4.84 (0.24), $P < 0.001$] (Fig. 1B).

We next investigated whether the induction of IFN mRNAs by RV-16 infection of BEAS-2B cells leads to production of detectable levels of type I and type III IFN proteins at 24 h after infection. We detected no significant induction of IFN- α protein, but IFN- β protein production was significantly increased [19.8 (2.3) pg/ml, $P < 0.01$, Fig. 1C] while IFN- λ was produced at greater levels [99.89 (2.03) pg/ml, $P < 0.001$, Fig. 1D] in RV-16 infected BEAS-2B cells in comparison to non-infected cells.

Rhinovirus induction of type I and type III interferons in primary BECs

As BEAS-2B cells are a transformed cell line, their responses may differ from primary cells. We therefore

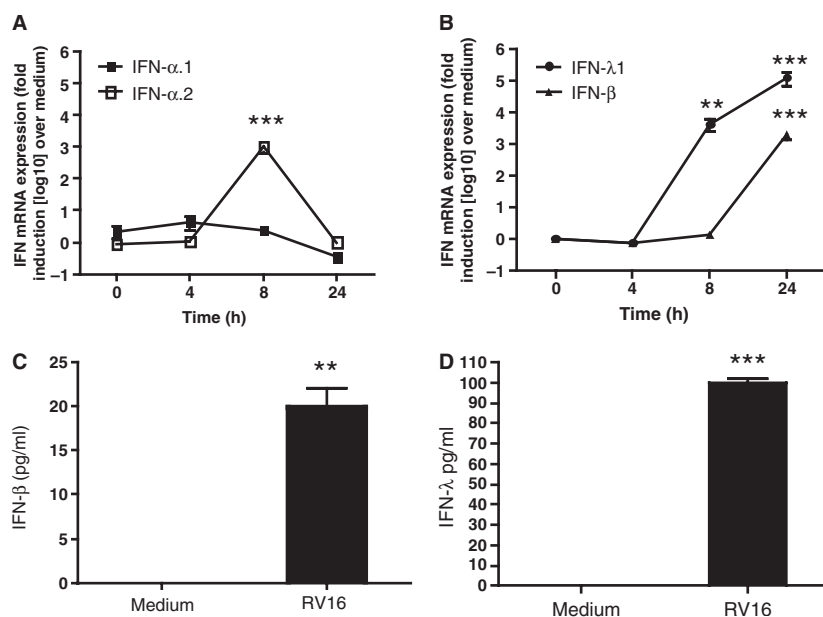


Figure 1. Time course of RV-16 induction of type I and type III IFNs in BEAS-2B cells. (A) The expression of IFN- α mRNAs was studied by Taqman PCR. For detection of various IFN- α subtypes two pairs of Taqman PCR primers and probes were selected. No significant induction of IFN- α subtypes by RV-16 was detected by primer/probe IFN- α .1, however significant induction was detected by IFN- α .2 at 8 h but not at other time points ($n = 5$, *** $P < 0.001$ compared with time point 0). (B) The expression of IFN- λ 1 and IFN- β mRNAs was studied by Taqman PCR. Significant induction of IFN- λ 1 mRNA expression by RV-16 was observed at 8 h ($n = 5$, ** $P < 0.01$ compared with time point 0) with a further increase observed at 24 h ($n = 5$, *** $P < 0.001$ compared with time point 0). IFN- β mRNA expression was induced by RV-16 only at 24 h ($n = 5$, *** $P < 0.001$ compared with time point 0). (C) Release of IFN- β into supernatants at 24 h was measured by ELISA. Significant induction of IFN- β protein was detected in RV-16 infected BEAS-2B cells ($n = 3$, ** $P < 0.01$ compared to medium control). (D) Release of IFN- λ s into supernatants at 24 h was measured by ELISA. Significant induction of IFN- λ protein was detected in RV-16 infected BEAS-2B cells ($n = 3$, *** $P < 0.001$ compared to medium control).

next investigated primary BECs expression of IFN- α , IFN- β , IFN- λ 1 mRNA expression during RV-16 infection. We also studied IFN- λ 2/3 expression to determine whether its time course and level of expression paralleled that of IFN- λ 1. Similar to BEAS-2B cells, there was no significant induction by RV-16 of IFN- α subtypes detected by IFN- α .1 at 4, 8 or 24 h, however, subtypes detected by IFN- α .2 were significantly induced in comparison to medium at 8 h [3.06 (0.24) log₁₀-fold induction compared to medium, $P < 0.001$] and remained elevated at 24 h, though at 24 h this was not statistically significant (Fig. 2A).

Also similar to BEAS-2B cells, IFN- β mRNA expression was induced by RV-16 only at 24 h [4.1 (0.44), $P < 0.001$, Fig. 2B]. IFN- λ 2/3 mRNA expression paralleled the increase in IFN- λ 1, both being induced by RV-16 at 8 h [IFN- λ 2/3 2.75 (0.23), $P < 0.001$ and IFN- λ 1 2.2 (1.23), $P = \text{NS}$] and further induced at 24 h [IFN- λ 2/3 4.2 (0.12), and IFN- λ 1 5.03 (0.21), both $P < 0.001$, Fig. 2C].

These levels of mRNA induction were not sufficient to lead to production of detectable levels of type I and type III IFN proteins at 24 h after infection, as each of α , β and λ IFNs were undetectable. We confirmed that another mediator induced by RV was significantly

induced in the same cells by assaying CCL5/RANTES and observing that this was significantly induced [42.67 (7.7) pg/ml, $P < 0.001$, Fig. 2D] in RV-16 infected primary BECs in comparison to non-infected cells.

Rhinovirus induction of type I and type III interferons in primary BECs is receptor independent and replication dependent

Rhinovirus-16 is a member of the major group of RVs using ICAM-1 as cellular receptor while RV-1B is the member of the minor group of RVs using low density lipoprotein receptor (26). To determine whether RV induction of type I and type III IFNs in primary BECs is receptor restricted or not we next investigated the effect of RV-1B.

Similar to RV-16, there was no significant induction by RV-1B of IFN- α subtypes detected by IFN- α .1 at 4, 8 or 24 h, however, subtypes detected by IFN- α .2 were significantly induced in comparison to medium at 8 h [2.96 (0.29) log₁₀-fold induction compared to medium] though these remained significantly elevated at 24 h [3.1 (0.4), both $P < 0.05$, Fig. 3A].

Also similar to RV-16, IFN- β mRNA expression was induced by RV-1B only at 24 h [4.12 (0.25), $P < 0.01$, Fig. 3B]. IFN- λ 2/3 mRNA expression again paralleled

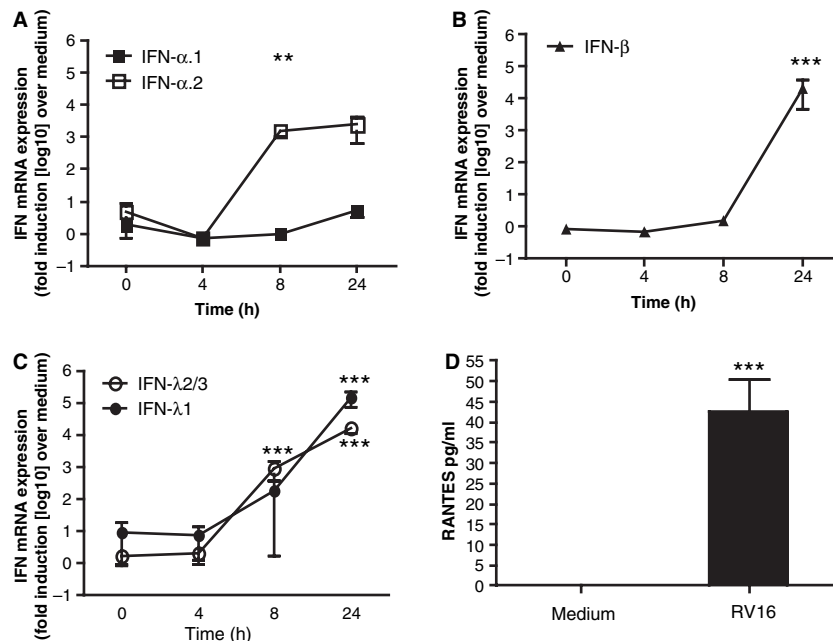


Figure 2. Time course of RV-16 induction of type I and type III IFNs in primary BECs. (A) The expression of IFN- α mRNAs was assessed by Taqman PCR. IFN- α s detected by IFN- α .1 primer pair were not induced by rhinovirus 16. With IFN- α .2 statistically significant induction was observed by 8 h ($n = 4$, $**P < 0.01$ compared to time point 0). (B) The expression of IFN- β mRNA was assessed by Taqman PCR. Significant induction of IFN- β mRNA by RV-16 was detected only at 24 h ($n = 4$, $***P < 0.001$ compared to time point 0). (C) The expression of IFN- λ 1 and IFN- λ 2/3 mRNAs was studied by Taqman PCR. IFN- β mRNA expression was up-regulated at 24 h ($n = 4$, $***P < 0.001$ compared to time point 0). IFN- λ 2/3 mRNA was significantly induced by RV-16 at 24 h ($n = 4$, $***P < 0.001$ compared to time point 0). (D) Release of CCL5/RANTES into supernatants at 24 h was measured by ELISA. Significant induction of CCL5/RANTES protein was detected in RV-16 infected primary BECs ($n = 4$, $***P < 0.001$ compared to medium control).

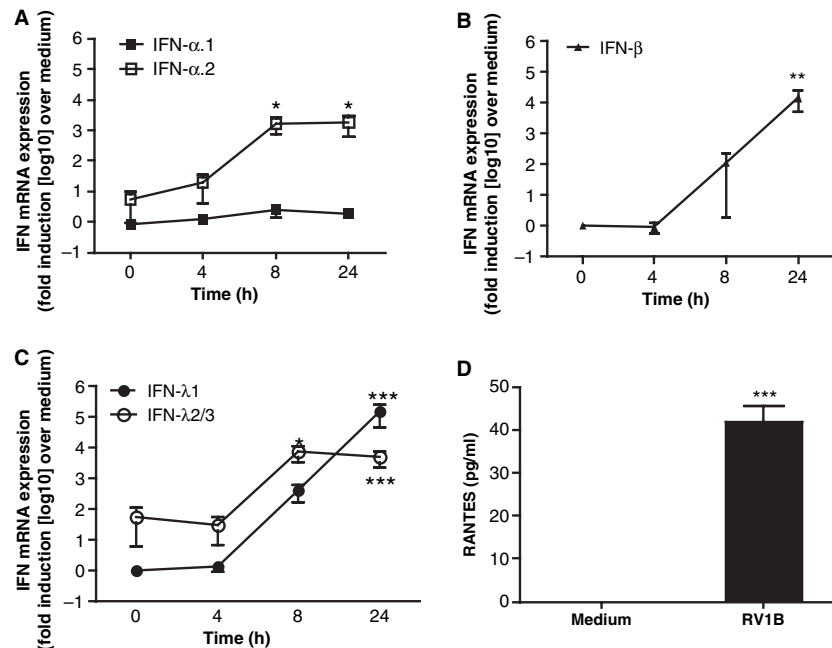


Figure 3. Time course of RV-1B induction of type I and type III IFNs in primary BECs. (A) IFN- α mRNA expression was observed by Taqman PCR. mRNAs of IFN- α subtypes detected by primer/probe IFN- α .1 were not induced by RV-1B. By IFN- α .2 significant induction was observed at 8 h ($n = 4$, $*P < 0.05$ compared to medium control) and 24 h ($n = 4$, $*P < 0.05$ compared to medium control). (B) The expression of IFN- β mRNA was assessed by Taqman PCR. Significant induction of IFN- β mRNA by RV-16 was detected only at 24 h ($n = 4$, $**P < 0.01$ compared to time point 0). (C) The expression of IFN- λ 1 and IFN- λ 2/3 mRNAs was studied by Taqman PCR. IFN- λ 1 mRNA expression was significantly induced at 24 h ($n = 4$, $***P < 0.001$). IFN- λ 2/3 mRNA was significantly up-regulated at 8 h ($n = 4$, $*P < 0.05$ compared to medium control) and 24 h ($n = 4$, $*P < 0.05$ compared to medium control). (D) Release of CCL5/RANTES into supernatants at 24 h was measured by ELISA. Significant induction of CCL5/RANTES protein was detected in RV-16 infected primary BECs ($n = 4$, $***P < 0.001$ compared to medium control).

the increase in IFN- λ 1, both being induced by RV-1B at 8 h [IFN- λ 2/3 3.76 (0.19), $P < 0.05$ and IFN- λ 1 1.83 (0.65), $P = \text{NS}$] remaining induced at 24 h [IFN- λ 2/3 3.6 (0.2) $P < 0.05$, and IFN- λ 1 5.01 (0.38), $P < 0.001$, Fig. 3C].

Again, these levels of mRNA induction were not sufficient to lead to production of detectable levels of type I and type III IFN proteins at 24 h after infection, as each of α , β and λ IFNs were again undetectable, while CCL5/RANTES was once more significantly induced [41.94 (3.57) pg/ml, $P < 0.001$, Fig. 3D] in RV-1B infected primary BECs.

We next investigated the effects of live and UV-inactivated RV-16 to determine whether IFN induction was replication dependent. In contrast to Figure 2A, in these experiments live RV-16 induction of IFN- α subtypes detected by IFN- α .2 was statistically significant at 24 h after infection [3.49 (0.28) log₁₀-fold induction compared to medium, $P < 0.05$, Fig. 4A], and as previously shown in Fig. 2B,C, significant induction of IFN- β and both IFN- λ 1 and IFN- λ 2/3 were again observed (Fig. 4B–D), however UV-inactivated RV-16 induced no type I or type III IFN mRNA (Fig. 4A–D), indicating that RV-induced expression of

type I and type III IFNs has a replication dependent mechanism.

Influenza virus induction of type I and type III interferons in primary BECs

Influenza viruses are important respiratory pathogens also implicated in asthma exacerbations (2, 4, 5), they are also known to suppress type I IFN responses (27–30). We therefore next investigated the effects of influenza virus type A on type I and type III IFNs in primary BECs.

Similar to RV induction, there was no significant induction by influenza virus of IFN- α subtypes detected by IFN- α .1 at 4, 8 or 24 h, however, subtypes detected by IFN- α .2 were significantly induced in comparison to medium at 24 h [2.46 (0.16) log₁₀-fold induction compared to medium, $P < 0.01$, Fig. 5A] though not at earlier time points.

Also similar to RV induction, IFN- β mRNA expression was induced by influenza virus only at 24 h [4.16 (0.14), $P < 0.001$, Fig. 5B] and IFN- λ 2/3 mRNA expression again paralleled the increase in IFN- λ 1, both being induced by influenza virus at 8 h [IFN- λ 2/3 3.48 (0.04), and IFN- λ 1 3.13 (0.36), both $P < 0.001$] and remaining

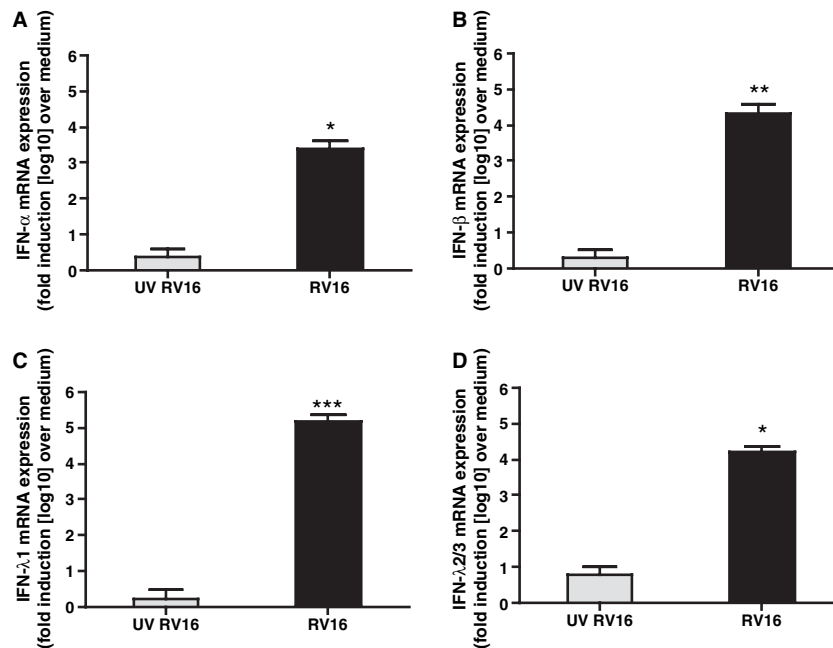


Figure 4. The influence of UV inactivation on the ability of RV-16 to induce type I and type III IFN expression. (A) IFN- α mRNA expression was assessed by Taqman PCR. In contrast to live virus, at 24 h UV-inactivated RV-16 did not induce mRNA expression of IFN- α subtypes detected by primer/probe IFN α .2 ($n = 4$, $*P < 0.05$ live compared to UV inactivated RV-16). (B) The expression of IFN- β mRNA was assessed by Taqman PCR. In contrast to live virus, at 24 h UV-inactivated RV-16 caused no induction of IFN- β mRNA expression ($n = 4$, $**P < 0.01$ live compared to UV-inactivated RV-16). (C) The expression of IFN- λ mRNA detected by primer/probe IFN- λ 1 was assessed by Taqman PCR. At 24 h UV-inactivated RV-16 caused no induction of IFN- λ 1 mRNA expression ($n = 4$, $***P < 0.001$ live compared to UV-inactivated RV-16). (D) The expression of IFN- λ mRNA detected by primer/probe IFN- λ 2/3 was assessed by Taqman PCR. At 24 h UV-inactivated RV-16 caused no induction of IFN- λ 2/3 mRNA expression ($n = 4$, $*P < 0.05$ live compared to UV-inactivated RV-16).

elevated at 24 h [IFN- λ 2/3 3.84 (0.17) $P < 0.001$, and IFN- λ 1 4.97 (0.09), $P = \text{NS}$, Fig. 5C].

Again, each of α , β and λ IFN proteins were undetectable at 24 h, while CCL5/RANTES was once more significantly induced [30.57 (6.3) pg/ml, $P < 0.001$, Fig. 5D] in influenza virus infected primary BECs.

Rhinovirus induction of type I and type III interferons in PBMCs

Having found IFN- λ s to be the major IFN type induced by virus infection of BECs, we next used PBMCs to elucidate the type of IFNs induced in monocytes during RV infections. RV-16 strongly induced mRNA expression of IFN- α subtypes detected by both IFN- α .1 and IFN- α .2 primer pairs peaking at 8 h [IFN- α .1 5.84 (0.27), and IFN- α .2 5.94 (0.55) log₁₀-fold induction compared to medium, both $P < 0.001$] and remaining elevated at 24 h [IFN- α .1 5.21 (0.33), and IFN- α .2 4.73 (0.1) log₁₀-fold induction compared to medium, both $P < 0.001$, $P < 0.001$ for IFN- α .1 and $P = \text{NS}$ for IFN- α .2, Fig. 6A]. Both IFN- β and IFN- λ 1 mRNAs were also induced strongly, in a similar manner, also peaking at 8 h [IFN- β 6.19 (0.34), and IFN- λ 1 5.55 (0.21), both $P < 0.001$] and remaining elevated at 24 h [IFN- β 4.66

(0.14), and IFN- λ 1 4.43 (0.34), $P < 0.001$ for IFN- β and $P < 0.01$ for IFN- λ 1, Fig. 6B].

These strong inductions of IFN mRNA expression were accompanied by protein release into supernatants of RV-16 infected cells for each IFN type [IFN- α 335.79 (5.23), and IFN- β 136.69 (6.33), both $P < 0.001$ and IFN- λ 79.23 (2.53) pg/ml, $P < 0.01$, Fig. 6C–E].

Discussion

Here we show that the BEC line BEAS-2B and primary BECs expressed increased levels of mRNAs of α -, β - and λ -IFNs in response to RV infection, but that λ -IFNs were the most strongly induced both early and during sustained induction. RV induction of IFN proteins were mostly below detection limits, but when proteins were detected in BEAS-2B cells, IFN- λ s were detected at the highest levels. Similar results were obtained with influenza A virus infection of primary BECs. We also investigated PBMCs and observed that mRNAs for all IFN types investigated were strongly induced by RV infection, but in this cell type, IFN- α s were the most strongly induced.

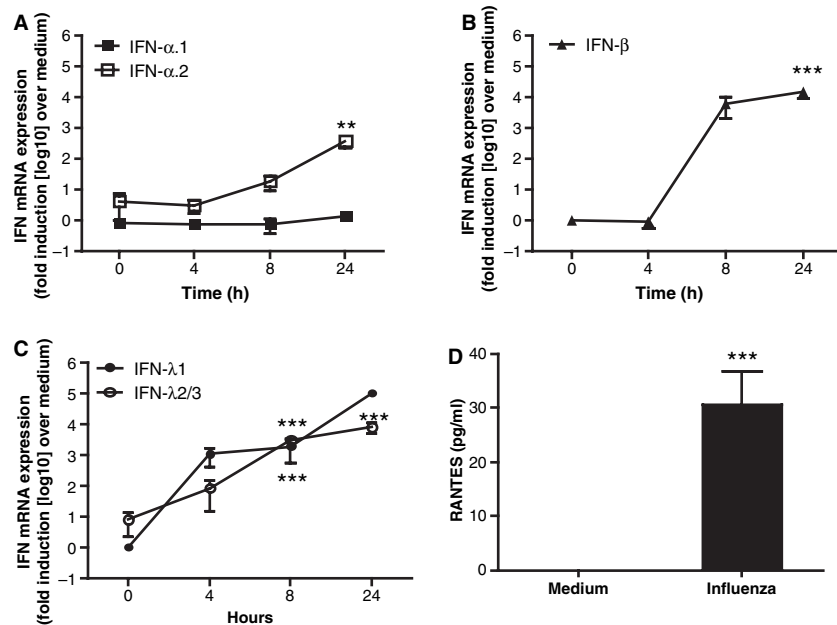


Figure 5. Time course of influenza virus induction of type I and type III IFNs in primary BECs. (A) The expression of IFN- α mRNA was assessed by Taqman PCR. IFN- α subtypes detected by primer/probe IFN- α .1 were not induced by rhinovirus 16. IFN- α .2 primer/probe detected significant induction at 24 h ($n = 4$, $**P < 0.01$ compared to time point 0). (B) The expression of IFN- β mRNA was assessed by Taqman PCR. Significant induction of IFN- β mRNA by influenza virus was detected at 24 h ($n = 6$, $***P < 0.001$ compared to time point 0). (C) The expression of IFN- λ 1 and IFN- λ 2/3 mRNAs was studied by Taqman PCR. IFN- λ 1 mRNA expression was up-regulated at 8 h ($n = 4$, $***P < 0.001$ compared to time point 0). IFN- λ 2/3 mRNA was significantly induced by influenza virus at 8 h ($n = 4$, $***P < 0.001$ compared to time point 0) and 24 h ($n = 4$, $***P < 0.001$ compared to time point 0). (D) Release of CCL5/RANTES into supernatants at 24 h was measured by ELISA. Significant induction of CCL5/RANTES protein was detected in influenza virus infected primary BECs ($n = 4$, $***P < 0.001$ compared to medium control).

Respiratory epithelial cells are the major site of RV replication as replication has been confirmed in both BEC lines (23, 24) and primary BECs (10, 19, 31) *in vitro*, and RV has been detected by *in situ* hybridization and immunohistochemistry within the BEC cell layer in bronchial biopsies *in vivo* (32). BEC innate responses to RV infection are therefore likely of major importance in protection against RV infections. This interpretation is supported by the recent evidence implicating impaired BEC innate responses in the pathogenesis of rhinovirus induced asthma exacerbations (10, 19). Our studies indicate both in a cell line and in primary cells, that IFN- λ s are likely the most abundant IFN type induced in response to RV infection of BECs. These data would support the development of IFN- λ s as candidate therapy for asthma exacerbations, as well as perhaps other viral diseases where BECs are the major site of virus replication.

We were unable to detect IFN proteins at 24 h in RV infected primary BECs. Both IFN- α and IFN- β mRNAs were expressed only at low levels (~ 3 – 4 logs), protein levels may thus have been below the detection limits of the assays. To determine whether later time points may have detected proteins, experiments were also carried out at 48 and 72 h, but again no proteins were detected. We believe that the levels of induction of IFN mRNA in

BEAS2B cells or primary BECs were not sufficient to generate detectable levels of protein production using the assays available. The levels of mRNA induced by RV in PBMCs at 8 h (the peak in most measurements) were 6–7 logs for IFN- α and - β , and 5–6 logs for IFN- λ 1. These are very high levels of mRNA induction and are associated with detectable protein levels. In contrast the levels of mRNA induced by RV in BEAS2B cells or primary BECs at 8 h were only 2–4 logs for each of IFN- α , - β , - λ 1 and - λ 2. These are very much lower levels of mRNA induction (difference of between 100-fold and 100 000-fold induction between BECs and PBMCs) and these were associated with protein levels below the detection limits of the assays.

For both α - and β -IFNs it is likely that IFN proteins are quickly taken up by the IFN- $\alpha\beta$ receptor as the affinity of the IFN AR2 subunit for IFN- α 2 alone is high ($K_D \sim 3$ nM) and increased up to 20-fold when complexed with IFN AR1 (33). The affinities of the IFN- λ receptor subunits for the IFN- λ s are not known and the assay for IFN- λ s was considerably less sensitive (25 pg/ml) than those for type I IFNs (0.63 and 2.5 pg/ml for α and β , respectively), potentially explaining why the IFN- λ s were not detectable despite mRNAs being induced to higher levels than the type I IFNs.

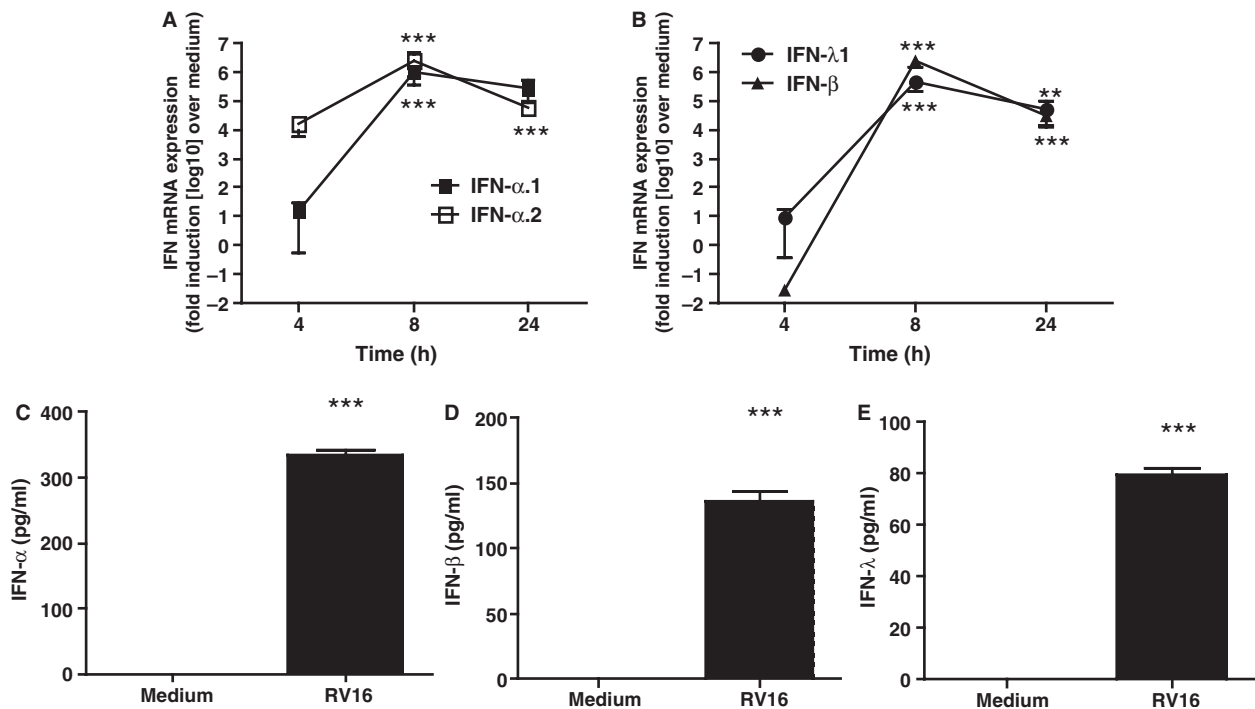


Figure 6. Time course of RV 16 induction of type I and type III IFNs in PBMCs. (A) The expression of IFN- α mRNA subtypes was studied in PBMCs infected by RV-16 by Taqman PCR. mRNA expression of IFN- α subtypes detected by primer/probe IFN- α .1 were induced at 8 h ($n = 3$, $***P < 0.001$ compared to time point 0) and 24 h ($n = 3$, $***P < 0.001$ compared to time point 0). mRNA of IFN- α subtypes detected by primer/probe IFN- α .2 were significantly up-regulated by 24 h ($n = 3$, $***P < 0.001$ compared to time point 0). (B) The expression of IFN- λ 1 and IFN- β mRNAs was studied by Taqman PCR. Significant induction of IFN- λ 1 mRNA expression by RV-16 was observed at 8 h ($n = 3$, both $***P < 0.001$ compared with time point 0) and was elevated at 24 h ($n = 3$, both $***P < 0.01$ compared with time point 0). IFN- β mRNA expression was induced by RV-16 also only at 8 h ($n = 3$, both $***P < 0.001$ compared with time point 0) and 24 h ($n = 3$, $***P < 0.001$ compared with time point 0). (C) Release of IFN- α into supernatants at 24 h was measured by ELISA. Significant induction of IFN- α protein was detected in RV-16 infected PBMCs ($n = 3$, $***P < 0.001$ compared to medium control). (D) Release of IFN- β into supernatants at 24 h was measured by ELISA. Significant induction of IFN- β protein was detected in RV-16 infected PBMCs ($n = 3$, $***P < 0.001$ compared to medium control). (E) Release of IFN- λ s into supernatants at 24 h was measured by ELISA. Significant induction of IFN- λ protein was detected in RV-16 infected PBMCs ($n = 3$, $***P < 0.001$ compared to medium control).

Experiments on PEBCs were carried out on cells derived from three different individuals (Cambrex). Small numbers of donors is a possible explanation for the lack of statistically significant results at some time points.

As previously published (34) epithelial cell type and differentiation status are likely important for IFN protein production. Chen et al. demonstrated that well differentiated primary human tracheobronchial epithelial cells infected with RV16 produced detectable amounts of IFN-beta protein (34). However it was also previously demonstrated that well differentiated cells have low susceptibility to RV infection in comparison to poorly differentiated cells (35). It is likely that Chen et al. succeeded in inducing IFN-beta protein production by using very high infective doses of concentrated virus, as they report infecting the cell layer with 200 μ L of virus at a very high concentration of 5×10^8 pfu/ml. Interestingly our data on the lack of IFN-alpha

production is consistent with their findings, as they were also unable to detect IFN- α protein using this very high virus dose (34). These findings do not follow the classical type I IFN literature which reports that IFN- α 4 along with IFN- β are the first type I IFNs to be expressed and produced upon virus infection, and that these then enhance virus-mediated induction of themselves and all the other IFN- α s through autocrine/paracrine mechanisms, signalling via the IFN- $\alpha\beta$ receptor and induction of IRF7 (13, 14). Therefore our findings together with already published data highlight that there are important differences in type I interferon production in different cell types.

This literature has not investigated the role of type III IFNs, and experiments were not performed with RVs, or in BECs. Our observations are also not consistent with the reported importance of early induction of IFN- β (13, 14), as we consistently observed that RV induction of IFN- β in BECs was late, after both IFN- λ 1 and - λ 2/3 as

well as IFN- α subtypes detected by the IFN- α .2 qRT-PCR. These data indicate that the relative timings of the different IFNs and their mechanisms of induction may vary in different cell types and with different virus types. Our data indicate that in the context of RV infection of their natural lower respiratory tract host cells (BECs), induction of IFN- λ s is likely important in both early induction events, as well as sustained induction consequent upon autocrine/paracrine signalling, however, further studies on RV infection in BECs and the interplay between type I and type III IFNs will be required to dissect these relationships further.

In contrast, our data are consistent with the reported importance of early induction of IFN- α 4 (13, 14), as in RV infected BECs we observed early induction of mRNAs detected by the primer/probe combination IFN- α .2 that detects IFN- α subtypes 2, 4, 5, 8, 10, 14, 17 and 21. It is thus possible that the early IFN- α induction was indeed IFN- α 4. Further studies with PCRs specific for each IFN- α subtype will be required to determine if this is the case.

We have recently reported limited replication of RVs also occurs in monocyte-derived macrophages, resulting in induction of both IFN- α s and IFN- β (20), as well as induction of IFN- λ s (19). It is therefore likely that airway macrophages are also an important source of RV induction of type I and type III IFNs *in vivo*. We therefore studied RV induction of interferons in PBMCs, as monocytes are likely to be the major source of interferons in PBMCs. We observed that mRNAs of all the IFN types studied were induced early and to similar strong degrees (~ 6 logs) by RV infection of PBMCs. However, unlike in BECs, where IFN- β and - λ responses increased between 8 and 24 h, responses in PBMCs peaked at 8 h and were declining at 24 h. The most likely explanation for this data obtained in PBMCs is that RV replication in PBMCs is very limited, probably because of these robust early IFN responses (20). Interferon proteins were induced by RV in these PBMC cultures, but in contrast to our observations in BECs, in PBMCs, IFN- α was induced most robustly, followed by IFN- β , with IFN- λ s being least strongly induced.

We also investigated influenza virus type A induction of IFNs in primary BECs as this virus type is known to suppress type I IFN production via its NS1 protein (27–30) and influenza induction may therefore differ from RV. The pattern of IFN- λ responses observed with influenza infection of BECs was similar to that observed with RV. However induction of IFN- α was different, as there was no early induction - both IFN- β and subtypes of IFN- α detected by primer/probe IFN- α .2 were only induced late at 24 h. It is likely the lack of an early IFN- $\alpha\beta$ response is a consequence of the known action of

influenza NS1 in suppressing type I IFN production (27–30). Influenza virus suppresses type I IFN production by inhibition of the transcription factors NF- κ B (30, 36) and IRF3 (29) which are essential for activation of $\alpha\beta$ IFN promoters. Influenza virus is also thought to inhibit type I IFN production by inhibiting double-stranded-RNA (dsRNA)-activated protein kinase (PKR) (27). The early induction of IFN- λ s by influenza suggests these IFNs may be less susceptible to the same mechanisms of viral suppression of IFN induction. Further work will be needed to investigate these possibilities.

We conclude that both type I and type III IFNs are induced by RV and influenza infection of BECs, but IFN- λ s appear to be the principal IFNs involved in responses to respiratory viruses in BECs, while in PBMCs, all IFN types were strongly induced by RV, to similar degrees, though levels of IFN- α protein exceeded - β , which itself exceeded - λ .

Competing interests

Professor Johnston has served as a consultant to, and/or has received research grant support from AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, MedImmune, Merck, Pfizer, Sanofi-Aventis, Schering Plough, and Synairgen. He holds patents on the use of interferons in asthma and COPD. The other authors declare that they have no competing interests.

Authors' contributions

Musa Khaitov performed all the laboratory (except where stated performed by others) and statistical analyses reported in this manuscript and wrote the first draft of the manuscript, Michael Edwards designed the interferon- λ quantitative PCRs and assisted in the conduct of the rest of the studies, Ross Walton assisted with influenza virus propagation and infection experiments, Gernot Rhode, Marco Contoli, Luminia A. Stanciu all assisted in the conduct of the studies, Sergei Kotenko advised on the scientific aspects of the interferon- λ studies and provided reagents for the studies and Sebastian Johnston conceived, designed and supervised all the studies, wrote the final draft of the manuscript and acts as guarantor for the studies. All authors contributed to the writing of the manuscript and have approved the final version for publication.

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