ORIGINAL ARTICLE

Dynamics of IFN-β Responses during Respiratory Viral Infection
Insights for Therapeutic Strategies

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Abstract

Rationale: Viral infections are major drivers of exacerbations and clinical burden in patients with asthma and chronic obstructive pulmonary disease (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 antiviral therapy.

Methods: Monocyte-derived macrophages (MDMs), alveolar macrophages, and primary bronchial epithelial cells (PBECs) were isolated from healthy control subjects and patients with COPD and infected with influenza virus either prior to or after IFN-β stimulation. Infection levels were measured by the percentage of nucleoprotein 1–positive cells using flow cytometry. Viral RNA shedding and IFN-stimulated gene expression were measured by quantitative PCR. Production of inflammatory cytokines was measured using MSD.

Measurements and Main Results: Adding IFN-β to MDMs, alveolar macrophages, and PBECs prior to, but not after, infection reduced the percentage of nucleoprotein 1–positive cells by 85, 56, and 66%, respectively ($P < 0.05$). Inhibition of infection lasted for 24 hours after removal of IFN-β and was maintained albeit reduced up to 1 week in MDMs and 72 hours in PBECs; this was similar between healthy control subjects and patients with 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* modeling 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.

Keywords: innate immunity; respiratory viruses; exacerbation; chronic obstructive pulmonary disease

At a Glance Commentary

Scientific Knowledge on the Subject: IFN-β is an important facet of the innate immune response to respiratory viral infection and may have therapeutic potential if administered optimally.

What This Study Adds to the Field: This study describes the dynamics of IFN-β responses to viral infection using lung infection models and highlights the potential of prophylactic therapy.

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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 Organization estimates influenza virus to be responsible for 290,000–650,000 deaths per year 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 antiviral IFN-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 antiviral response are 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 Djukanović and colleagues 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 (A Study in Asthma Patients to Evaluate Efficacy, Safety and Tolerability of 14 Days Once Daily Inhaled Interferon Beta-1a after the Onset of Symptoms of an Upper Respiratory Tract Infection for the Prevention of Severe Exacerbations) 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 action and its ability to modulate influenza infection using appropriate *in vitro* models. In this study, we first used monocyte-derived macrophages (MDMs) 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 (Local Research Ethics Committee no: 13/SC/0416 and 15/SC/0528). Cell culture methodology is described in the online supplement. Volunteer demographics and clinical characteristics are summarized in Table E1 in the online supplement; all volunteers gave written informed consent.

Infection with Influenza

Cells were incubated for 2 hours with 30,000 plaque-forming units (pfu) per milliliter (MDMs), 360,000 pfu/ml (AMs), or 36,000 pfu/ml (PBECs) of influenza A/Wisconsin/67/2005 (H3N2) (supplied at tissue culture infective dose [$TCID_{50}$] of 3.8×10^8 international units (IU)/ml; Virapur) (multiplicity of infection of 0.03, 0.72 or 0.25, respectively). Cells were washed and incubated for 22 hoursbefore 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 solubilized in PBS (National Institute for Biological Standards and Control, United Kingdom). IFN-β was administered to model treatment for 22 hours (2 h after infection) or prophylaxis (for 2 or 16 h before infection).

After modeling prophylaxis by incubating with IFN-β for 16 hours, 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 analyze 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 hours before infection.

Flow Cytometry

Infected cells were detected by flow cytometry using an antiinfluenza A virus nucleoprotein 1 (fluorescein isothiocyanate) antibody [431] (ab20921; Abcam), as previously described (16).

RNA Isolation and Quantitative PCR

RNA isolation was performed using an RNeasy Micro kit (Qiagen), according to manufacturer's instructions. Quantitative PCR was performed as previously described (16). Expression levels were detected using gene-specific primers (Table E2) normalized to β-actin expression using the $2^{-\Delta\Delta Ct}$ method (17) (*see* online supplement).

Mesoscale Discovery Multiplex Cytokine Analysis

Concentrations of GM-CSF (granulocyte–macrophage colony–stimulating factor), TSLP (thymic stromal lymphopoietin), IL-33, IL-25, TNF (tumor necrosis factor), IL-1β, IL-6, CCL17 (thymus and activation-regulated chemokine), CCL22, and RANTES (regulated on activation, normal T cell expressed and secreted) in culture supernatants were measured by R-plex (RANTES) combined as a multiplex U-plex (all others) immunoassay (Mesoscale Discovery), according to the manufacturer's protocol. The lower limits of detection of the assays are provided in Table E3.

Statistical Analysis

Mann-Whitney *U* tests were used to analyze 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.). Results were considered significant if $P < 0.05$.

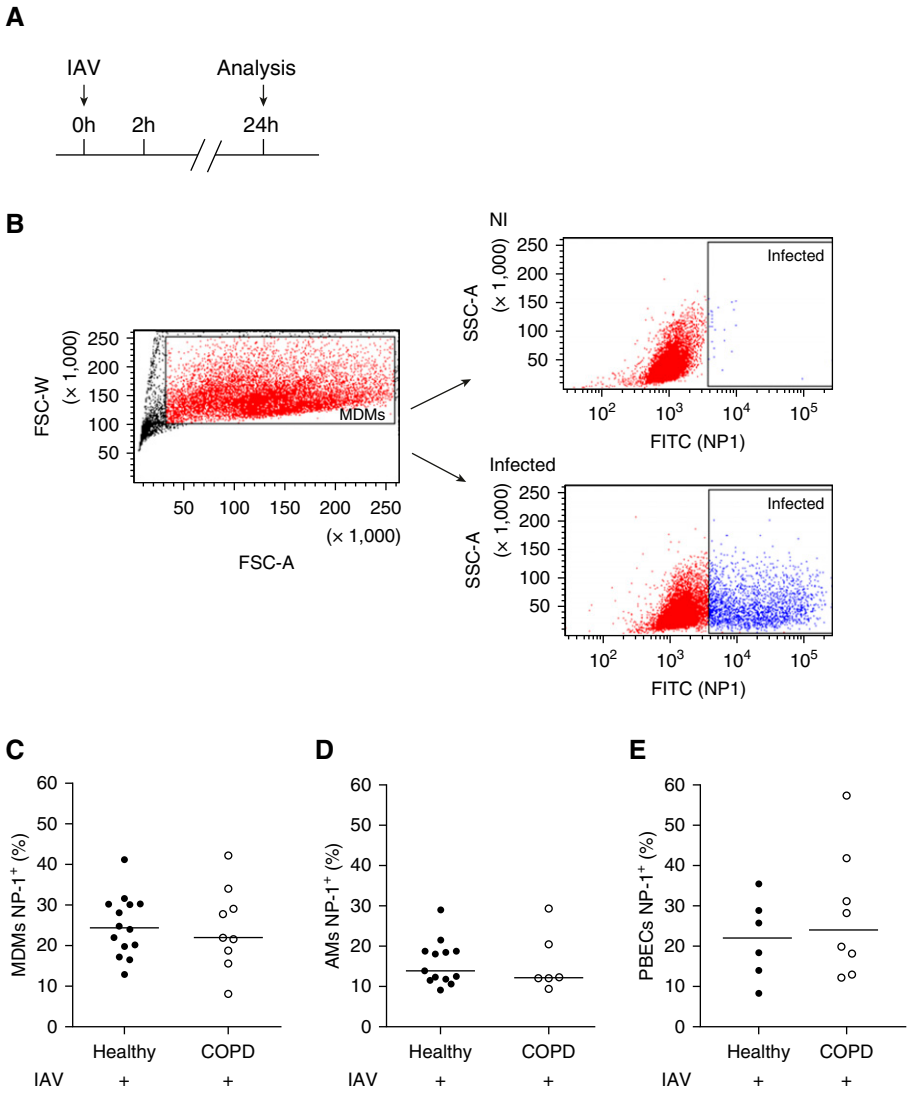

Figure 1. Influenza infected macrophages and epithelial cells similarly between healthy subjects and patients with COPD. (*A*) A schematic demonstrating the infection of cells with influenza A virus (IAV-H3N2) for 2 hours, removal of the virus, and subsequent analysis by flow cytometry at 24 hours. (*B*) The gating strategy for infected differentiated monocyte-derived macrophages (MDMs). Forward scatter-area (FSC-A) and -width (FSC-W) were used to define MDMs by size using flow cytometry. Infection was detected using an anti-IAV nucleoprotein (NP)-1 fluorescein isothiocyanate (FITC)-labeled antibody plotted against side scatter-area (SSC-A). Noninfected (NI) cells in red are clearly distinguishable from infected cells ($NP1^+$) in blue. (*C*) Differentiated MDMs, (*D*) alveolar macrophages (AMs), or (*E*) primary bronchial epithelial cells (PBECs) from healthy subjects or patients with COPD 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 analyzed using Mann-Whitney *U* tests. COPD = chronic obstructive pulmonary disease.

Results

Influenza Infects Macrophages and PBECs Similarly between Healthy Subjects and Patients with 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 (Figures 1B and 1C). 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 the percentage of $NP1^+$ cells between healthy (24.4%) subjects and subjects with COPD (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 subjects compared with 12.1% for subjects with COPD, $P = 0.92$) and PBECs (22.1% $NP1^+$ cells for healthy subjects compared with 24.1% for subjects with COPD, $P = 0.66$) (Figures 1D and 1E, 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 hours after infection with influenza and incubated for a further 22 hours (Figure 2A). Treatment after infection was not effective at modulating influenza infection at 24 hours, either as characterized by the percentage of $NP1^+$ cells, detected by flow cytometry, or by viral RNA load in the culture supernatants, detected by quantitative PCR (Figures 2B and 2C, 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 hours 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 hours before infection (Figure 2A). This significantly reduced the percentage of $NP1^+$ 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 IFN and ISG Expression in MDMs

Upon infection with influenza (Figure 3A), MDM expression of steady-state mRNA levels of *IFNA1*

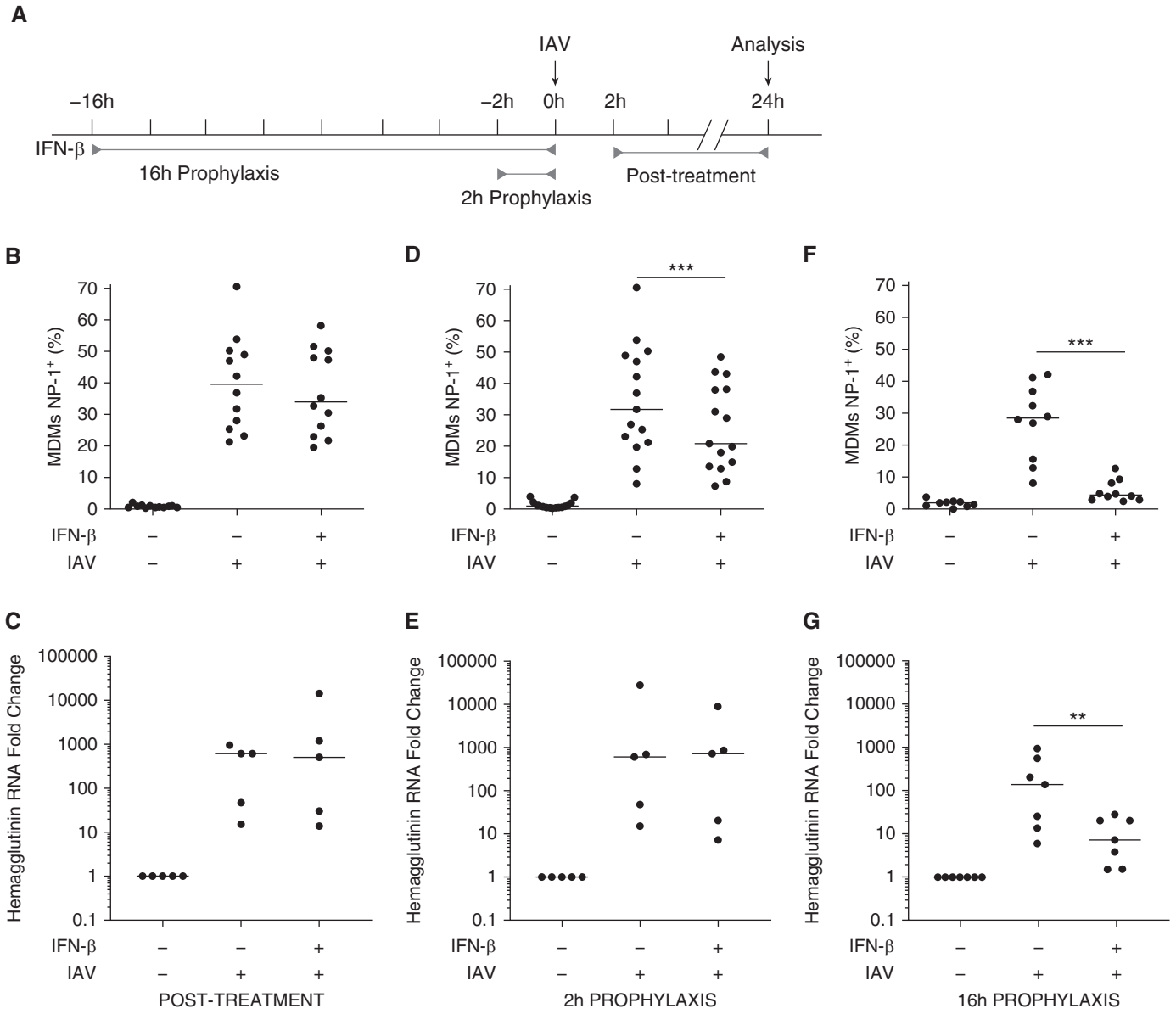

Figure 2. IFN-β prophylaxis but not treatment modulates influenza infection of macrophages. (*A*) A schematic illustrating the experimental setup of differentiated monocyte-derived macrophages (MDMs) from healthy volunteers being administered IFN-β or PBS vehicle 2 hours after infection ($n = 12$), 2 hours before infection ($n = 15$), or 16 hours before infection ($n = 10$), with outputs measured 24 hours after infection. Differentiated MDMs were infected with 30,000 plaque-forming units per milliliter of influenza A virus (IAV-H3N2) (multiplicity of infection of 0.03). (*B* and *C*) Results for MDMs treated with IFN-β after infection, (*D* and *E*) results for MDMs given IFN-β 2 hours before infection, and (*F* and *G*) results for MDMs given IFN-β 16 hours 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 hemagglutinin RNA levels in the culture supernatant using quantitative PCR (normalized to RNA at 0 hours after removal of the virus) ($n > 5$). Data were analyzed using Wilcoxon signed-rank tests (**$P < 0.01$ and ***$P < 0.001$).

and *IFNB1* was rapidly detectable from 1 hour, *IFNL1* from 2 hours, and *IFNL2/3* from 8 hours; maximal expression was reached at 16 hours after infection (Figure 3B). However, the up-regulation of ISGs including *MX1, OAS1*, and *DDX58* was detectable at 4 hours and was significant at 16 hours after infection (Figure 3C).

Exogenous IFN-β did not induce endogenous *IFNA1, IFNB1, IFNL1*, or *IFNL2/3* mRNA expression at any concentration or time point assessed (Figure E2C and data not shown), but there was rapid detection of ISG expression 1 hour after stimulation that also peaked at 16 hours (Figure 3E). Notably, IFN-β treatment after infection of cells with influenza did not further increase the expression of IFNs or ISGs (Figures E2C and E2D).

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

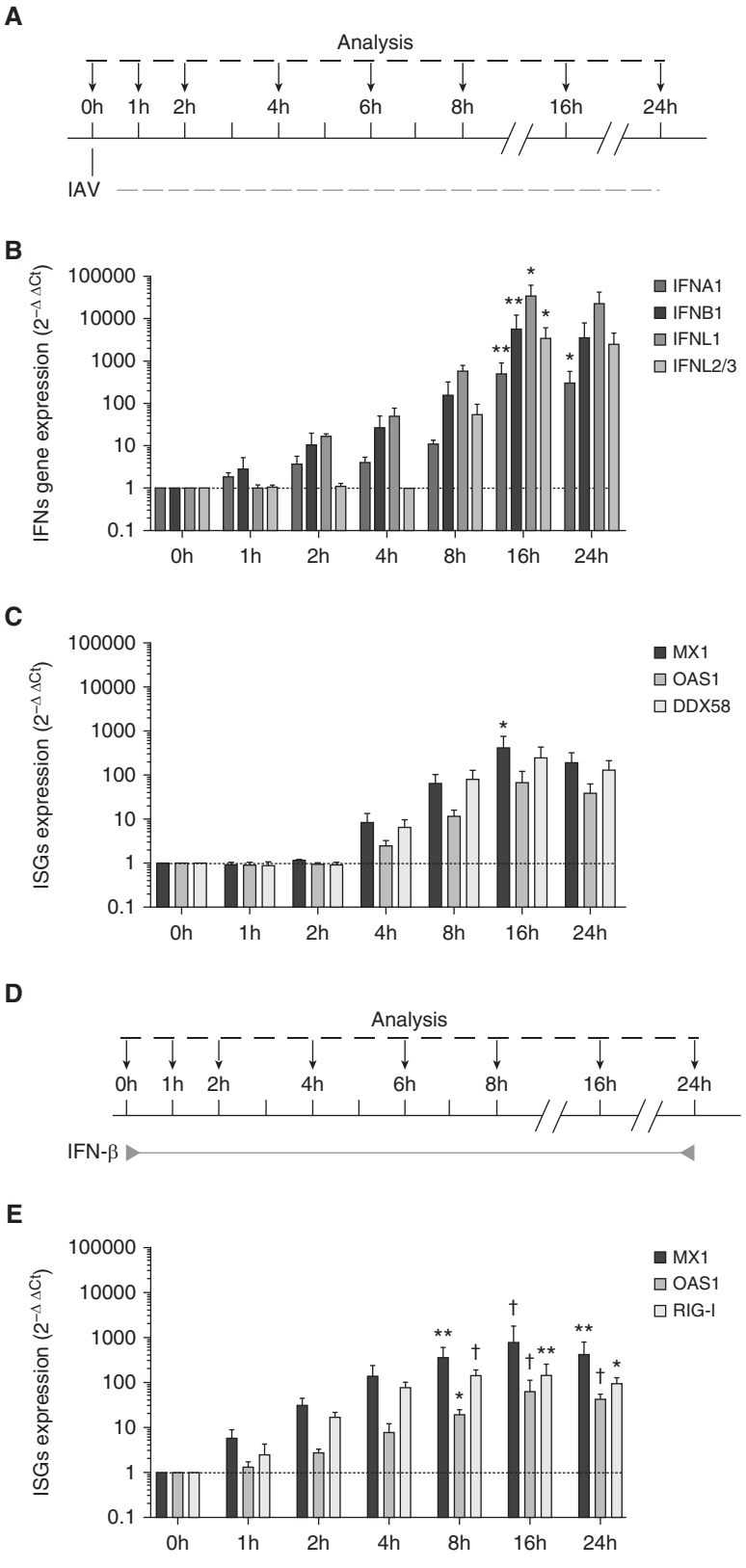

Figure 3. The dynamics of IFN and IFN-stimulated gene (ISG) expression in macrophages. (*A* and *D*) Schematics showing the measurement of the endogenous expression of IFNs and ISGs in monocyte-derived macrophages from healthy volunteers at different time points (0–24 h) after (*A*) influenza A

IFN-β for 16 hours, removing the IFN-β, and, subsequently, culturing for up to a further 2 weeks before either infection or measurement of ISG expression (Figure 4A). When IFN-β was administered 16 hours before infection, the percentage of $NP1^+$ cells was reduced by 76.9% (Figure 4B). This level of inhibition was maintained 48 hours after IFN-β removal, with a 74.1% reduction of the percentage 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 subjects and subjects with COPD.

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 (Figures 4C and 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, CCL22 (macrophage-derived chemokine), CCL17, IL-25, or RANTES in MDMs from healthy subjects or subjects with COPD, as detected by mesoscale discovery multiplex; 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 hours, 48 hours, 72 hours, 1 week, and 2 weeks after IFN-β removal.

Chronically Stimulated MDMs Remain Sensitive to Exogenous IFN-β Prophylaxis

To understand if repeated doses of IFN-β desensitize 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 hours before influenza infection (Figure 4D). IFN-β administration in MDMs after 3 weeks of chronic stimulation reduced the percentage of $NP1^+$ cells by 84.1% ($P < 0.05$), as compared with 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 before infection (Figure 5A). AMs and PBECs were likely productively infected, with influenza RNA being released into the culture supernatant 24 hours after infection (Figures 5D and 5G). Similarly to MDMs, treatment of AMs and PBECs after infection did not significantly modulate influenza A virus infection (Figures 5B and 5E). However, administration of IFN-β 16 hours prior to infection of AMs reduced the percentage of $NP1^+$ cells by 55.7% ($P < 0.005$) and viral RNA release by 2.3-fold ($P < 0.05$) (Figures 5C and 5D). Similarly, IFN-β reduced the percentage of $NP1^+$ PBECs by 66.5% ($P < 0.0005$) and shedding by 200-fold when administered 16 hours prior to infection ($P < 0.05$) (Figures 5F and 5G). Furthermore, pretreatment of an immortalized bronchial epithelial cell line with IFN-β for 16 hours significantly reduced the infection of these cells by Memphis 37 respiratory syncytial virus (RSV) as measured by RSV N gene expression (Figure E4).

Exogenous IFN-β Modulates Influenza Infection in PBECs 72 hours after Administration

We further investigated the duration of the IFN-β response in PBECs by incubating with IFN-β for 16 hours, removing the IFN-β and subsequently culturing for up to a further week before infection (Figure 6A). IFN-β maintained the capacity to significantly inhibit influenza infection of PBECs up to 72 hours after its removal, with the percentage of $NP1^+$ cells being reduced by 38.8% at 72 hours ($P < 0.05$) (Figure 6B). However, this protective effect of IFN-β was lost after 1 week of IFN-β removal. These dynamics were similar between healthy subjects and subjects with COPD.

Similarly to MDMs, treatment with exogenous IFN-β induced expression of ISGs including *MX1*, *OAS1*, and *DDX58* (Figures 6C and E5B). ISG expression remained induced 24 hours 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; CCL22, CCL17, 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 hours before infection ($P = 0.0222$) and was maintained for a further 24 hours ($P = 0.0462$) (Figure 6D).

Discussion

The recent INEXAS clinical trial evaluated the use of on-demand IFN-β to treat patients with asthma with upper-respiratory-tract infection 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 the present study, we have modeled 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 postinfection 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 before infection. Although reduced in magnitude, this modulation of infection lasted up to 72 hours 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 hours 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 subjects and subjects with COPD, an observation supported by previously published work using resected lung tissue (16). Patients with COPD 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 human rhinovirus (18). However, a deficiency in the ability of BAL cells to produce IFN-β upon infection with human rhinovirus has been described in patients with COPD (9). Therefore, we initially modeled the dynamics of IFN-β therapy in macrophages, with subsequent confirmation in PBECs. The delay of 4 hours 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

Figure 3. (Continued). virus (IAV) infection or (*D*) exogenous IFN-β treatment with 50 international units/ml. (*B*, *C*, and *E*) Expression was measured at the RNA level by quantitative PCR after IAV infection ($n = 3$; *B* and *C*) or exogenous IFN treatment ($n = 5$; *E*). RNA was analyzed 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 per milliliter of IAV (H3N2) (multiplicity of infection of 0.03). Data are expressed as means (±SD) and further analyzed using a Friedman ANOVA with a Dunn's *post hoc* test compared with 0 hour (*$P < 0.05$, **$P < 0.01$, and $^{\dagger}P < 0.001$). ΔΔCt = change in Cycle threshold (Ct; treated sample) – change in Ct (untreated sample).

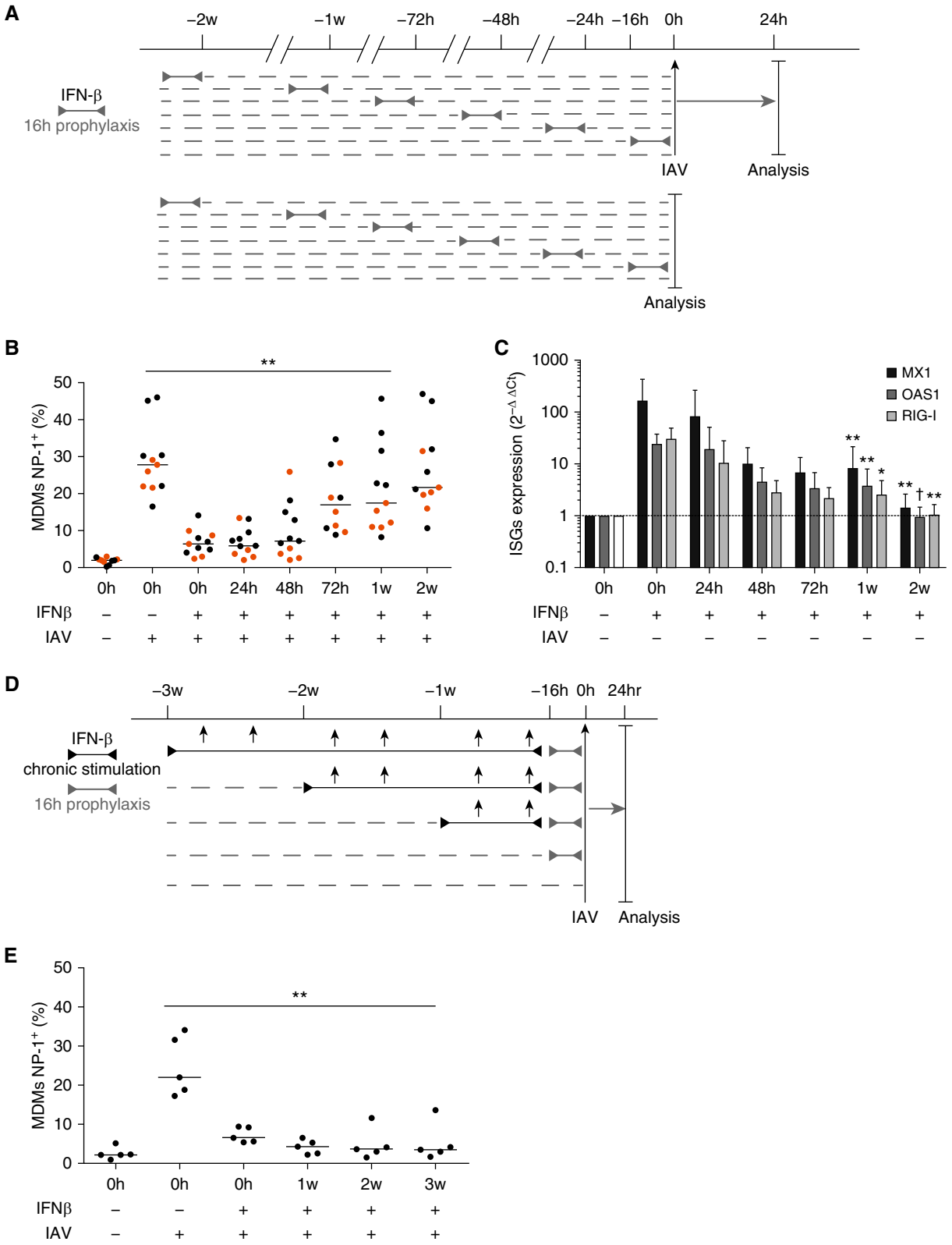

Figure 4. Exogenous IFN-β modulates influenza infection in macrophages 1 week after administration. Macrophages remain sensitive to IFN-β after 3 weeks of chronic stimulation. (*A*) A schematic demonstrating the administration (or not) of 50 IU/ml of IFN-β to differentiated monocyte-derived macrophages (MDMs) from healthy volunteers for 16 hours. MDMs were subsequently cultured for varying lengths of time (0 h, 24 h, 48 h, 72 h, 1 wk, or 2 wk) after IFN-β removal prior to infection or measurement of IFN-stimulated gene (ISG) expression levels. (*B*) MDMs from healthy volunteers (black, $n = 6$)

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 antiviral functions from 1 hour after stimulation with IFN-β, peaking at 16 hours, 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 hours preadministration of IFN-β before viral infection (10, 19). These models led to the recent clinical trials of treating patients with asthma with upper-respiratory-tract infection symptoms with IFN-β on-demand (11, 12). However, these *in vitro* models may not have accurately modeled the dynamics of on-demand IFN-β treatment, as it is unlikely that patients would be able to identify a cold and be treated before 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, before the peak of symptoms, suggesting that a respiratory viral infection has likely become established before 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 before 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 before 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 before 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 before the onset of an established viral infection in COPD may be challenging, but, if feasible, could offer therapeutic opportunity (9). In the present study, we effectively model this treatment *in vitro*, highlighting that treatment of MDMs, AMs, and PBECs with IFN-β after influenza infection is not effective, but administration before infection is. In addition to the established literature (10), our experimental data with both RSV and influenza suggest a potentially generalizable effect of IFN-β prophylaxis across virally induced events and hence potential as an exacerbation therapy that 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 hours after its removal, respectively, although this effect was reduced compared with 24 hours after 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 patients with multiple sclerosis with IFN-β every other day suggests that repeated systemic administration of IFN-β does not desensitize patients to IFN-β treatment (24, 25). However, it is unknown as to whether repeated administration of IFN-β could desensitize cells in the lung to IFN-β. Here we demonstrate that MDMs chronically stimulated with IFN-β for 3 weeks were not desensitized 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 while in culture; MDMs do not undergo cell division while 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 help in understanding 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

Figure 4. (Continued). or patients with chronic obstructive pulmonary disease (red, $n = 5$) were infected or not with 30,000 plaque-forming units per milliliter 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 analyzed using a Friedman ANOVA with a Dunn's *post hoc* test compared with 16 hours before treatment ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\dagger}P < 0.001$). (*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, 1, 2, or 3 wk). After chronic stimulation, there was a further 16-hour 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 analyzed using Wilcoxon signed-rank tests ($^{**}P < 0.01$). ΔΔCt = change in Cycle threshold (Ct; treated sample) – change in Ct (untreated sample); IU = international units.

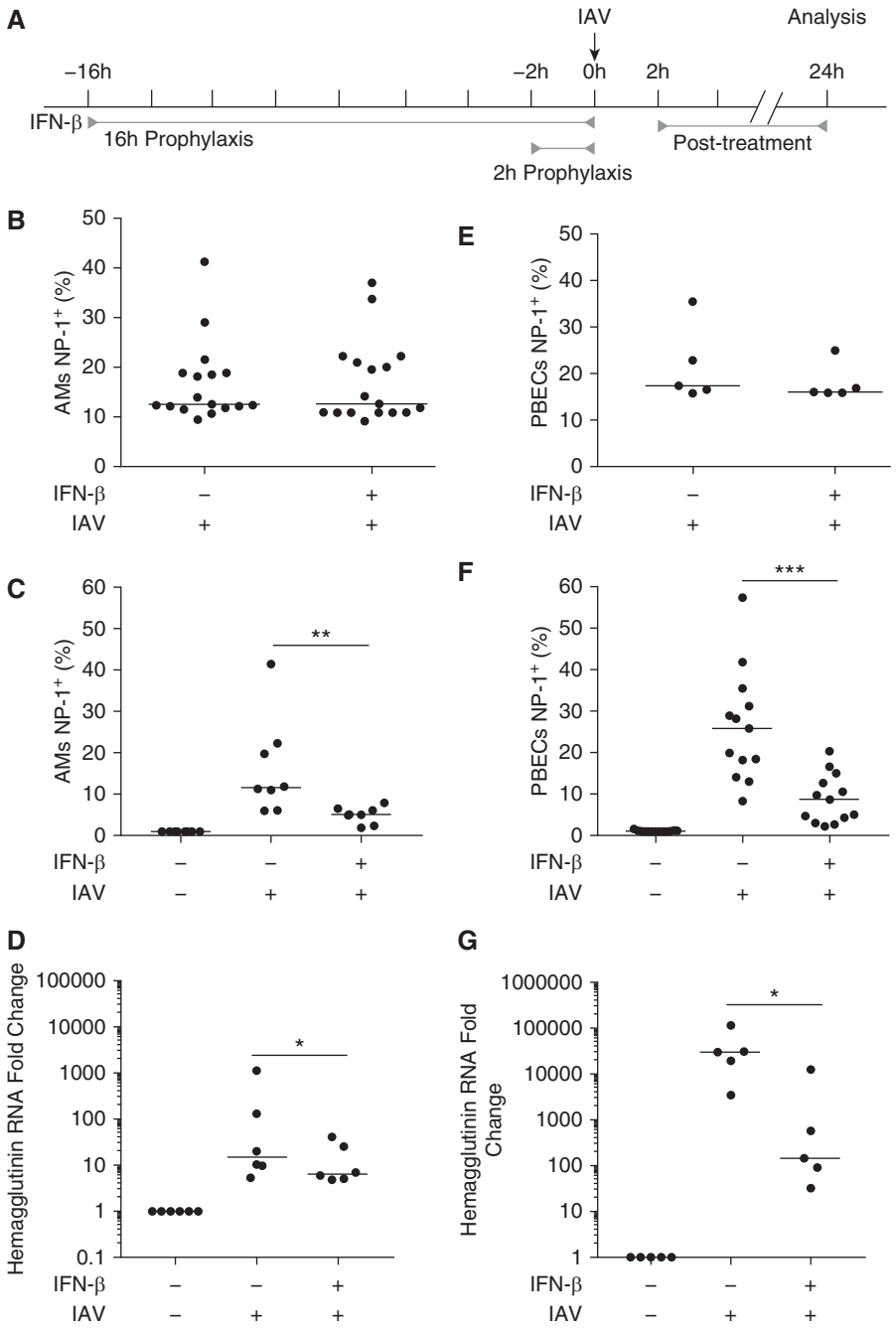

Figure 5. Prophylactic IFN-β modulates influenza infection in lung-derived alveolar macrophages (AMs) and primary bronchial epithelial cells (PBECs). (*A*) A schematic illustrating the experimental setup of AMs (from BAL fluid) or PBECs (from bronchial brushings) being administered IFN-β or phosphate-buffered saline vehicle 2 hours after infection (*B* and *E*) or 16 hours before infection (*C*, *D*, *F*, and *G*). Outputs were measured 24 hours after infection with 360,000 plaque-forming units (pfu) per milliliter (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 hemagglutinin RNA levels in the culture supernatant at 24 hours using quantitative PCR (normalized to RNA at 0 h after removal of the virus). All experiments show the median. Data were analyzed using Wilcoxon signed-rank tests (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

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 the 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 mega/million IU and found no evidence of increased sputum CXCL8 and CCL4 mRNA levels upon IFN-β treatment (11, 12).

We recognize 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 healthy subjects and patients with 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 modeled *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 antiviral activities, it is likely that IFN-β may have broad-spectrum antiviral 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

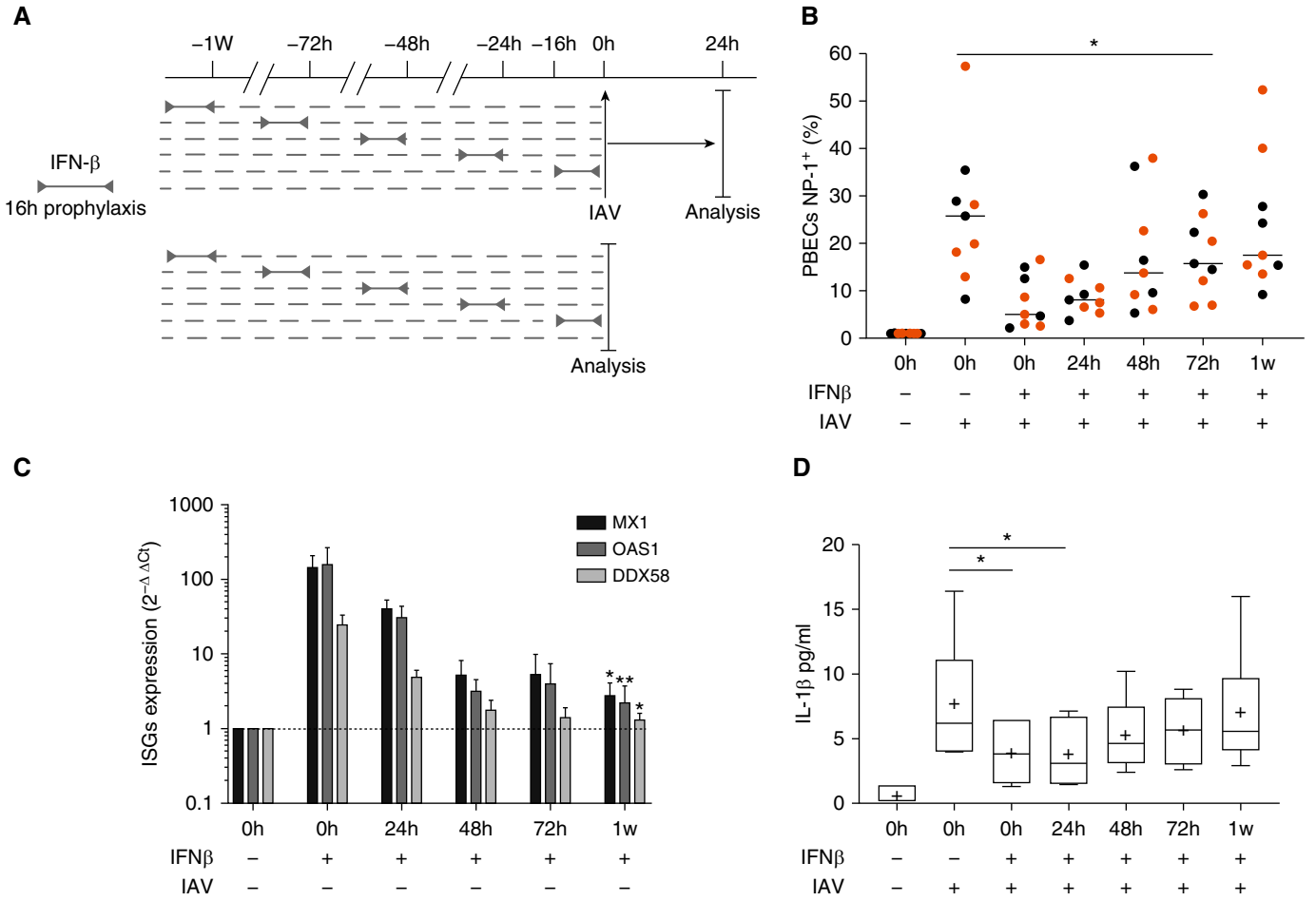

Figure 6. Exogenous IFN-β modulates influenza infection in primary bronchial epithelial cells (PBECs) 24 hours after administration and reduces expression of IL-1β. (*A*) A schematic demonstrating the administration (or not) of 50 IU/ml of IFN-β to PBECs for 16 hours. PBECs were subsequently cultured for varying lengths of time (0 h, 24 h, 48 h, 72 h, or 1 wk) after IFN-β removal prior to measurement of IFN-stimulated gene (ISG) expression levels, or infection and measurement of the percentage of viral nucleoprotein (NP)-1$^+$ cells or IL-1β release 24 hours 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 plaque-forming units per milliliter of influenza A virus (IAV-H3N2) (multiplicity of infection of 0.25). (*B*) PBECs infected with replicating virus from healthy volunteers (black, $n = 4$) or subjects with COPD (red, $n = 5$) 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 noninfected 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 analyzed using (*B*) Wilcoxon signed-rank tests or (*C* and *D*) a Friedman ANOVA with Dunn's *post hoc* analysis (*$P < 0.05$ and **$P < 0.01$). COPD = chronic obstructive pulmonary disease; ΔΔCt = change in Cycle threshold (Ct; treated sample) − change in Ct (untreated sample); IQR = interquartile range; IU = international units.

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 down-regulation of negative regulators such as SOCS1 and SOCS3 which have been shown to prevent IFN-β signaling (35). Targeting negative regulators in tandem with the administration of exogenous IFN-β or induction through TLR (Toll-like receptor) stimulation could be effective at modulating viral infection. Similarly, augmenting antiviral 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 antiviral benefits of prophylaxis over treatment is driven by delayed ISG expression; however, eliciting the exact mechanism for this is complex, not least due to significant redundancy in signaling 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 antiviral therapeutics (43).

The *in vitro* experimental work was limited to a description of viral and immune response in cell culture models. While 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 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 action 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 patients at risk during viral seasons. This modeling work has generated novel insights into viral defense 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. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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