Dynamics of IFN-β responses during respiratory viral infection: insights for therapeutic strategies

Alastair Watson1,3, C. Mirella Spalluto1,3, Christopher McCrae4,5, Doriana Cellura1, Hannah Burke1,2, Danen Cunoosamy6, Anna Freeman1,2, Alex Hicks1,2, Michael Hühn7, Kristoffer Ostridge1,2, Karl J. Staples1,3, Outi Vaarala8, Tom Wilkinson1,2,3.#.

1Clinical & Experimental Sciences, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, UK; 2Southampton NIHR Respiratory Biomedical Research Unit, Southampton General Hospital, Southampton, UK; 3Wessex Investigational Sciences Hub, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, UK; 4Respiratory, Inflammation and Autoimmunity, IMED Biotech Unit, AstraZeneca Gothenburg, Sweden; 5Krefting Research Centre, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden; 6Respiratory, Inflammation and Autoimmunity, IMED Biotech Unit, AstraZeneca Gothenburg, Sweden; 7Target & Translational Science, Respiratory, Inflammation and Autoimmunity IMED Biotech Unit, AstraZeneca, Gothenburg, Sweden; 8Respiratory, Inflammation and Autoimmunity, IMED Biotech Lung Immunity, AstraZeneca Gothenburg, Sweden

# Corresponding Author

Prof Tom Wilkinson, MA Cantab MBBS PhD FRCP,
Professor of Respiratory Medicine and Honorary NHS Consultant Physician, Clinical and Experimental Sciences, Southampton University Faculty of Medicine, Mailpoint 810, Level F, South Block, Southampton General Hospital, Southampton SO16 6YD United Kingdom
Running title:

IFN-β responses during respiratory viral infection

Total body word count: 4012
ABSTRACT

RATIONALE:
Viral infections are major drivers of exacerbations and clinical burden in patients with asthma and COPD. IFN-β is a key component of the innate immune response to viral infection. To date studies of inhaled IFN-β treatment have not demonstrated a significant effect on asthma exacerbations.

OBJECTIVES:
The dynamics of exogenous IFN-β activity were investigated to inform on future clinical indications for this potential anti-viral therapy.

METHODS:
Monocyte-derived macrophages (MDMs), alveolar macrophages (AMs) and primary bronchial epithelial cells (PBECs) were isolated from healthy controls and COPD patients and infected with influenza virus either prior to or after IFN-β stimulation. Infection levels were measured by % nucleoprotein 1 positive (NP1+) cells using flow cytometry. Viral RNA shedding and interferon stimulated gene expression were measured by qPCR. Production of inflammatory cytokines was measured using MSD.

MEASUREMENTS AND MAIN RESULTS:
Adding IFN-β to MDMs, AMs and PBECs prior to, but not after, infection reduced %NP1+ cells by 85%, 56% and 66%, respectively (p<0.05). Inhibition of infection lasted for 24h following removal of IFN-β and was maintained albeit reduced up to 1 week in MDMs and 72h in PBECs;
this was similar between health and COPD. IFN-β did not induce inflammatory cytokine production by MDMs or PBECs but reduced influenza-induced IL-1β production by PBECs.

CONCLUSIONS:

In vitro modelling of IFN-β dynamics highlights the potential for intermittent prophylactic doses of exogenous IFN-β to modulate viral infection. This provides important insights to aid the future design of clinical trials of IFN-β in asthma and COPD.

Abstract word count: 249

Key Words:

Innate Immunity, Respiratory Viruses, Exacerbation, Chronic Obstructive Pulmonary Disease.
INTRODUCTION

Acute respiratory infections lead to around 4 million deaths per year and are an enormous burden socioeconomically and to health systems globally (1). The World Health Organisation estimates influenza virus to be responsible for 290,000-650,000 mortalities per annum worldwide (2). Influenza infects both macrophages and the epithelium and is a major driver of clinical burden, particularly in patients with underlying respiratory disease such as asthma and chronic obstructive pulmonary disease (COPD) (2-6).

IFN-β is an integral part of lung innate immunity and is essential for controlling the infection and spread of viruses such as influenza by inducing the expression of an array of anti-viral interferon stimulated genes (ISGs) (7). These ISGs’ products have diverse functions including sensing viral single stranded RNA (e.g. RIG-I), preventing viral genome replication (e.g. MX1) and cleaving host and viral RNA (through the OAS1/RNase L system) (8).

The dynamics of induction and maintenance of this anti-viral response is complex and poorly understood. However, a defect in IFN-β expression in response to viral infection has been demonstrated in both the asthmatic epithelium and BAL macrophages of patients with COPD (9, 10). These observations have generated interest in the potential of exogenous IFN-β as a treatment to prevent virally-induced asthma and COPD exacerbations, a major cause of disease progression (11, 12). A previous study by Djukanovic et al showed no effect of inhaled IFN-β treatment on the primary endpoint of asthma exacerbation symptoms but did demonstrate a small but significant effect on morning peak flow (11). In contrast in a subgroup analysis of difficult-to-treat asthma, there were significant improvements in both symptoms and morning peak flow (11). The recent INEXAS phase 2 trial also reported some improvement in morning peak flow, but this was in a limited number of subjects as the trial was stopped.
early (13). Thus the clinical efficacy of inhaled IFN-β on asthma exacerbations still remains to be proven.

We therefore sought to inform future clinical trials of inhaled IFN-β by investigating the dynamics of exogenous IFN-β and its ability to modulate influenza infection using appropriate in vitro models. In this study, we first used monocyte derived macrophages (MDM) to model the utility of IFN-β as either a treatment or prophylactic to modulate influenza infection. We subsequently confirmed these data using alveolar macrophages (AMs) and primary bronchial epithelial cells (PBECs) from human lung, with the aim of generating novel insights for the optimal design of future clinical trials using IFN-β. Some of the results of these studies have been previously reported in the form of abstracts (14, 15)

METHODS

Isolation of primary cells

The collection of bronchoscopy samples and blood was approved by and performed in accordance with the ethical standards of the National Research Ethics Service South Central – Hampshire A and Oxford C Committees (LREC no: 13/SC/0416 & 15/SC/0528). Cell culture methodology is described in the online supplement. Volunteer demographics and clinical characteristics are summarised in Table E1; all volunteers gave written informed consent.

Infection with influenza

Cells were incubated for 2 h with 30,000 pfu/ml (MDMs), 360,000 pfu/ml (AMs) or 36,000 pfu/ml (PBECs) of influenza A/Wisconsin/67/2005 (H3N2) (Virapur, San Diego, USA: supplied
at TCID$_{50}$ of 3.8 x 10$^8$IU/ml (MOI of 0.03, 0.72 or 0.25, respectively). Cells were washed and incubated for 22 h, prior to harvesting and analysis by flow cytometry.

Stimulation with IFN-β

Cells were incubated with either PBS or 50 IU/ml of recombinant glycosylated human IFN-β solubilised in PBS (NIBSC, UK). IFN-β was administered to model treatment for 22 h (2 h after infection) or prophylaxis (for 2 h or 16 h prior to infection).

After modelling prophylaxis by incubating with IFN-β for 16 h, PBECs and MDMs were also incubated for up to a further 1 or 2 weeks after IFN-β removal, respectively. Cells were then infected or lysed in Qiazol to analyse gene expression. MDMs were chronically stimulated with IFN-β by administration of 50 IU/ml twice weekly without washing. MDMs were subsequently administered IFN-β 16 h prior to infection.

Flow cytometry

Infected cells were detected by flow cytometry using an anti-influenza A virus nucleoprotein 1 (FITC) antibody [431] (Abcam, Cambridge, UK: ab20921), as previously described (16).

RNA isolation and quantitative PCR

RNA isolation was performed using an RNeasy Micro kit (Qiagen), according to manufacturer’s instructions. qPCR was performed as previously described (16). Expression levels were detected using gene specific primers (Table E2) normalised to β-actin expression using the $2^{-\Delta\Delta Ct}$ method (17) (See online protocols).
Concentrations of GM-CSF, TSLP, IL-33, IL-25, TNF, IL-1β, IL-6, CCL17, CCL22 and RANTES in culture supernatants were measured by R-plex (RANTES) combined as a multiplex U-plex (all others) immunoassay (Mesoscale Discovery, Rockville, MD, USA), according to the manufacturer’s protocol. The lower limits of detection of the assays are provided in supplementary table E3.

**Statistical analysis**

Mann-Whitney U tests were used to analyse unpaired data. Wilcoxon signed rank tests and Friedman ANOVA with Dunn’s post hoc analysis were used to test significance of paired data (GraphPad Prism v6, GraphPad Software Inc., San Diego, USA). Results were considered significant if p<0.05.

**RESULTS**

**Influenza infects macrophages and PBECs similarly between health and COPD**

We first differentiated MDMs and infected them with Influenza A/Wisconsin/67/2005 virus (H3N2) (Figure 1A). MDMs were productively infected, as detected by a median of 24.0% of cells being positive for viral nucleoprotein 1 (NP1+) by flow cytometry (Figure 1B and C). This indicated infection with replicating virus, as no NP1+ cells were detected when exposed to UV-inactivated virus (data not shown). We detected no significant differences in %NP1+ cells between healthy (24.4%) and COPD subjects (22.0%) (p=0.79) (Figure 1C). Similarly, we detected no differences in infection in lung derived cells, including AMs (13.9% NP1+ cells for
healthy compared with 12.1% for COPD, p=0.92) and PBECs (22.1% NP1+ cells for healthy compared with 24.1% for COPD, p=0.66) (Figure 1D and E, respectively).

IFN-β prophylaxis but not treatment modulates influenza infection of MDMs

MDMs have previously been shown to produce up to 50 IU/ml of IFN-β upon influenza infection (4). To assess the impact of IFN-β on an already established infection, we added 50 IU/ml of exogenous IFN-β 2 h after infection with influenza and incubated for a further 22 h (Figure 2A). Treatment after infection was not effective at modulating influenza infection at 24 h, either as characterised by %NP1+ cells, detected by flow cytometry, or by viral RNA load in the culture supernatants, detected by qPCR (Figure 2B and C, respectively). This was not dependent on the concentration, as higher concentrations of exogenous IFN-β (up to 2,000 IU/ml) did not significantly modulate influenza infection (Figure E1). We next administered IFN-β to MDMs 2 h before infection (Figure 2A). This reduced the proportion of NP1+ cells by 34.3% (p<0.001) (Figure 2D). However, viral RNA release into the supernatant was not affected (Figure 2E).

To model prophylaxis, 50 IU/ml of exogenous IFN-β was added to MDMs 16 h prior to infection (Figure 2A). This significantly reduced the %NP1+ cells by 84.7% (p=0.001) (Figure 2F). Similarly, shedding of viral RNA into the culture supernatant was reduced 20-fold (p<0.001) (Figure 2G).

The dynamics of interferon and interferon stimulated gene (ISG) expression in MDMs

Upon infection with influenza (Figure 3A), MDM expression of steady state mRNA levels of IFNA1 and IFNB1 was rapidly detectable from 1 h, IFNL1 from 2 h and IFNL2/3 from 8 h; maximal expression was reached at 16 h after infection (Figure 3B). However, the upregulation of ISGs including MX1, OAS1 and DDX58 was detectable at 4 h and was significant at 16 h after infection (Figure 3C).
Exogenous IFN-β did not induce endogenous IFNA1, IFNB1, IFNL1 or IFNL2/3 mRNA expression at any concentration or timepoint assessed (Figure E2C and data not shown), but there was rapid detection of ISG expression 1 h after stimulation, which also peaked at 16 h (Figure 3E). Notably, IFN-β treatment after infection of cells with influenza did not further increase the expression of interferons or ISGs (Figure E2C and D).

Exogenous IFN-β modulates influenza infection in MDMs 1 week after administration

We next investigated the duration of the IFN-β response by incubating MDMs with IFN-β for 16 h, removing the IFN-β and subsequently culturing for up to a further 2 weeks prior to either infection or measurement of ISG expression (Figure 4A). When IFN-β was administered 16 h before infection, the %NP1+ cells was reduced by 76.9% (Figure 4B). This level of inhibition was maintained 48 h after IFN-β removal, with a 74.1% reduction of %NP1+ cells. At 1 week after IFN-β removal, a reduced but still statistically significant reduction of 37.1% was seen (p<0.01). However, this effect was lost 2 weeks after IFN-β removal. Notably, these dynamics were similar for MDMs derived from both healthy and COPD subjects.

The induction of ISG expression mirrored these dynamics and MX1, OAS1 and DDX58 remained elevated at 1 week after IFN-β removal but returned to baseline after 2 weeks of culture (Figure 4C and Figure E5A).

Exogenous IFN-β does not stimulate inflammatory cytokine production in MDMs

IFN-β did not significantly induce the release of acute phase inflammatory cytokines including GM-CSF, TNF-α, IL-6, MDC, TARC, IL-25 or RANTES in MDMs from healthy or COPD subjects, as detected by MSD; IL-1β, TSLP and IL-33 were below the detection limit (Figure E3). This was similar both immediately after administration of IFN-β and 24 h, 48 h, 72 h, 1 week and 2 week after IFN-β removal.
Chronically stimulated MDMs remain sensitive to exogenous IFN-β prophylaxis

To understand if repeated doses of IFN-β desensitise macrophages to IFN-β treatment, we chronically stimulated MDMs with IFN-β for up to 3 weeks, followed by a final administration of IFN-β 16 h before influenza infection (Figure 4D). IFN-β administration in MDMs after 3 weeks of chronic stimulation reduced %NP1+ cells by 84.1% (p<0.05), as compared to 70.5% for MDMs administered IFN-β without prior chronic stimulation (Figure 4E).

IFN-β prophylaxis modulates influenza infection in lung derived macrophages and epithelial cells

To validate our findings in lung derived cells, we next confirmed the efficacy of IFN-β in modulating influenza infection in both AMs and PBECs when administered prior to infection (Figure 5A). AMs and PBECs were likely productively infected with influenza RNA being released into the culture supernatant 24 h after infection (Figure 5D and 5G). Similarly to MDMs, treatment of AMs and PBECs after infection did not significantly modulate IAV infection (Figure 5B & 5E). However, administration of IFN-β 16 h prior to infection of AM reduced %NP1+ cells by 55.7% (p<0.005) and viral RNA release by 2.3 fold (p<0.05) (Figure 5C and D). Similarly, IFN-β reduced %NP1+ PBECs by 66.5% (p<0.0005) and shedding by 200 fold when administered 16 h prior to infection (p<0.05) (Figure 5F and G). Furthermore, 16 h IFN-β pretreatment significantly reduced Memphis 37 RSV infection of an immortalised bronchial epithelial cell as measured by RSV N gene expression (Figure E4).

Exogenous IFN-β modulates influenza infection in PBECs 72 h after administration

We further investigated the duration of the IFN-β response in PBECs by incubating with IFN-β for 16 h, removing the IFN-β and subsequently culturing for up to a further week prior to infection (Figure 6A). IFN-β maintained the capacity to significantly inhibit influenza infection of PBECs up to 72 h after its removal, with %NP1+ cells being reduced by 38.8% at 72 h (p<0.05)
However, this protective effect of IFN-β was lost after 1 week of IFN-β removal. These dynamics were similar between healthy and COPD subjects. Similarly to MDMs, treatment with exogenous IFN-β induced expression of ISGs including MX1, OAS1 and DDX58 (Figure 6C and Figure E5B). ISG expression remained induced 24 h after removal of IFN-β and was gradually lost over time with minimal induction of ISG genes after 1 week.

Exogenous IFN-β treatment does not stimulate inflammatory cytokine production by epithelial cells but reduces IL-1β during influenza infection

Similarly to MDMs, IFN-β did not induce the general production of acute phase inflammatory mediators from PBECs including GM-CSF, IL-1β, TNF-α, IL-6, and RANTES; MDC, TARC, IL-25, IL-33 and TSLP were below the detection limit. Notably, infection with influenza virus induced IL-1β release into the culture supernatant; this was significantly decreased by 39.0% when stimulated with IFN-β 16 h prior to infection (p=0.0222) and was maintained for a further 24 h (p=0.0462) (Figure 6D).

DISCUSSION

The recent INEXAS clinical trial evaluated the use of on-demand IFN-β to treat asthmatics with upper respiratory tract infection (URTI) symptoms but the trial did not meet its primary endpoint of a reduction in the rate of severe exacerbations due to the low number of severe exacerbations experienced by this cohort(13). However there was some evidence of IFN-β treatment improving morning peak flow despite no effect on asthma symptoms or reliever medication use. In this present study, we have modelled the dynamics of IFN-β therapy in vitro to try to provide insights into future clinical evaluation of inhaled IFN-β. We have
demonstrated that post-infection treatment with IFN-β was ineffective at modulating influenza virus in both macrophages and epithelial cells *in vitro*. However, IFN-β did effectively modulate influenza infection when administered prior to infection. Although reduced in magnitude, this modulation of infection lasted up to 72 h after IFN-β removal, without inducing the secretion of acute phase inflammatory mediators. Thus, we have highlighted that the timing of IFN-β administration seems to be crucial in modulating at least the first 24 h of respiratory viral infection, suggesting efficacy when used prophylactically.

As well as asthma, IFN-β also has potential for prevention of exacerbations in COPD. In the present study, we initially tested the infection levels of MDMs, AMs and PBECs and found them to be similar between healthy and COPD subjects, an observation supported by previously published work using resected lung tissue (16). COPD patients are more prone to suffer from lower respiratory tract symptoms, suggesting a defect in lung mucosal immune responses. However, *in vivo* this is more likely related to the complex interplay of immune cells, mediators and the microbiome, rather than the ability of the individual cells to be infected (9).

Unlike in asthma, a deficiency in IFN-β production has not been found in the epithelial cells of patients with COPD. In fact, in one study, expression of IFN-β was found to be increased at the RNA level in epithelial cells from COPD upon infection with HRV (18). However, a deficiency in the ability of BAL cells to produce IFN-β upon infection with HRV has been described in COPD patients (9). Therefore, we initially modelled the dynamics of IFN-β therapy in macrophages, with subsequent confirmation in PBECs. The delay of 4 h for MDMs to induce expression of ISGs including, *MX1*, *OAS1* and *DDX58* after influenza infection likely provides a window of opportunity for the virus to infect, replicate and take control of the cell. After infection, it may
be too late for ISGs to effectively modulate the course of infection, hence we found treatment of macrophages and epithelial cells with IFN-β after infection to be ineffective. However, the rapid induction of these ISGs with anti-viral functions from 1 h after stimulation with IFN-β, peaking at 16 h, suggests that prophylactic IFN-β therapy may close this window of opportunity for the virus. Indeed, we have demonstrated that stimulation of lung derived macrophages and epithelial cells with IFN-β before infection effectively modulates influenza infection, suggesting IFN-β could have potential in preventing virally induced exacerbations, if given prophylactically.

These results support previous *in vitro* work in PBECs and macrophages where models have demonstrated the efficacy of 24 h pre-administration of IFN-β prior to viral infection (10, 19). These models led to the recent clinical trials of treating asthmatic patients with URTI symptoms with IFN-β on-demand (11, 12). However, these *in vitro* models may not have accurately modelled the dynamics of on-demand IFN-β treatment as it is unlikely that patients would be able to identify a cold and be treated prior to an established viral infection taking hold. A human experimental model of rhinovirus infection in asthma has previously shown that both the upper and lower respiratory tract infection symptoms peaked simultaneously 4 days after inoculation (20). Furthermore, in this study, viral titres peaked 3 days after inoculation, prior to peak of symptoms, suggesting that a respiratory viral infection has likely become established prior to identification of a cold (20). This raises questions as to whether IFN-β treatment can be given quickly enough to effectively prevent the establishment or spread of a viral infection (21, 22). In the recent INEXAS trial, the highest asthma symptom scores and reliever medication use in the placebo group were observed prior to treatment at the time of randomization, with a steady decline throughout the treatment period (13). This coincided with the highest serum concentration of the IFN response biomarker, CXCL10, being
found immediately before the first dose. This suggests that an established infection was reached prior to delivery of on-demand IFN-β treatment, despite treatment being started as early as possible (within 48 h of cold symptoms).

IFN-β also has potential in the prevention of virally induced exacerbations in COPD. However, the COPD prodrome prior to exacerbation is uncertain but has previously been reported as a median of 4 days for “gradual onset exacerbations”, with “sudden exacerbations” occurring on the same day of initial symptom presentation (23). A further recent study found that 50% of all COPD exacerbations began in the first 3 days of a cold (12). This suggests that administration of IFN-β prior to the onset of an established viral infection in COPD may be challenging but if feasible could offer therapeutic opportunity (9). In this present study, we effectively model this treatment in vitro, highlighting that treatment of MDM, AM and PBECs with IFN-β after influenza infection is not effective but administration prior to infection is. In addition to the established literature (10), our experimental data with both RSV and influenza suggests a potentially generalizable effect of IFN-β prophylaxis across virally induced events and hence potential as an exacerbation therapy which requires investigation in clinical trials.

To give IFN-β prophylactically, it is first important to understand the duration of the IFN-β response. We demonstrated in vitro that IFN-β prophylaxis caused a statistically significant decrease in influenza infection in MDMs and PBECs 1 week and 72 h after its removal, respectively, although this effect was reduced compared to 24 h following infection. Thus, there may be potential for repeated intermittent prophylactic doses of IFN-β twice weekly throughout the winter months to modulate respiratory viral infections. Evidence from treatment of MS patients with IFN-β every other day suggests that repeated systemic administration of IFN-β does not desensitise patients to IFN-β treatment (24, 25). However, it
is unknown as to whether repeated administration of IFN-β could desensitise cells in the lung to IFN-β. Here we demonstrate that MDMs chronically stimulated with IFN-β for 3 weeks were not desensitised to IFN-β prophylaxis in vitro but actually influenza infection modulation was increased. The differences in dynamics between MDMs and PBECs could be due to the PBECs undergoing cell division whilst in culture; MDMs do not undergo cell division whilst in culture (26). This could account for the more rapid loss of ISG expression induced by IFN-β in PBECs. MDMs are, however, cultured from blood and have had no prior exposure to previous stimuli, unlike PBECs which originate from the lung. Unfortunately, due to both sample availability and viability, these dynamics could not be confirmed in AMs. Future work, looking at differentiated epithelial cells in air-liquid interface cultures which no longer undergo cell division could provide further insight into the duration of the IFN-β prophylactic effect more closely to the in vivo system. This could give help understand the required frequency of dosing to modulate viral infection through IFN-β prophylaxis.

We found that 50 IU/ml of IFN-β did not induce acute phase inflammatory mediators. Furthermore, we found that IFN-β prophylaxis reduced IL-1β production by PBECs after infection with influenza. This decrease of IL-1β is likely due to the modulation of influenza infection and could be important in preventing the proliferation and differentiation of inflammatory cells and immunopathology in vivo (27). In this present study we used a physiological concentration of IFN-β, comparable to the 100 IU/ml and 250 IU/ml used in previous in vitro studies (10, 19). The recent clinical trials have administered a dose of 6 MIU and found no evidence of increased sputum CXCL8 and CCL4 mRNA levels upon IFN-β treatment (11, 12).
We recognise that the present study has limitations, not least that our study consisted of patients with mild and moderate COPD due to the necessity for a bronchoscopy. Furthermore, in some of our in vitro analyses there is a small sample size with the associated limited statistical power. Both of these factors may have impacted on our ability to discern differences in infection between health and COPD. In addition, although we used clinically relevant strains of H3N2 influenza and RSV, these were only used at one concentration and it would therefore be important to confirm these results using other influenza strains as well as other viruses including rhinovirus and other strains of RSV, an important respiratory pathogen in both COPD and young children (28, 29). Rhinovirus is also an important driver of virally induced exacerbations in asthma and COPD (30, 31) and the efficacy of IFN-β prophylaxis in modulating rhinovirus infection has previously been modelled in vitro (10, 19). Although, the infection time course and ability to subvert the IFN-β system may be different between pathogens, due to the induction of a large number of ISGs by IFN-β with diverse anti-viral activities, it is likely that IFN-β may have broad spectrum anti-viral efficacy when administered prophylactically (8). Viruses have previously been shown to impact macrophage recruitment, phagocytosis and clearance of bacteria in the lung (32, 33). Thus, it is also now important to investigate the impact of IFN-β on macrophage function in the context of respiratory viruses and secondary bacterial infections (30, 31, 34).

Alongside administration of exogenous IFN-β, there may be other potential ways to augment the antiviral IFN responses. These include targeted downregulation of negative regulators such as SOCS1 and SOCS3 which have been shown to prevent IFN-β signalling (35). Targeting negative regulators in tandem with the administration of exogenous IFN-β or induction through TLR stimulation could be effective at modulating viral infection. Similarly, augmenting anti-viral responses through targeting other IFN pathways such as IFN-λ could also be effective.
at modulating viral infections (36-39). We postulate that the mechanism underpinning the anti-viral benefits of prophylaxis over treatment is driven by delayed ISG expression, however eliciting the exact mechanism for this is complex not least due significant redundancy in signalling in parallel ISG associated pathways (40-42). Additional experiments using high throughput methodologies would be required to investigate this more fully and may identify alternative targets for anti-viral therapeutics (43).

The *in vitro* experimental work was limited to a description of viral and immune response in cell culture models. Whilst this approach offers insights into infection dynamics at a cellular level it does not fully explain the dynamics of infection in the airway as a complex structure. Here regional variations in timing of infection may mean that IFN-β may have some clinical benefit as a treatment but the earlier this can be given after infection of the individual the more likely it is to protect uninfected cells in the airway.

In this present study, following the inconclusive results of the recent clinical trials in asthma, we model the treatment of virally infected cells with exogenous IFN-β with an aim of providing insights into the further development of inhaled IFN-β. We describe the dynamics of endogenous and exogenous IFN-β and highlight its lack of efficacy at modulating viral infection in already infected cells. We further show the effectiveness of IFN-β as a prophylactic and its potential for repeated intermittent dosing to at risk patients during viral seasons. This modelling work has generated novel insights into viral defence dynamics to inform the optimal design of future clinical trials of IFN-β and other related therapies, to prevent exacerbations in both asthma and COPD.
REFERENCES


FIGURE LEGENDS

Figure 1 Influenza infected macrophages and epithelial cells similarly between health and COPD. (A) A schematic demonstrating the infection of cells with influenza A virus (IAV-H3N2) for 2 h, removal of the virus and subsequent analysis by flow cytometry at 24 h. (B) The gating strategy for infected differentiated monocyte-derived macrophages (MDMs). Forward Scatter-Area (FSC-A) and -Width (FSC-W) were used to define MDM by size using flow cytometry. Infection was detected using an anti-IAV nucleoprotein (NP)-1 fluorescein isothiocyanate (FITC)-labelled antibody plotted against Side Scatter-Area (SSC-A). Non-infected (NI) cells in red are clearly distinguishable from infected cells (NP-1+) in blue. (C) Differentiated MDMs (D) alveolar macrophages (AMs) or (E) primary bronchial epithelial cells (PBECs) from healthy or COPD patients were infected or not with 30,000 plaque forming units (pfu)/ml (multiplicity of infection (MOI) of 0.03), 360,000 pfu/ml (MOI of 0.72) or 36,000 pfu/ml (MOI of 0.25) of IAV (H3N2), respectively (showing medians, n>5). Data were analysed using Mann-Whitney U tests.

Figure 2 Interferon (IFN)-β prophylaxis but not treatment modulates influenza infection of macrophages. (A) A schematic illustrating the experimental set up of differentiated monocyte-derived macrophages (MDMs) from healthy volunteers being administered IFN-β or phosphate buffered saline vehicle 2 h post-infection (n=12), 2 h before infection (n=15) or 16 h before infection (n=10) with outputs measured 24 h after infection. Differentiated MDMs were infected with 30,000 plaque forming units (pfu)/ml of influenza A virus (IAV - H3N2) (multiplicity of infection of 0.03). (B) and (C) Results for MDMs treated with IFN-β post-infection, (D) and (E) results for MDMs given IFN-β 2 h before infection and (F) and (G) results
for MDMs given IFN-β 16 h before infection. (B), (D) and (F) Flow cytometry results, detecting
cells infected with replicating virus using an anti-IAV nucleoprotein (NP)-1 antibody. (C), (E)
and (G) Viral shedding was quantified by influenza haemagglutinin RNA levels in the culture
supernatant using quantitative PCR (normalised to RNA at 0 h after removal of the virus) (n>5)
Data were analysed using Wilcoxon signed rank tests (**P<0.01, ***P<0.001).

Figure 3  The dynamics of interferon (IFN) and interferon stimulated gene (ISG) expression
in macrophages. Schematics showing the measurement of the endogenous expression of IFNs
and ISGs in monocyte-derived macrophages (MDMs) from healthy volunteers at different time
points (0-24h) following (A) influenza A virus (IAV) infection or (D) exogenous IFN-β treatment
with 50 IU/ml. (B) and (C) Expression was measured at the RNA level by quantitative PCR after
IAV infection (n=3) or (E) exogenous IFN treatment (n=5). RNA was analysed for expression of
(B) IFNA, IFNB, IFNL1 or IFNL2/3 or (C) and (E) the ISGs MX1, OAS1 or DDX58. (B) and (C) MDMs
were infected with 30,000 plaque forming units (pfu)/ml of IAV (H3N2) (multiplicity of
infection of 0.03). Data are expressed as means (± SD) and further analysed using a Friedman
ANOVA with a Dunn’s post hoc test compared to 0 h (*P<0.05, **P<0.01, ***P<0.001).

Figure 4 Exogenous interferon (IFN)-β modulates influenza infection in macrophages 1 week
after administration. Macrophages remain sensitive to IFN-β after 3 weeks of chronic
stimulation. (A) A schematic demonstrating the administration (or not) of 50 IU/ml of IFN-β
to differentiated monocyte-derived macrophages (MDMs) from healthy volunteers for 16 h.
MDMs were subsequently cultured for varying lengths of time (0 h, 24 h, 48 h, 72 h, 1 week
or 2 weeks) after IFN-β removal prior to infection or measurement of interferon-stimulated
gene (ISG) expression levels. (B) MDMs from (black, n=6) healthy or (red, n=5) chronic obstructive pulmonary disease (COPD) patients were infected or not with 30,000 plaque forming units (pfu)/ml of influenza A virus (IAV - H3N2) (multiplicity of infection of 0.03). (C) After IFN-β treatment and culture for up to 2 weeks after IFN-β removal, MDMs were lysed and the expression of ISGs (including MX1, OAS1 and DDX58) was assessed by quantitative PCR (n=7). Data are expressed as means (± SD) and further analysed using a Friedman ANOVA with a Dunn’s post hoc test compared to 16 h pretreatment (*P<0.05, **P<0.01, ***P<0.001).

(D) A schematic showing the chronic stimulation (or not) of MDMs with 50 IU/ml of IFN-β. New media and IFN-β were added twice weekly for the indicated culture period (0 weeks, 1 week, 2 weeks or 3 weeks). After chronic stimulation, there was a further 16 h period of IFN-β prophylaxis prior to the MDMs being infected as above (n=5). (B) and (E) Cells infected with replicating virus were detected using flow cytometry with an anti-IAV nucleoprotein (NP)-1 antibody. For all experiments, MDMs were treated in a staggered approach to ensure all cells were cultured for the same length of time. In (B) and (E), bars indicate median values and data were analysed using Wilcoxon signed rank tests (**P<0.01).

Figure 5  Prophylactic interferon (IFN)-β modulates influenza infection in lung derived alveolar macrophages (AMs) and primary bronchial epithelial cells (PBECs). (A) A schematic illustrating the experimental set up of AMs (from bronchoalveolar lavage fluid) or PBECs (from bronchial brushings) being administered IFN-β or phosphate buffered saline vehicle 2 h post-infection (B) and (E) or 16 h before infection (C), (D), (F) and (G). Outputs were measured 24 h after infection with 360,000 plaque forming units (pfu)/ml (multiplicity of infection (MOI) of 0.72) or 36,000 pfu/ml (MOI of 0.25) of influenza A virus (IAV - H3N2) for AMs or PBECs,
respectively. (B, n=17) and (C, n=8) AMs or (E, n=5) and (F, n=13) PBECs infected with replicating virus were detected using flow cytometry with an anti-IAV nucleoprotein (NP)-1 antibody. Viral shedding from (D, n=6) AMs or (G, n=5) PBECs was quantified by influenza haemagglutinin RNA levels in the culture supernatant at 24 h using quantitative PCR (normalised to RNA at 0 h after removal of the virus). All experiments show the median. Data were analysed using a Wilcoxon signed rank tests (*P<0.05, **P<0.01, ***P<0.001).

Figure 6 Exogenous interferon (IFN)-β modulates influenza infection in primary bronchial epithelial cells (PBECs) 24 h after administration and reduces expression of interleukin (IL)-1β. (A) A schematic demonstrating the administration (or not) of 50 IU/ml of IFN-β to PBECs for 16 h. PBECs were subsequently cultured for varying lengths of time (0 h, 24 h, 48 h, 72 h or 1 week) after IFN-β removal prior to measurement of interferon stimulated gene (ISG) expression levels, or infection and measurement of % viral nucleoprotein (NP)-1+ cells or IL-1β release 24 h later. PBECs were treated with IFN-β using a staggered approach to ensure that all cells were cultured for the same length of time. (B and D) PBECs were infected or not with 36,000 pfu/ml of influenza A virus (H3N2) (IAV - MOI of 0.25). (B) PBECs from (black, n=4) healthy or (red, n=5) COPD subjects infected with replicating virus were detected using flow cytometry with an anti-IAV NP-1 antibody (showing medians). (C) The expression of ISGs including MX1, OAS1 and DDX58 was assessed in non-infected PBECs using quantitative PCR (n=5, showing mean ± SD). (D) The expression of IL-1β was quantified using multiplex ELISA (n=6, showing medians ± IQR). Data were analysed using (B) Wilcoxon signed rank tests or (C) and (D) a Friedman ANOVA with Dunn’s post hoc analysis (*P<0.05, **P<0.01).
Figure 1
Figure 2

POST-TREATMENT

2h PROPHYLAXIS

16h PROPHYLAXIS
Figure 4
Figure 5
Figure 6
Dynamics of IFN-β responses during respiratory viral infection: insights for therapeutic strategies

Alastair Watson, C. Mirella Spalluto, Christopher McCrae, Doriana Cellura, Hannah Burke, Danen Cunoosamy, Anna Freeman, Alex Hicks, Michael Hühn, Kristoffer Ostridge, Karl J. Staples, Outi Vaarala, Tom Wilkinson.
SUPPLEMENTARY METHODS

Culturing macrophages and epithelial cells

MDMs were differentiated from human blood mononuclear cells, as described previously (1, 2). AMs and PBECs were isolated from subjects undergoing bronchoscopy. AMs were selected from BAL by adherence to plastic and cultured overnight in RPMI with 10% heat inactivated fetal calf serum, prior to infection the following day. PBECs were isolated from bronchial brushes and grown in culture using PneumaCult™-Ex Medium, (STEMCELL Technologies, Cambridge, UK) in flasks coated with collagen (PureCol, Nutacon, the Netherlands) until passage 2 or 3.

The immortalized human airway BC cell line termed BCi-NS1 was provided by Walters et al.(3). BCi-NS1 were similarly cultured using PneumaCult™-Ex Medium in flasks coated with collagen between passage 20-25.

RSV infection of BCi

BCi-NS1 cells were incubated for 2 h with RSV subtype A Memphis 37 (4) with 2.4 x 10^4 pfu/ml at a multiplicity of infection (MOI) of 0.1 (Amsbio, Abingdon, UK: supplied at 2.9 x 10^6 pfu/ml measured by plaque assay using Vero cells). Cells were washed and incubated for 72 h, prior to harvesting with Qiazol and analysis by qPCR. RNA isolation was performed using an RNeasy Micro kit (Qiagen), according to manufacturer’s instructions. qPCR was performed as previously described (5, 6). Expression levels were detected using RSV N gene specific primers (7) (Table E2) normalised to HPRT expression using the 2^{ΔΔCt} method (8).
Figure E1 Higher doses of interferon (IFN)-β do not modulate influenza infection of macrophages when administered post infection. Differentiated monocyte-derived macrophages (MDMs) from healthy volunteers were either not infected (NI) or infected with 30,000 plaque forming units/ml of influenza A virus (IAV - H3N2) (multiplicity of infection of 0.03). MDMs were treated with varying doses of IFN-β (0, 50, 200, 500, 1,000 or 2,000 IU/ml) for either (A) 2 h pre infection or (B) 2 h post-infection (n=5). Infection levels were characterised by the % viral nucleoprotein (NP)-1+ cells using flow cytometry using anti-IAV NP-1 antibody. Bars indicate medians and data are further analysed using a Friedman ANOVA with a Dunn’s post hoc test (*P<0.05, **P<0.01). Comparison of the effects of 50 IU IFN-β and 200 IU IFN-β administered to MDM or PBECs 2 h pre-infection (C) and (E) or 2 h post infection (D) and (F). Bars indicate medians and data are further analysed using a Wilcoxon signed rank test.

Figure E2 The expression of interferons (IFNs) and interferon-stimulated genes (ISGs) upon treatment with IFN-β and infection. Monocyte-derived macrophages (MDMs) from healthy volunteers were either (A) treated with 50 IU/ml IFN-β for up to 24 (n=5) or (B) with concentrations of IFN-β from 0.05 to 500 IU/ml for 24 h only (n=3) and ISG expression measured by qPCR. MDMs were infected with 30,000 plaque forming units/ml of live or ultraviolet light (UV) inactivated influenza A virus (IAV - H3N2) (multiplicity of infection of 0.03) and/or treated with 50 IU/ml of exogenous IFN-β. Quantitative PCR was then used to assess the RNA expression levels of (C) IFNA1, IFNB1 and IFNL1 (n=3) or (D) CXCL10, IFIT1, ISG15, MX1, OAS1, DDX58 and RSAD2 (n=4). Data are expressed as means (± SD). Data were further
analysed using a Friedman ANOVA with Dunn’s post hoc test. For figure clarity asterisks are
not shown but in (A) there was significant gene upregulation from the 8 h timepoint onwards.
For (B) significant gene regulation is observed from 50 IU/ml IFN-β upwards. In (C) and (D) the
IFN genes and ISGs are significantly upregulated by IAV infection but there is no significant
difference upon treatment with IFN-β.

Figure E3 Cytokine and chemokine release from macrophages is not altered by incubation
with interferon (IFN)-β. The pro-inflammatory cytokines (A) GM-CSF, (B) TNF-α, (C) IL-6, (D)
IL-25, (E) IL-1β, and the chemokines (F) MDC, (G) TARC and (H) RANTES release into the
supernatants of monocyte-derived macrophages (MDMs) from healthy volunteers were not
infected (NI) and chronically stimulated with 50 IU/ml of IFN-β for 24 h, 48 h, 72 h, 1 week or
2 weeks. New media and IFN-β were added twice weekly for the indicated culture period.
Bars indicate median values (n=8). Data are analysed using a Friedman ANOVA with Dunn’s
post hoc test (**P<0.01).

Figure E4 Interferon (IFN)-β pretreatment inhibits respiratory syncytial virus (RSV) infection
of immortalised bronchial epithelial cells (BCi-NS1). (A) A schematic illustrating BCi-NS1 cells
being administered IFN-β 16 h prior to the infection of cells with RSV for 2 h, removal of the
virus and subsequent analysis by quantitative PCR after 72 h. (B) BCi-NS1 cells were treated
with 50 IU/ml IFN-β for 16 h after which the cells were washed and the IFN-β removed. BCi-
NS1s were then infected with 2.4 x 10⁴ pfu/ml of M37 RSV (MOI of 0.1) for 72 h. Cells were
then lysed and RNA extracted and RSV nucleoprotein (N) gene expression was analysed using
quantitative PCR (n=5) and normalised to HPRT expression using the $2^{-\Delta\Delta Ct}$ method. Bar indicates median values. Data were analysed using Wilcoxon signed rank tests (*P<0.05)

**Figure E5** The duration of interferon-stimulated gene (ISG) induction of macrophages and primary bronchial epithelial cells (PBECs) after interferon (IFN)-β administration. (A) Differentiated monocyte-derived macrophages (MDMs) or (B) PBECs were administered 50 IU/ml of IFN-β for 16 h. IFN-β was subsequently removed and cells were cultured for up to a further 2 weeks or 1 week for MDMs or PBECs, respectively. The expression of various ISGs was subsequently analysed including CXCL10, IFIT1, ISG15, MX1, OAS1, DDX58, RSAD2, EIF2AK2 and CH25H. Data are expressed as means (± SD, n=5) and further analysed using a Friedman ANOVA with a Dunn’s post hoc test compared to 0 h IFN-β treatment. For figure clarity asterisks are not shown, but in both figures there are significant differences in ISG expression from 1 week following IFN-β removal.
**Supplementary Table E1** Clinical Characteristics of Included Subjects

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>COPD</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Number of patients</td>
<td>27</td>
<td>12</td>
<td>-</td>
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<tr>
<td>Age, yr</td>
<td>66.0 (61.0-70.0)</td>
<td>70.5 (65.0-75.25)</td>
<td>0.1851†</td>
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<tr>
<td>M/F</td>
<td>12/15</td>
<td>10/2</td>
<td>0.0365‡</td>
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<tr>
<td>BMI, kg/m²</td>
<td>27.2 (25.2-28.9)</td>
<td>27.6 (25.1-29.1)</td>
<td>0.9587†</td>
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<tr>
<td>Smoker, never/ex</td>
<td>14/13</td>
<td>0/12</td>
<td>-</td>
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<tr>
<td>Pack-years of smoking</td>
<td>0.0 (0.0-21.3)</td>
<td>22.5 (18.8-39.4)</td>
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<tr>
<td>FEV₁ (%)</td>
<td>102.0 (94.0-108.0)</td>
<td>78.5 (65.8-92.8)</td>
<td>0.0002†</td>
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<tr>
<td>FEV₁/FVC ratio</td>
<td>0.79 (0.76-0.82)</td>
<td>0.63 (0.52-0.65)</td>
<td>&lt;0.0001†</td>
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Definition of abbreviations: BMI = body mass index; COPD = chronic obstructive pulmonary disease. Healthy ex-smokers and COPD patients had stopped smoking for more than 6 months. Data are presented as median (interquartile range). †Mann-Whitney U test. ‡Fisher’s exact test.
**Supplementary Table E2** List of Taqman Gene expression assays used for analysis of gene expression by quantitative PCR (ThermoFisher)

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<td>CH25H</td>
<td>Cholesterol 25-hydroxylase</td>
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<td>DDX58</td>
<td>RIG-I</td>
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<td>EIF2AK2</td>
<td>PKR</td>
<td>Hs00169345_m1</td>
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<td>FluA/Wisconsin Haemaggutinin</td>
<td>AP47VVA</td>
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<td>HPRT</td>
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**Supplementary Table E3** MSD cytokine and chemokine multiplex assay lower limits of detection

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<tr>
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<tr>
<td>TSLP</td>
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</tr>
<tr>
<td>RANTES</td>
<td>0.64</td>
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</tbody>
</table>
References


Figure E1
Figure E2A-B
Figure E2C-D
Figure E3A-E
Figure E3F-H
Figure E4
Figure E5