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

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1 **ABSTRACT**

2 **RATIONALE:**

3 Viral infections are major drivers of exacerbations and clinical burden in patients with asthma

4 and COPD. IFN-β is a key component of the innate immune response to viral infection. To date

5 studies of inhaled IFN-β treatment have not demonstrated a significant effect on asthma

6 exacerbations.

7 **OBJECTIVES:**

8 The dynamics of exogenous IFN-β activity were investigated to inform on future clinical

9 indications for this potential anti-viral therapy.

10 **METHODS:**

11 Monocyte-derived macrophages (MDMs), alveolar macrophages (AMs) and primary bronchial

12 epithelial cells (PBECs) were isolated from healthy controls and COPD patients and infected

13 with influenza virus either prior to or after IFN-β stimulation. Infection levels were measured

14 by % nucleoprotein 1 positive (NP1+) cells using flow cytometry. Viral RNA shedding and

15 interferon stimulated gene expression were measured by qPCR. Production of inflammatory

16 cytokines was measured using MSD.

17 **MEASUREMENTS AND MAIN RESULTS:**

18 Adding IFN-β to MDMs, AMs and PBECs prior to, but not after, infection reduced %NP1$^+$ cells

19 by 85%, 56% and 66%, respectively ($p<0.05$). Inhibition of infection lasted for 24h following

20 removal of IFN-β and was maintained albeit reduced up to 1 week in MDMs and 72h in PBECs;

21   this was similar between health and COPD. IFN-β did not induce inflammatory cytokine

22   production by MDMs or PBECs but reduced influenza-induced IL-1β production by PBECs.

23   **CONCLUSIONS:**

24   *In vitro* modelling of IFN-β dynamics highlights the potential for intermittent prophylactic

25   doses of exogenous IFN-β to modulate viral infection. This provides important insights to aid

26   the future design of clinical trials of IFN-β in asthma and COPD.

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28   **Abstract word count:** 249

29

30   **Key Words:**

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

32

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

56 early (13). Thus the clinical efficacy of inhaled IFN-β on asthma exacerbations still remains to

57 be proven.

58 We therefore sought to inform future clinical trials of inhaled IFN-β by investigating the

59 dynamics of exogenous IFN-β and its ability to modulate influenza infection using appropriate

60 *in vitro* models. In this study, we first used monocyte derived macrophages (MDM) to model

61 the utility of IFN-β as either a treatment or prophylactic to modulate influenza infection. We

62 subsequently confirmed these data using alveolar macrophages (AMs) and primary bronchial

63 epithelial cells (PBECs) from human lung, with the aim of generating novel insights for the

64 optimal design of future clinical trials using IFN-β. Some of the results of these studies have

65 been previously reported in the form of abstracts (14, 15)

66

67 **METHODS**

68 Isolation of primary cells

69 The collection of bronchoscopy samples and blood was approved by and performed in

70 accordance with the ethical standards of the National Research Ethics Service South Central –

71 Hampshire A and Oxford C Committees (LREC no: 13/SC/0416 & 15/SC/0528). Cell culture

72 methodology is described in the online supplement. Volunteer demographics and clinical

73 characteristics are summarised in Table E1; all volunteers gave written informed consent.

74 Infection with influenza

75 Cells were incubated for 2 h with 30,000 pfu/ml (MDMs), 360,000 pfu/ml (AMs) or 36,000

76 pfu/ml (PBECs) of influenza A/Wisconsin/67/2005 (H3N2) (Virapur, San Diego, USA: supplied

77   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

78   incubated for 22 h, prior to harvesting and analysis by flow cytometry.

79   Stimulation with IFN-β

80   Cells were incubated with either PBS or 50 IU/ml of recombinant glycosylated human IFN-β

81   solubilised in PBS (NIBSC, UK). IFN-β was administered to model treatment for 22 h (2 h after

82   infection) or prophylaxis (for 2 h or 16 h prior to infection).

83   After modelling prophylaxis by incubating with IFN-β for 16 h, PBECs and MDMs were also

84   incubated for up to a further 1 or 2 weeks after IFN-β removal, respectively. Cells were then

85   infected or lysed in Qiazol to analyse gene expression. MDMs were chronically stimulated with

86   IFN-β by administration of 50 IU/ml twice weekly without washing. MDMs were subsequently

87   administered IFN-β 16 h prior to infection.

88   Flow cytometry

89   Infected cells were detected by flow cytometry using an anti-influenza A virus nucleoprotein

90   1 (FITC) antibody [431] (Abcam, Cambridge, UK: ab20921), as previously described (16).

91   RNA isolation and quantitative PCR

92   RNA isolation was performed using an RNeasy Micro kit (Qiagen), according to manufacturer's

93   instructions. qPCR was performed as previously described (16). Expression levels were

94   detected using gene specific primers (Table E2) normalised to β-actin expression using the 2^{-}

95     $^{\Delta\Delta Ct}$method (17) (See online protocols).

96   MSD

97   Concentrations of GM-CSF, TSLP, IL-33, IL-25, TNF, IL-1β, IL-6, CCL17, CCL22 and RANTES in

98   culture supernatants were measured by R-plex (RANTES) combined as a multiplex U-plex (all

99   others) immunoassay (Mesoscale Discovery, Rockville, MD, USA), according to the

100   manufacturer's protocol. The lower limits of detection of the assays are provided in

101   supplementary table E3

102   Statistical analysis

103   Mann-Whitney U tests were used to analyse unpaired data. Wilcoxon signed rank tests and

104   Friedman ANOVA with Dunn's post hoc analysis were used to test significance of paired data

105   (GraphPad Prism v6, GraphPad Software Inc., San Diego, USA). Results were considered

106   significant if $p < 0.05$.

107

108   **RESULTS**

109   Influenza infects macrophages and PBECs similarly between health and COPD

110   We first differentiated MDMs and infected them with Influenza A/Wisconsin/67/2005 virus

111   (H3N2) (Figure 1A). MDMs were productively infected, as detected by a median of 24.0% of

112   cells being positive for viral nucleoprotein 1 (NP1+) by flow cytometry (Figure 1B and C). This

113   indicated infection with replicating virus, as no NP1+ cells were detected when exposed to UV-

114   inactivated virus (data not shown). We detected no significant differences in %NP1+ cells

115   between healthy (24.4%) and COPD subjects (22.0%) (p=0.79) (Figure 1C). Similarly, we

116   detected no differences in infection in lung derived cells, including AMs (13.9% NP1+ cells for

6

117   healthy compared with 12.1% for COPD, p=0.92) and PBECs (22.1% NP1^{+} cells for healthy

118   compared with 24.1% for COPD, p=0.66) (Figure 1D and E, respectively).

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

120   MDMs have previously been shown to produce up to 50 IU/ml of IFN-β upon influenza

121   infection (4). To assess the impact of IFN-β on an already established infection, we added 50

122   IU/ml of exogenous IFN-β 2 h after infection with influenza and incubated for a further 22 h

123   (Figure 2A). Treatment after infection was not effective at modulating influenza infection at

124   24 h, either as characterised by %NP1^{+} cells, detected by flow cytometry, or by viral RNA load

125   in the culture supernatants, detected by qPCR (Figure 2B and C, respectively). This was not

126   dependent on the concentration, as higher concentrations of exogenous IFN-β (up to 2,000

127   IU/ml) did not significantly modulate influenza infection (Figure E1). We next administered

128   IFN-β to MDMs 2 h before infection (Figure 2A). This reduced the proportion of NP1^{+} cells by

129   34.3% (p<0.001) (Figure 2D). However, viral RNA release into the supernatant was not affected

130   (Figure 2E).

131   To model prophylaxis, 50 IU/ml of exogenous IFN-β was added to MDMs 16 h prior to infection

132   (Figure 2A). This significantly reduced the %NP1^{+} cells by 84.7% (p=0.001) (Figure 2F). Similarly,

133   shedding of viral RNA into the culture supernatant was reduced 20-fold (p<0.001) (Figure 2G).

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

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

136   *IFNA1* and *IFNB1* was rapidly detectable from 1 h, *IFNL1* from 2 h and *IFNL2/3* from 8 h;

137   maximal expression was reached at 16 h after infection (Figure 3B). However, the upregulation

138   of ISGs including *MX1, OAS1* and *DDX58* was detectable at 4 h and was significant at 16 h after

139   infection (Figure 3C).

140 Exogenous IFN-β did not induce endogenous *IFNA1, IFNB1, IFNL1* or *IFNL2/3* mRNA expression

141 at any concentration or timepoint assessed (Figure E2C and data not shown), but there was

142 rapid detection of ISG expression 1 h after stimulation, which also peaked at 16 h (Figure 3E).

143 Notably, IFN-β treatment after infection of cells with influenza did not further increase the

144 expression of interferons or ISGs (Figure E2C and D).

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

146 We next investigated the duration of the IFN-β response by incubating MDMs with IFN-β for

147 16 h, removing the IFN-β and subsequently culturing for up to a further 2 weeks prior to either

148 infection or measurement of ISG expression (Figure 4A). When IFN-β was administered 16 h

149 before infection, the %NP1$^+$ cells was reduced by 76.9% (Figure 4B). This level of inhibition

150 was maintained 48 h after IFN-β removal, with a 74.1% reduction of %NP1$^+$ cells. At 1 week

151 after IFN-β removal, a reduced but still statistically significant reduction of 37.1% was seen

152 (p<0.01). However, this effect was lost 2 weeks after IFN-β removal. Notably, these dynamics

153 were similar for MDMs derived from both healthy and COPD subjects.

154 The induction of ISG expression mirrored these dynamics and *MX1*, *OAS1* and *DDX58*

155 remained elevated at 1 week after IFN-β removal but returned to baseline after 2 weeks of

156 culture (Figure 4C and Figure E5A).

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

158 IFN-β did not significantly induce the release of acute phase inflammatory cytokines including

159 GM-CSF, TNF-α, IL-6, MDC, TARC, IL-25 or RANTES in MDMs from healthy or COPD subjects,

160 as detected by MSD; IL-1β, TSLP and IL-33 were below the detection limit (Figure E3). This was

161 similar both immediately after administration of IFN-β and 24 h, 48 h, 72 h, 1 week and 2 week

162 after IFN-β removal.

8

163   Chronically stimulated MDMs remain sensitive to exogenous IFN-β prophylaxis

164   To understand if repeated doses of IFN-β desensitises macrophages to IFN-β treatment, we

165   chronically stimulated MDMs with IFN-β for up to 3 weeks, followed by a final administration

166   of IFN-β 16 h before influenza infection (Figure 4D). IFN-β administration in MDMs after 3

167   weeks of chronic stimulation reduced %NP1$^+$ cells by 84.1% (p<0.05), as compared to 70.5%

168   for MDMs administered IFN-β without prior chronic stimulation (Figure 4E).

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

170   To validate our findings in lung derived cells, we next confirmed the efficacy of IFN-β in

171   modulating influenza infection in both AMs and PBECs when administered prior to infection

172   (Figure 5A). AMs and PBECs were likely productively infected with influenza RNA being

173   released into the culture supernatant 24 h after infection (Figure 5D and 5G). Similarly to

174   MDMs, treatment of AMs and PBECs after infection did not significantly modulate IAV

175   infection (Figure 5B & 5E). However, administration of IFN-β 16 h prior to infection of AM

176   reduced %NP1$^+$ cells by 55.7% (p<0.005) and viral RNA release by 2.3 fold (p<0.05) (Figure 5C

177   and D). Similarly, IFN-β reduced %NP1$^+$ PBECs by 66.5% (p<0.0005) and shedding by 200 fold

178   when administered 16 h prior to infection (p<0.05) (Figure 5F and G). Furthermore, 16 h IFN-

179   β pretreatment significantly reduced Memphis 37 RSV infection of an immortalised bronchial

180   epithelial cell as measured by RSV N gene expression (Figure E4).

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

182   We further investigated the duration of the IFN-β response in PBECs by incubating with IFN-β

183   for 16 h, removing the IFN-β and subsequently culturing for up to a further week prior to

184   infection (Figure 6A). IFN-β maintained the capacity to significantly inhibit influenza infection

185   of PBECs up to 72 h after its removal, with %NP1$^+$ cells being reduced by 38.8% at 72 h (p<0.05)

186   (Figure 6B). However, this protective effect of IFN-β was lost after 1 week of IFN-β removal.

187   These dynamics were similar between healthy and COPD subjects.

188   Similarly to MDMs, treatment with exogenous IFN-β induced expression of ISGs including

189   *MX1*, *OAS1* and *DDX58* (Figure 6C and Figure E5B). ISG expression remained induced 24 h after

190   removal of IFN-β and was gradually lost over time with minimal induction of ISG genes after 1

191   week.

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

194   Similarly to MDMs, IFN-β did not induce the general production of acute phase inflammatory

195   mediators from PBECs including GM-CSF, IL-1β, TNF-α, IL-6, and RANTES; MDC, TARC, IL-25,

196   IL-33 and TSLP were below the detection limit. Notably, infection with influenza virus induced

197   IL-1β release into the culture supernatant; this was significantly decreased by 39.0% when

198   stimulated with IFN-β 16 h prior to infection (p=0.0222) and was maintained for a further 24

199   h (p=0.0462) (Figure 6D).

200

201   **DISCUSSION**

202   The recent INEXAS clinical trial evaluated the use of on-demand IFN-β to treat asthmatics with

203   upper respiratory tract infection (URTI) symptoms but the trial did not meet its primary

204   endpoint of a reduction in the rate of severe exacerbations due to the low number of severe

205   exacerbations experienced by this cohort(13). However there was some evidence of IFN-β

206   treatment improving morning peak flow despite no effect on asthma symptoms or reliever

207   medication use. In this present study, we have modelled the dynamics of IFN-β therapy *in vitro*

208   to try to provide insights into future clinical evaluation of inhaled IFN-β. We have

209 demonstrated that post-infection treatment with IFN-β was ineffective at modulating

210 influenza virus in both macrophages and epithelial cells *in vitro*. However, IFN-β did effectively

211 modulate influenza infection when administered prior to infection. Although reduced in

212 magnitude, this modulation of infection lasted up to 72 h after IFN-β removal, without

213 inducing the secretion of acute phase inflammatory mediators. Thus, we have highlighted that

214 the timing of IFN-β administration seems to be crucial in modulating at least the first 24 h of

215 respiratory viral infection, suggesting efficacy when used prophylactically.

216 As well as asthma, IFN-β also has potential for prevention of exacerbations in COPD. In the

217 present study, we initially tested the infection levels of MDMs, AMs and PBECs and found

218 them to be similar between healthy and COPD subjects, an observation supported by

219 previously published work using resected lung tissue (16). COPD patients are more prone to

220 suffer from lower respiratory tract symptoms, suggesting a defect in lung mucosal immune

221 responses. However, *in vivo* this is more likely related to the complex interplay of immune

222 cells, mediators and the microbiome, rather than the ability of the individual cells to be

223 infected (9).

224 Unlike in asthma, a deficiency in IFN-β production has not been found in the epithelial cells of

225 patients with COPD. In fact, in one study, expression of IFN-β was found to be increased at the

226 RNA level in epithelial cells from COPD upon infection with HRV (18). However, a deficiency in

227 the ability of BAL cells to produce IFN-β upon infection with HRV has been described in COPD

228 patients (9). Therefore, we initially modelled the dynamics of IFN-β therapy in macrophages,

229 with subsequent confirmation in PBECs. The delay of 4 h for MDMs to induce expression of

230 ISGs including, *MX1*, *OAS1* and *DDX58* after influenza infection likely provides a window of

231 opportunity for the virus to infect, replicate and take control of the cell. After infection, it may

232   be too late for ISGs to effectively modulate the course of infection, hence we found treatment

233   of macrophages and epithelial cells with IFN-β after infection to be ineffective. However, the

234   rapid induction of these ISGs with anti-viral functions from 1 h after stimulation with IFN-β,

235   peaking at 16 h, suggests that prophylactic IFN-β therapy may close this window of

236   opportunity for the virus. Indeed, we have demonstrated that stimulation of lung derived

237   macrophages and epithelial cells with IFN-β before infection effectively modulates influenza

238   infection, suggesting IFN-β could have potential in preventing virally induced exacerbations, if

239   given prophylactically.

240   These results support previous *in vitro* work in PBECs and macrophages where models have

241   demonstrated the efficacy of 24 h pre-administration of IFN-β prior to viral infection (10, 19).

242   These models led to the recent clinical trials of treating asthmatic patients with URTI

243   symptoms with IFN-β on-demand (11, 12). However, these *in vitro* models may not have

244   accurately modelled the dynamics of on-demand IFN-β treatment as it is unlikely that patients

245   would be able to identify a cold and be treated prior to an established viral infection taking

246   hold. A human experimental model of rhinovirus infection in asthma has previously shown

247   that both the upper and lower respiratory tract infection symptoms peaked simultaneously 4

248   days after inoculation (20). Furthermore, in this study, viral titres peaked 3 days after

249   inoculation, prior to peak of symptoms, suggesting that a respiratory viral infection has likely

250   become established prior to identification of a cold (20). This raises questions as to whether

251   IFN-β treatment can be given quickly enough to effectively prevent the establishment or

252   spread of a viral infection (21, 22). In the recent INEXAS trial, the highest asthma symptom

253   scores and reliever medication use in the placebo group were observed prior to treatment at

254   the time of randomization, with a steady decline throughout the treatment period (13). This

255   coincided with the highest serum concentration of the IFN response biomarker, CXCL10, being

256 found immediately before the first dose. This suggests that an established infection was

257 reached prior to delivery of on-demand IFN-β treatment, despite treatment being started as

258 early as possible (within 48 h of cold symptoms).

259 IFN-β also has potential in the prevention of virally induced exacerbations in COPD. However,

260 the COPD prodrome prior to exacerbation is uncertain but has previously been reported as a

261 median of 4 days for "gradual onset exacerbations", with "sudden exacerbations" occurring

262 on the same day of initial symptom presentation (23). A further recent study found that 50%

263 of all COPD exacerbations began in the first 3 days of a cold (12). This suggests that

264 administration of IFN-β prior to the onset of an established viral infection in COPD may be

265 challenging but if feasible could offer therapeutic opportunity (9). In this present study, we

266 effectively model this treatment *in vitro*, highlighting that treatment of MDM, AM and PBECs

267 with IFN-β after influenza infection is not effective but administration prior to infection is. In

268 addition to the established literature (10), our experimental data with both RSV and influenza

269 suggests a potentially generalizable effect of IFN-β prophylaxis across virally induced events

270 and hence potential as an exacerbation therapy which requires investigation in clinical trials.

271 To give IFN-β prophylactically, it is first important to understand the duration of the IFN-β

272 response. We demonstrated *in vitro* that IFN-β prophylaxis caused a statistically significant

273 decrease in influenza infection in MDMs and PBECs 1 week and 72 h after its removal,

274 respectively, although this effect was reduced compared to 24 h following infection. Thus,

275 there may be potential for repeated intermittent prophylactic doses of IFN-β twice weekly

276 throughout the winter months to modulate respiratory viral infections. Evidence from

277 treatment of MS patients with IFN-β every other day suggests that repeated systemic

278 administration of IFN-β does not desensitise patients to IFN-β treatment (24, 25). However, it

279   is unknown as to whether repeated administration of IFN-β could desensitise cells in the lung

280   to IFN-β. Here we demonstrate that MDMs chronically stimulated with IFN-β for 3 weeks were

281   not desensitised to IFN-β prophylaxis *in vitro* but actually influenza infection modulation was

282   increased. The differences in dynamics between MDMs and PBECs could be due to the PBECs

283   undergoing cell division whilst in culture; MDMs do not undergo cell division whilst in culture

284   (26). This could account for the more rapid loss of ISG expression induced by IFN-β in PBECs.

285   MDMs are, however, cultured from blood and have had no prior exposure to previous stimuli,

286   unlike PBECs which originate from the lung. Unfortunately, due to both sample availability and

287   viability, these dynamics could not be confirmed in AMs. Future work, looking at differentiated

288   epithelial cells in air-liquid interface cultures which no longer undergo cell division could

289   provide further insight into the duration of the IFN-β prophylactic effect more closely to the

290   *in vivo* system. This could give help understand the required frequency of dosing to modulate

291   viral infection through IFN-β prophylaxis.

292   We found that 50 IU/ml of IFN-β did not induce acute phase inflammatory mediators.

293   Furthermore, we found that IFN-β prophylaxis reduced IL-1β production by PBECs after

294   infection with influenza. This decrease of IL-1β is likely due to the modulation of influenza

295   infection and could be important in preventing the proliferation and differentiation of

296   inflammatory cells and immunopathology *in vivo* (27). In this present study we used a

297   physiological concentration of IFN-β, comparable to the 100 IU/ml and 250 IU/ml used in

298   previous *in vitro* studies (10, 19). The recent clinical trials have administered a dose of 6 MIU

299   and found no evidence of increased sputum CXCL8 and CCL4 mRNA levels upon IFN-β

300   treatment (11, 12).

301 We recognise that the present study has limitations, not least that our study consisted of

302 patients with mild and moderate COPD due to the necessity for a bronchoscopy. Furthermore

303 in some of our *in vitro* analyses there is a small sample size with the associated limited

304 statistical power. Both of these factors may have impacted on our ability to discern differences

305 in infection between health and COPD. In addition, although we used clinically relevant strains

306 of H3N2 influenza and RSV, these were only used at one concentration and it would therefore

307 be important to confirm these results using other influenza strains as well as other viruses

308 including rhinovirus and other strains of RSV, an important respiratory pathogen in both COPD

309 and young children (28, 29). Rhinovirus is also an important driver of virally induced

310 exacerbations in asthma and COPD (30, 31) and the efficacy of IFN-β prophylaxis in modulating

311 rhinovirus infection has previously been modelled *in vitro* (10, 19). Although, the infection

312 time course and ability to subvert the IFN-β system may be different between pathogens, due

313 to the induction of a large number of ISGs by IFN-β with diverse anti-viral activities, it is likely

314 that IFN-β may have broad spectrum anti-viral efficacy when administered prophylactically

315 (8). Viruses have previously been shown to impact macrophage recruitment, phagocytosis and

316 clearance of bacteria in the lung (32, 33). Thus, it is also now important to investigate the

317 impact of IFN-β on macrophage function in the context of respiratory viruses and secondary

318 bacterial infections (30, 31, 34).

319 Alongside administration of exogenous IFN-β, there may be other potential ways to augment

320 the antiviral IFN responses. These include targeted downregulation of negative regulators

321 such as SOCS1 and SOCS3 which have been shown to prevent IFN-β signalling (35). Targeting

322 negative regulators in tandem with the administration of exogenous IFN-β or induction

323 through TLR stimulation could be effective at modulating viral infection. Similarly, augmenting

324 anti-viral responses through targeting other IFN pathways such as IFN-λ could also be effective

325　at modulating viral infections (36-39). We postulate that the mechanism underpinning the

326　anti-viral benefits of prophylaxis over treatment is driven by delayed ISG expression, however

327　eliciting the exact mechanism for this is complex not least due significant redundancy in

328　signalling in parallel ISG associated pathways (40-42). Additional experiments using high

329　throughput methodologies would be required to investigate this more fully and may identify

330　alternative targets for anti-viral therapeutics (43).

331　The *in vitro* experimental work was limited to a description of viral and immune response in

332　cell culture models. Whilst this approach offers insights into infection dynamics at a cellular

333　level it does not fully explain the dynamics of infection in the airway as a complex structure.

334　Here regional variations in timing of infection may mean that IFN- β may have some clinical

335　benefit as a treatment but the earlier this can be given after infection of the individual the

336　more likely it is to protect uninfected cells in the airway.

337　In this present study, following the inconclusive results of the recent clinical trials in asthma,

338　we model the treatment of virally infected cells with exogenous IFN-β with an aim of providing

339　insights into the further development of inhaled IFN-β. We describe the dynamics of

340　endogenous and exogenous IFN-β and highlight its lack of efficacy at modulating viral infection

341　in already infected cells. We further show the effectiveness of IFN-β as a prophylactic and its

342　potential for repeated intermittent dosing to at risk patients during viral seasons. This

343　modelling work has generated novel insights into viral defence dynamics to inform the optimal

344　design of future clinical trials of IFN-β and other related therapies, to prevent exacerbations

345　in both asthma and COPD.

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469

471 **Figure 1 Influenza infected macrophages and epithelial cells similarly between health and**

472 **COPD.** (A) A schematic demonstrating the infection of cells with influenza A virus (IAV -H3N2)

473 for 2 h, removal of the virus and subsequent analysis by flow cytometry at 24 h. (B) The gating

474 strategy for infected differentiated monocyte-derived macrophages (MDMs). Forward

475 Scatter-Area (FSC-A) and -Width (FSC-W) were used to define MDM by size using flow

476 cytometry. Infection was detected using an anti-IAV nucleoprotein (NP)-1 fluorescein

477 isothiocyanate (FITC)-labelled antibody plotted against Side Scatter-Area (SSC-A). Non-

478 infected (NI) cells in red are clearly distinguishable from infected cells (NP-1$^+$) in blue. (C)

479 Differentiated MDMs (D) alveolar macrophages (AMs) or (E) primary bronchial epithelial cells

480 (PBECs) from healthy or COPD patients were infected or not with 30,000 plaque forming units

481 (pfu)/ml (multiplicity of infection (MOI) of 0.03), 360,000 pfu/ml (MOI of 0.72) or 36,000

482 pfu/ml (MOI of 0.25) of IAV (H3N2), respectively (showing medians, n>5). Data were analysed

483 using Mann-Whitney U tests.

484

485 **Figure 2 Interferon (IFN)-β prophylaxis but not treatment modulates influenza infection of**

486 **macrophages.** (A) A schematic illustrating the experimental set up of differentiated monocyte-

487 derived macrophages (MDMs) from healthy volunteers being administered IFN-β or

488 phosphate buffered saline vehicle 2 h post-infection (n=12), 2 h before infection (n=15) or 16

489 h before infection (n=10) with outputs measured 24 h after infection. Differentiated MDMs

490 were infected with 30,000 plaque forming units (pfu)/ml of influenza A virus (IAV - H3N2)

491 (multiplicity of infection of 0.03). (B) and (C) Results for MDMs treated with IFN-β post-

492 infection, (D) and (E) results for MDMs given IFN-β 2 h before infection and (F) and (G) results

493 for MDMs given IFN-β 16 h before infection. (B), (D) and (F) Flow cytometry results, detecting

494 cells infected with replicating virus using an anti-IAV nucleoprotein (NP)-1 antibody. (C), (E)

495 and (G) Viral shedding was quantified by influenza haemagglutinin RNA levels in the culture

496 supernatant using quantitative PCR (normalised to RNA at 0 h after removal of the virus) (n>5)

497 Data were analysed using Wilcoxon signed rank tests (**P<0.01, ***P<0.001).

498

499 **Figure 3 The dynamics of interferon (IFN) and interferon stimulated gene (ISG) expression**

500 **in macrophages.** Schematics showing the measurement of the endogenous expression of IFNs

501 and ISGs in monocyte-derived macrophages (MDMs) from healthy volunteers at different time

502 points (0-24h) following (A) influenza A virus (IAV) infection or (D) exogenous IFN-β treatment

503 with 50 IU/ml. (B) and (C) Expression was measured at the RNA level by quantitative PCR after

504 IAV infection (n=3) or (E) exogenous IFN treatment (n=5). RNA was analysed for expression of

505 (B) *IFNA*, *IFNB*, *IFNL1* or *IFNL2/3* or (C) and (E) the ISGs *MX1, OAS1* or *DDX58*. (B) and (C) MDMs

506 were infected with 30,000 plaque forming units (pfu)/ml of IAV (H3N2) (multiplicity of

507 infection of 0.03). Data are expressed as means (± SD) and further analysed using a Friedman

508 ANOVA with a Dunn's post hoc test compared to 0 h (*P<0.05, **P<0.01, ***P<0.001).

509

510 **Figure 4 Exogenous interferon (IFN)-β modulates influenza infection in macrophages 1 week**

511 **after administration. Macrophages remain sensitive to IFN-β after 3 weeks of chronic**

512 **stimulation.** (A) A schematic demonstrating the administration (or not) of 50 IU/ml of IFN-β

513 to differentiated monocyte-derived macrophages (MDMs) from healthy volunteers for 16 h.

514 MDMs were subsequently cultured for varying lengths of time (0 h, 24 h, 48 h, 72 h, 1 week

515 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,

539 respectively. (B, n=17) and (C, n=8) AMs or (E, n=5) and (F, n=13) PBECs infected with

540 replicating virus were detected using flow cytometry with an anti-IAV nucleoprotein (NP)-1

541 antibody. Viral shedding from (D, n=6) AMs or (G, n=5) PBECs was quantified by influenza

542 haemagglutinin RNA levels in the culture supernatant at 24 h using quantitative PCR

543 (normalised to RNA at 0 h after removal of the virus). All experiments show the median. Data

544 were analysed using a Wilcoxon signed rank tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

545

546 **Figure 6 Exogenous interferon (IFN)-β modulates influenza infection in primary bronchial**

547 **epithelial cells (PBECs) 24 h after administration and reduces expression of interleukin (IL)-**

548 **1β.** (A) A schematic demonstrating the administration (or not) of 50 IU/ml of IFN-β to PBECs

549 for 16 h. PBECs were subsequently cultured for varying lengths of time (0 h, 24 h, 48 h, 72 h

550 or 1 week) after IFN-β removal prior to measurement of interferon stimulated gene (ISG)

551 expression levels, or infection and measurement of % viral nucleoprotein (NP)-1+ cells or IL-

552 1β release 24 h later. PBECs were treated with IFN-β using a staggered approach to ensure

553 that all cells were cultured for the same length of time. (B and D) PBECs were infected or not

554 with 36,000 pfu/ml of influenza A virus (H3N2) (IAV - MOI of 0.25). (B) PBECs from (black, n=4)

555 healthy or (red, n=5) COPD subjects infected with replicating virus were detected using flow

556 cytometry with an anti-IAV NP-1 antibody (showing medians). (C) The expression of ISGs

557 including *MX1, OAS1* and *DDX58* was assessed in non-infected PBECs using quantitative PCR

558 (n=5, showing mean ± SD). (D) The expression of IL-1β was quantified using multiplex ELISA

559 (n=6, showing medians ± IQR). Data were analysed using (B) Wilcoxon signed rank tests or (C)

560 and (D) a Friedman ANOVA with Dunn's post hoc analysis (*$P < 0.05$, **$P < 0.01$).
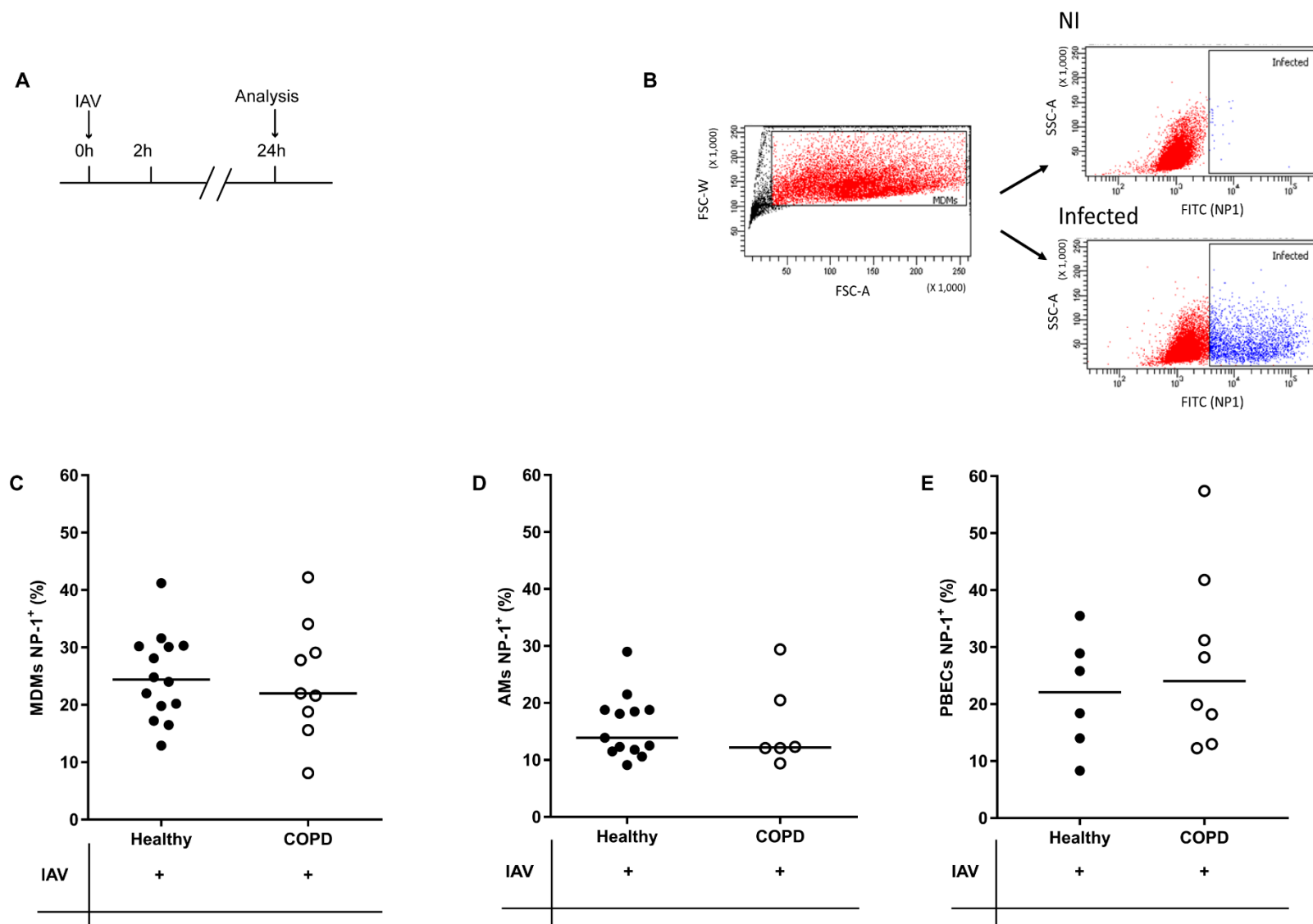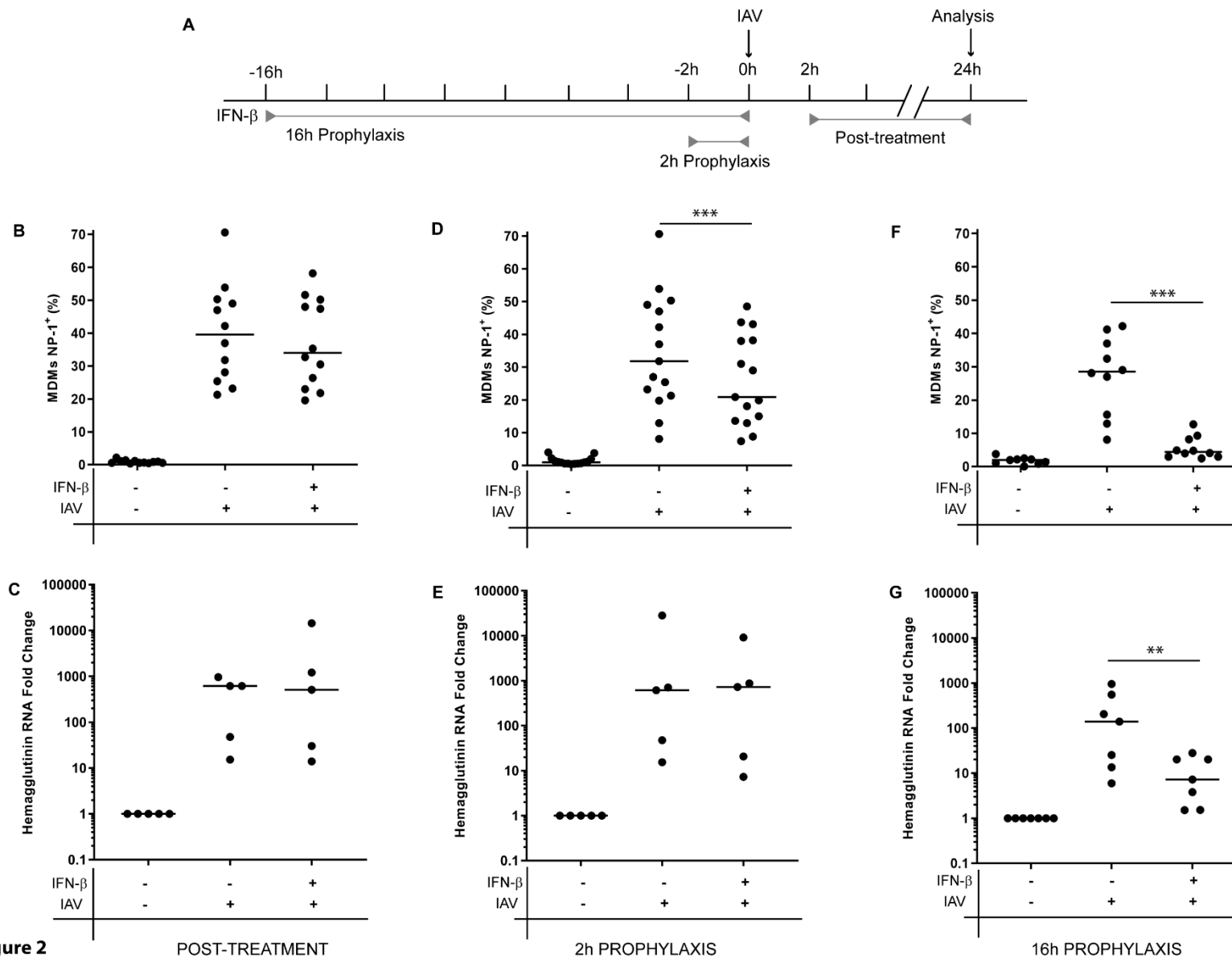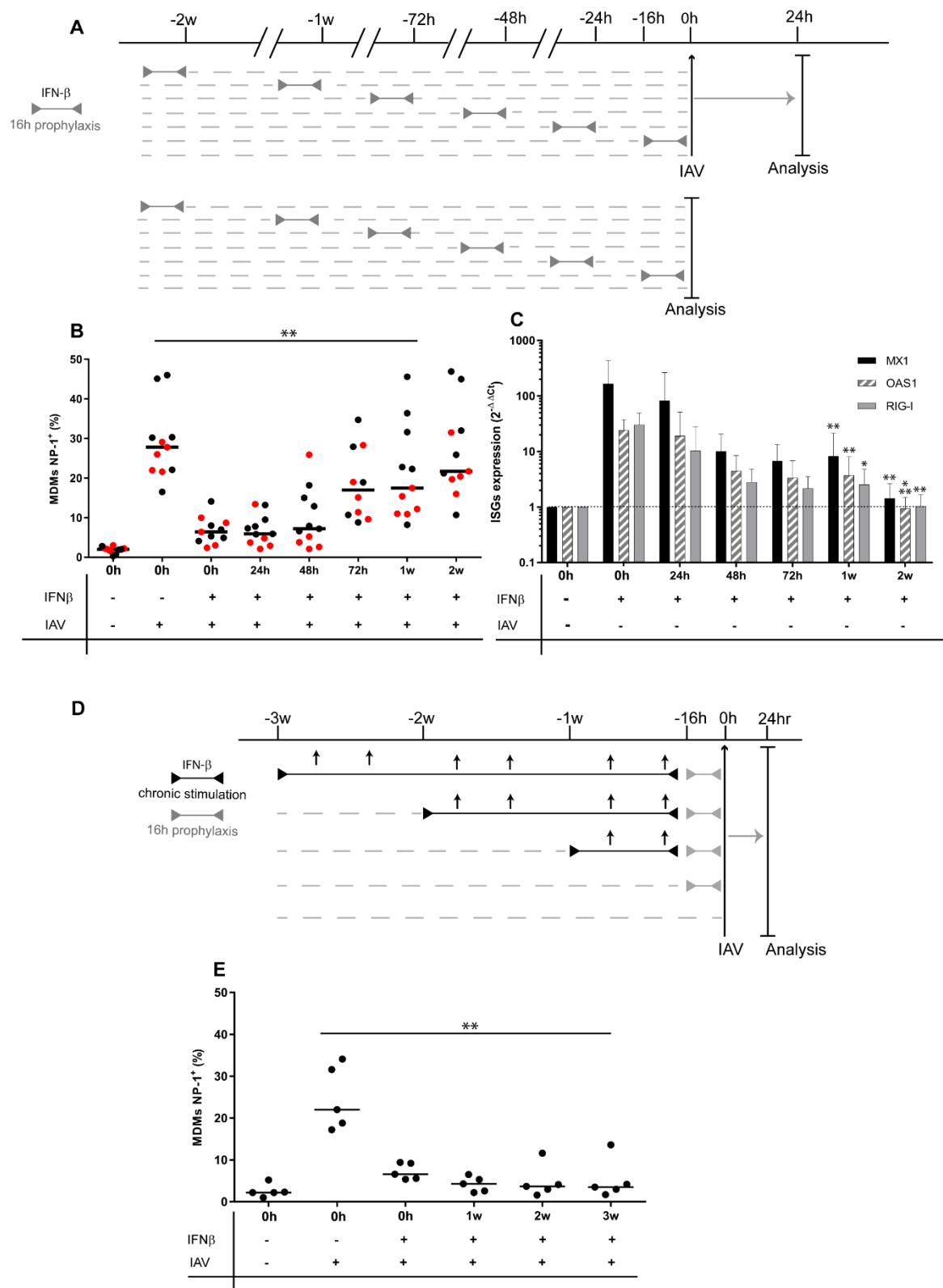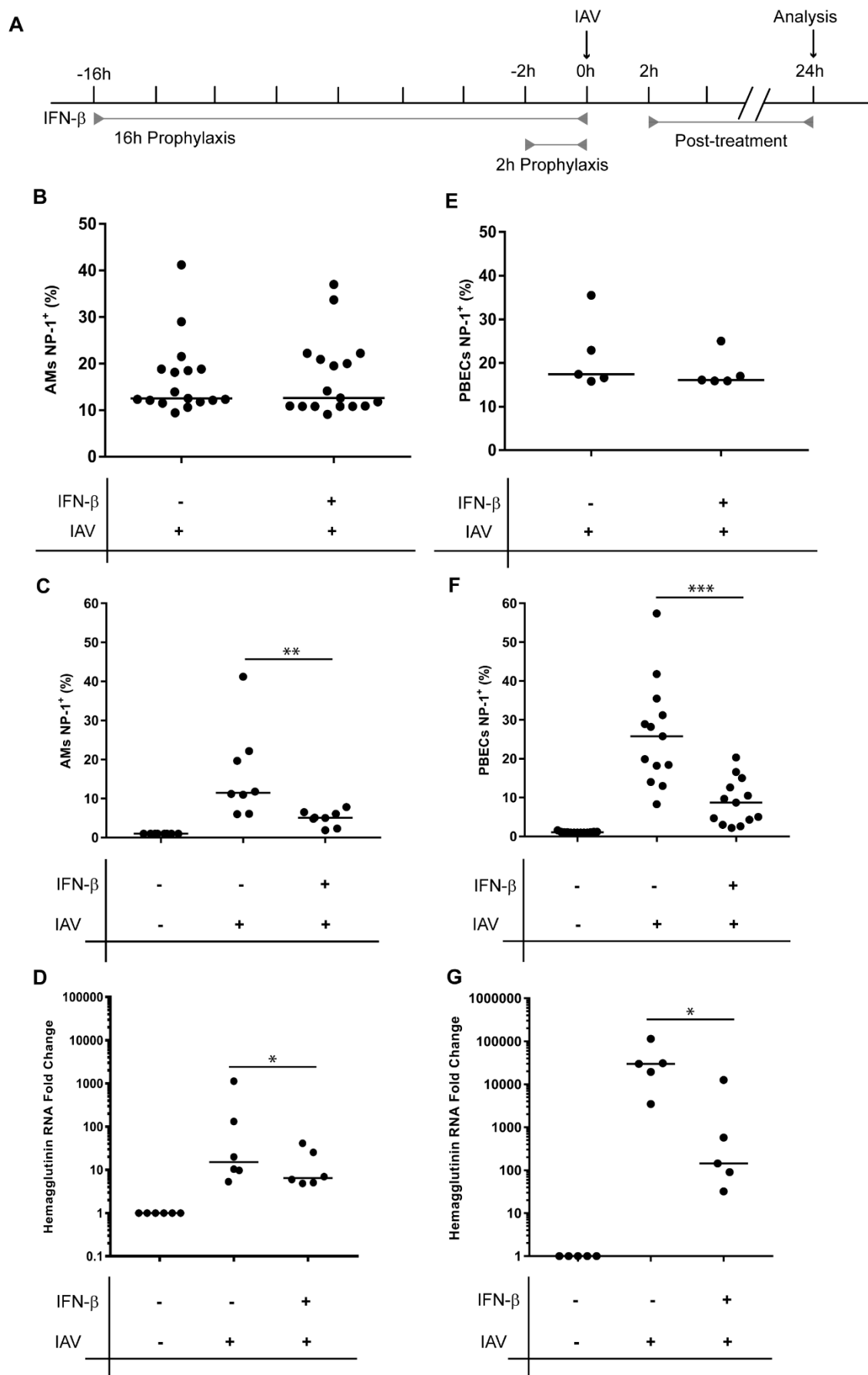
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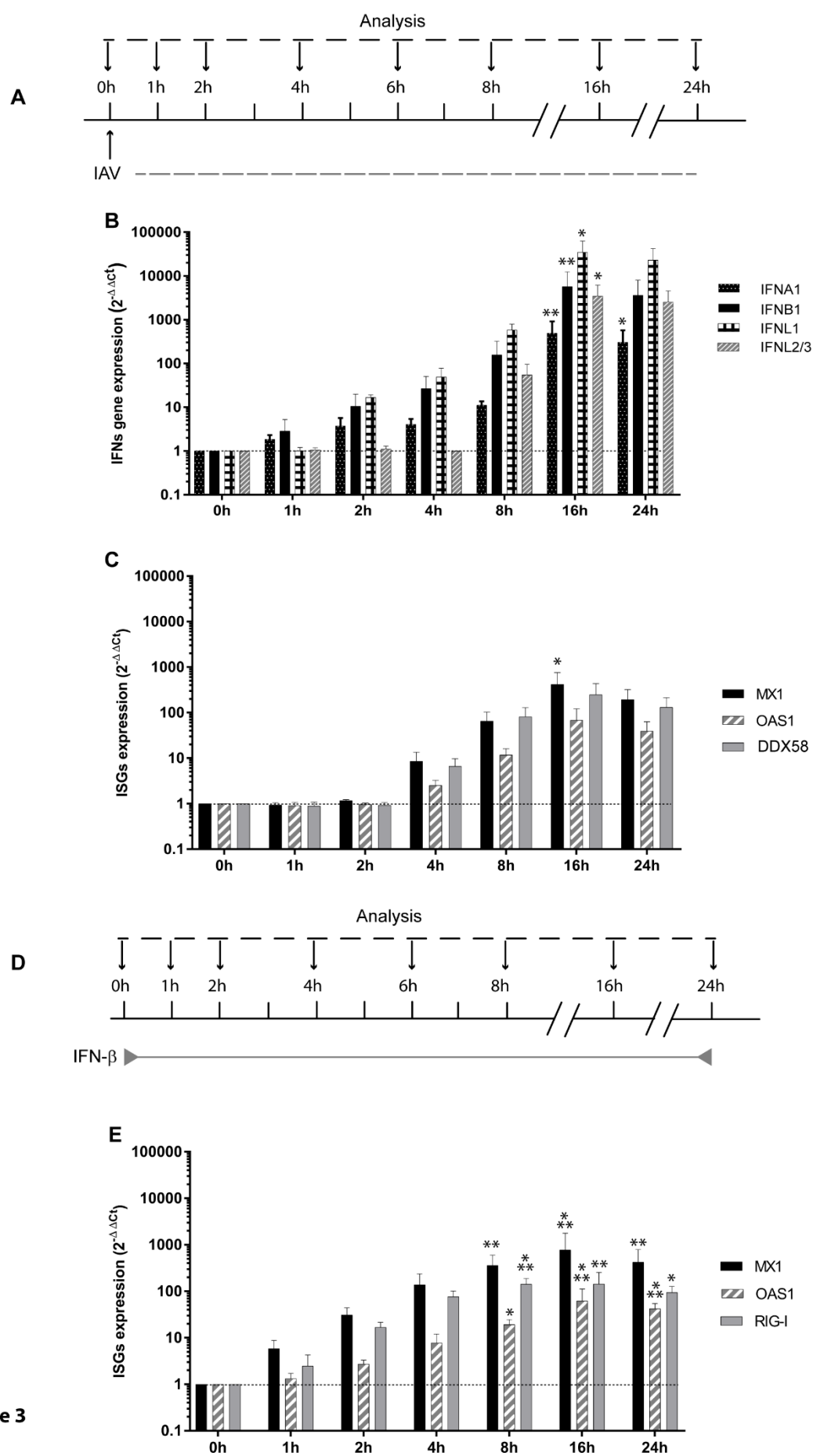
Figure 2

Figure 3

Figure 4
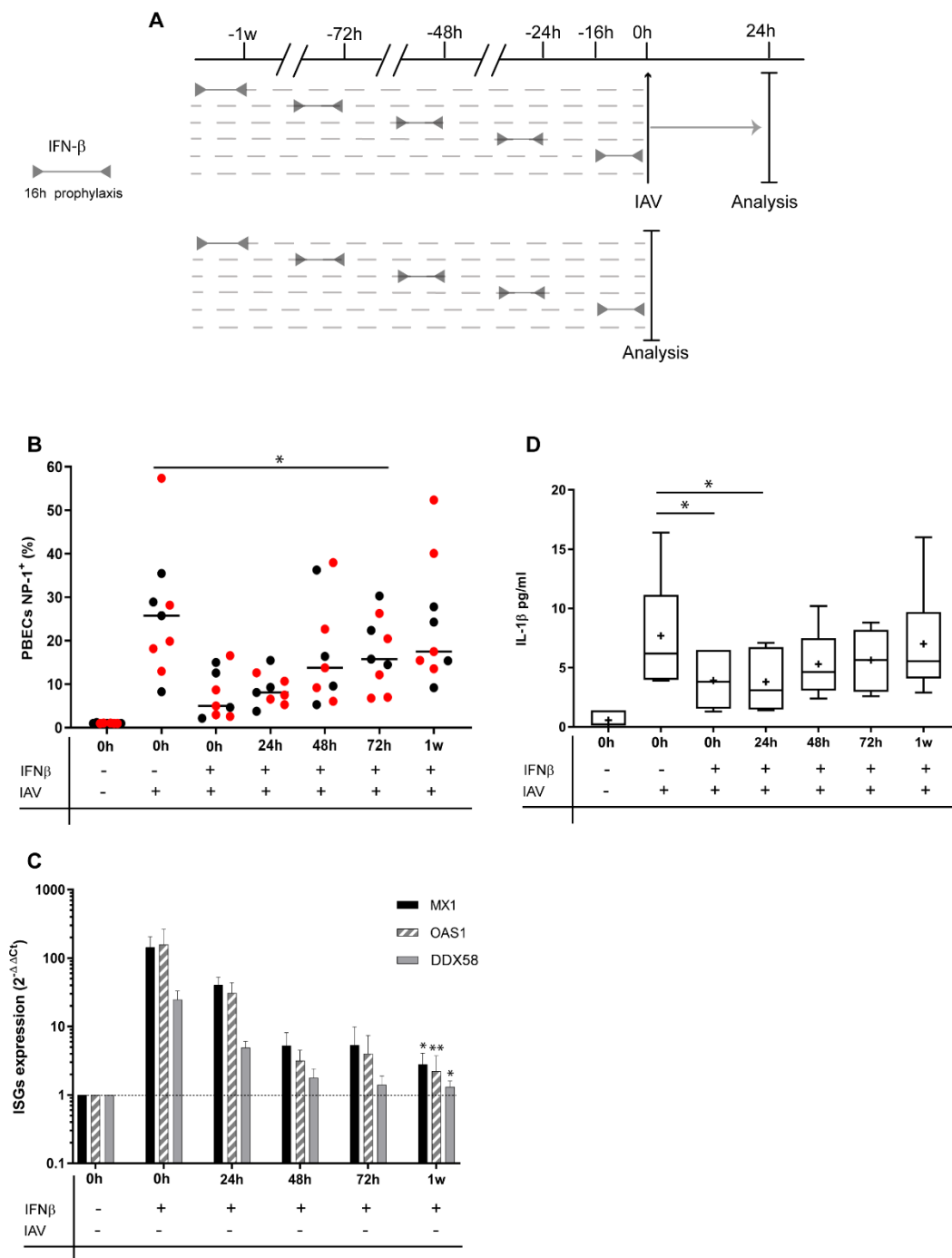
Figure 5

Figure 6

568   **ONLINE DATA SUPPLEMENT**

569

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

571   **strategies**

572   Alastair Watson[1,3], C. Mirella Spalluto[1,3], Christopher McCrae[4,5], Doriana Cellura[1], Hannah

573   Burke[1,2], Danen Cunoosamy[6], Anna Freeman[1,2], Alex Hicks[1,2], Michael Hühn[7], Kristoffer

574   Ostridge[1,2], Karl J. Staples[1,3], Outi Vaarala[8], Tom Wilkinson[1,2,3,#].

575

576

SUPPLEMENTARY METHODS

578 Culturing macrophages and epithelial cells

579 MDMs were differentiated from human blood mononuclear cells, as described previously (1,

580 2). AMs and PBECs were isolated from subjects undergoing bronchoscopy. AMs were selected

581 from BAL by adherence to plastic and cultured overnight in RPMI with 10% heat inactivated

582 fetal calf serum, prior to infection the following day. PBECs were isolated from bronchial

583 brushes and grown in culture using PneumaCult™-Ex Medium, (STEMCELL Technologies,

584 Cambridge, UK) in flasks coated with collagen (PureCol, Nutacon, the Netherlands) until

585 passage 2 or 3.

586 The immortalized human airway BC cell line termed BCi-NS1 was provided by Walters et al.(3).

587 BCi-NS1 were similarly cultured using PneumaCult™-Ex Medium in flasks coated with collagen

588 between passage 20-25.

589 RSV infection of BCi

590 BCi-NS1 cells were incubated for 2 h with RSV subtype A Memphis 37 (4) with 2.4×10^4 pfu/ml

591 at a multiplicity of infection (MOI) of 0.1 (Amsbio, Abingdon, UK: supplied at 2.9×10^6 pfu/ml

592 measured by plaque assay using Vero cells). Cells were washed and incubated for 72 h, prior

593 to harvesting with Qiazol and analysis by qPCR. RNA isolation was performed using an RNeasy

594 Micro kit (Qiagen), according to manufacturer's instructions. qPCR was performed as

595 previously described (5, 6). Expression levels were detected using RSV N gene specific primers

596 (7) (Table E2) normalised to *HPRT* expression using the $2^{-\Delta Ct}$ method (8).

597

SUPPLEMENTARY FIGURE LEGENDS

599

600 **Figure E1 Higher doses of interferon (IFN)-β do not modulate influenza infection of**

601 **macrophages when administered post infection.** Differentiated monocyte-derived

602 macrophages (MDMs) from healthy volunteers were either not infected (NI) or infected with

603 30,000 plaque forming units/ml of influenza A virus (IAV - H3N2) (multiplicity of infection of

604 0.03). MDMs were treated with varying doses of IFN-β (0, 50, 200, 500, 1,000 or 2,000 IU/ml)

605 for either (A) 2 h pre infection or (B) 2 h post-infection (n=5). Infection levels were

606 characterised by the % viral nucleoprotein (NP)-1+ cells using flow cytometry using anti-IAV

607 NP-1 antibody. Bars indicate medians and data are further analysed using a Friedman ANOVA

608 with a Dunn's post hoc test (*P<0.05, **P<0.01). Comparison of the effects of 50 IU IFN-β and

609 200 IU IFN-β administered to MDM or PBECs 2 h pre-infection (C) and (E) or 2 h post infection

610 (D) and (F). Bars indicate medians and data are further analysed using a Wilcoxon signed rank

611 test.

612

613 **Figure E2 The expression of interferons (IFNs) and interferon-stimulated genes (ISGs) upon**

614 **treatment with IFN-β and infection.** Monocyte-derived macrophages (MDMs) from healthy

615 volunteers were either (A) treated with 50 IU/ml IFN-β for up to 24 (n=5) or (B) with

616 concentrations of IFN-β from 0.05 to 500 IU/ml for 24 h only (n=3) and ISG expression

617 measured by qPCR. MDMs were infected with 30,000 plaque forming units/ml of live or ultra-

618 violet light (UV) inactivated influenza A virus (IAV - H3N2) (multiplicity of infection of 0.03)

619 and/or treated with 50 IU/ml of exogenous IFN-β. Quantitative PCR was then used to assess

620 the RNA expression levels of (C) *IFNA1, IFNB1 and IFNL1* (n=3) or (D) *CXCL10, IFIT1, ISG15,*

621 *MX1, OAS1, DDX58* and *RSAD2* (n=4). Data are expressed as means (± SD). Data were further

622   analysed using a Friedman ANOVA with Dunn's post hoc test. For figure clarity asterisks are

623   not shown but in (A) there was significant gene upregulation from the 8 h timepoint onwards.

624   For (B) significant gene regulation is observed from 50 IU/ml IFN-β upwards. In (C) and (D) the

625   IFN genes and ISGs are significantly upregulated by IAV infection but there is no significant

626   difference upon treatment with IFN-β.

627

628   **Figure E3 Cytokine and chemokine release from macrophages is not altered by incubation**

629   **with interferon (IFN)-β.** The pro-inflammatory cytokines (A) GM-CSF, (B) TNF-α, (C) IL-6, (D)

630   IL-25, (E) IL-1β, and the chemokines (F) MDC, (G) TARC and (H) RANTES release into the

631   supernatants of monocyte-derived macrophages (MDMs) from healthy volunteers were not

632   infected (NI) and chronically stimulated with 50 IU/ml of IFN-β for 24 h, 48, h, 72 h, 1 week or

633   2 weeks. New media and IFN-β were added twice weekly for the indicated culture period.

634   Bars indicate median values (n=8). Data are analysed using a Friedman ANOVA with Dunn's

635   post hoc test (**P<0.01).

636

637   **Figure E4 Interferon (IFN)-β pretreatment inhibits respiratory syncytial virus (RSV) infection**

638   **of immortalised bronchial epithelial cells (BCi-NS1).** (A) A schematic illustrating BCi-NS1 cells

639   being administered IFN-β 16 h prior to the infection of cells with RSV for 2 h, removal of the

640   virus and subsequent analysis by quantitative PCR after 72 h. (B) BCi-NS1 cells were treated

641   with 50 IU/ml IFN-β for 16 h after which the cells were washed and the IFN-β removed. BCi-

642   NS1s were then infected with 2.4×10^4 pfu/ml of M37 RSV (MOI of 0.1) for 72 h. Cells were

643   then lysed and RNA extracted and RSV nucleoprotein (N) gene expression was analysed using

644 quantitative PCR (n=5) and normalised to *HPRT* expression using the $2^{-\Delta Ct}$ method. Bar

645 indicates median values. Data were analysed using Wilcoxon signed rank tests (*P<0.05)

646

647 **Figure E5 The duration of interferon-stimulated gene (ISG) induction of macrophages and**

648 **primary bronchial epithelial cells (PBECs) after interferon (IFN)-β administration.** (A)

649 Differentiated monocyte-derived macrophages (MDMs) or (B) PBECs were administered 50

650 IU/ml of IFN-β for 16 h. IFN-β was subsequently removed and cells were cultured for up to a

651 further 2 weeks or 1 week for MDMs or PBECs, respectively. The expression of various ISGs

652 was subsequently analysed including *CXCL10, IFIT1, ISG15, MX1, OAS1, DDX58, RSAD2,*

653 *EIF2AK2 and CH25H*. Data are expressed as means (± SD, n=5) and further analysed using a

654 Friedman ANOVA with a Dunn's post hoc test compared to 0 h IFN-β treatment. For figure

655 clarity asterisks are not shown, but in both figures there are significant differences in ISG

656 expression from 1 week following IFN-β removal.

657
658

659   **SUPPLEMENTARY TABLES**

660   **Supplementary Table E1** Clinical Characteristics of Included Subjects

661

	Control	COPD	P Value
Number of patients	27	12	-
Age, yr	66.0 (61.0-70.0)	70.5 (65.0-75.25)	0.1851†
M/F	12/15	10/2	0.0365‡
BMI, kg/m^2	27.5 (25.2-28.9)	27.6 (25.1-29.1)	0.9587†
Smoker, never/ex	14/13	0/12	-
Pack-years of smoking	0.0 (0.0-21.3)	22.5 (18.8-39.4)	0.0036†
FEV$_1$%	102.0 (94.0-108.0)	78.5 (65.8-92.8)	0.0002†
FEV$_1$/FVC ratio	0.79 (0.76-0.82)	0.63 (0.52-0.65)	<0.0001†

662

663   Definition of abbreviations: BMI = body mass index; COPD = chronic obstructive pulmonary
664   disease.

665   Healthy ex-smokers and COPD patients had stopped smoking for more than 6 months.

666   Data are presented as median (interquartile range).

667   †Mann-Whitney *U* test.

668   ‡ Fisher's exact test.

669

670

6

671   **Supplementary Table E2** List of Taqman Gene expression assays used for analysis of gene
672   expression by quantitative PCR (ThermoFisher)

673

Gene name	Protein name	Assay ID
ACTB	β-actin	Hs99999903_m1
CH25H	Cholesterol 25-hydroxylase	Hs02379634_s1
CXCL10	CXCL10/IP-10	Hs00171042_m1
DDX58	RIG-I	Hs01061436_m1
EIF2AK2	PKR	Hs00169345_m1
FLUWisn	FluA/Wisconsin Haemaggutinin	AP47VVA
IFIT1	IFIT1	Hs03027069_s1
IFNA1	IFN-α	Hs00256882_s1
IFNB1	IFN-β	Hs01077958_s1
IFNL1	IFN-λ1	Hs00601677_g1
IFNL2/3	IFN-λ2/3	Hs04193047_gH
ISG15	ISG15	Hs01921425_s1
MX1	Mx1	Hs00895608_m1
OAS1	OAS1	Hs00973635_m1
RSAD2	viperin	Hs00187842_m1
RSVN	RSV N protein	AIT95TB
HPRT	Hypoxanthine-guanine E3 phosphoribosyltransferase	Hs02800695_m1

674

675   **Supplementary Table E3** MSD cytokine and chemokine multiplex assay lower limits of
676   detection

Analyte	LLOQ (pg/ml)
GM-CSF	0.63
IL-1beta	0.25
IL-6	0.68
IL-17E/IL-25	0.59
IL-33	0.67
TNF-alpha	1.09
CCL17	0.95
CCL22	32.16
TSLP	0.65
RANTES	0.64

677

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678

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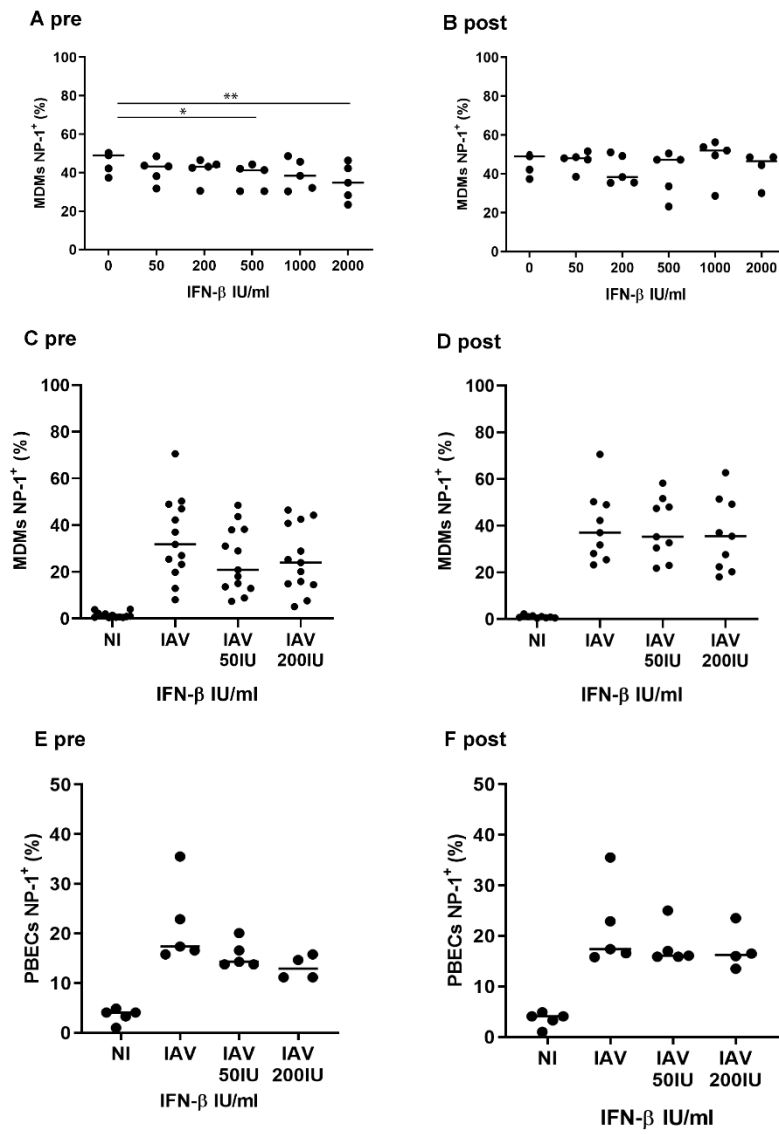703 quantitative PCR and the 2(T)(-Delta Delta C) method. Methods. 2001;25(4):402-8.

704

705

706 Figure E1

707

708

A

B

709 **Figure E2A-B**

710

C

Figure showing IFNs gene expression (2^{-ΔΔCt}) with legend:
- IFNA1
- IFNB1
- IFNL1

IFNβ	−	+	−	−	+
IAV	−	−	−	+	+
UV IAV	−	−	+	−	−

D

Figure showing ISGs expression (2^{-ΔΔCt}) with legend:
- CXCL10
- IFIT1
- ISG15
- MX1
- OAS1
- DDX58
- RSAD2

IFNβ	−	+	−	−	+
IAV	−	−	−	+	+
UV IAV	−	−	+	−	−

711   **Figure E2C-D**

712

A

GM-CSF pg/ml (y-axis, 0–5000)

x-axis: NI, NI +IFNb, NI +IFNb +24hr, NI +IFNb +48hr, NI +IFNb +72hr, NI +IFNb +1Week, NI +IFNb +2Weeks

B

TNF-α pg/ml (y-axis, 0.01–100)

x-axis: NI, NI +IFNb, NI +IFNb +24hr, NI +IFNb +48hr, NI +IFNb +72hr, NI +IFNb +1Week, NI +IFNb +2Weeks

C

IL-6 pg/ml (y-axis, 0–600)

x-axis: NI, NI +IFNb, NI +IFNb +24hr, NI +IFNb +48hr, NI +IFNb +72hr, NI +IFNb +1Week, NI +IFNb +2Weeks

D

IL-25 pg/ml (y-axis, 0.0–1.0)

x-axis: NI, NI +IFNb, NI +IFNb +24hr, NI +IFNb +48hr, NI +IFNb +72hr, NI +IFNb +1Week, NI +IFNb +2Weeks

E

IL-1β pg/ml (y-axis, 0.0–2.5)

x-axis: NI, NI +IFNb, NI +IFNb +24hr, NI +IFNb +48hr, NI +IFNb +72hr, NI +IFNb +1Week, NI +IFNb +2Weeks

713 **Figure E3A-E**

714

F

MDC pg/ml

G

TARC pg/ml

H

RANTES pg/ml

Figure E3F-H

715

716

A

RSV

Analysis

-16h   -2h 0h 2h   72h

IFN-β ▶

16h Prophylaxis

B

RSV N RNA $2^{-\Delta Ct}$

| IFN-β | - | + |
| RSV | + | + |

*

16h PROPHYLAXIS

717   **Figure E4**

718

A

B

719   **Figure E5**