

Prevention and treatment of swine-origin influenza virus with interferon: an in vivo and ex vivo study

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KEY MESSAGES

1. Prophylactic interferon reduces infection with influenza H1N1pdm in lung tissue. Therapeutic interferon is beneficial in lung tissue but not in bronchial tissue.
2. Exogenous interferon appears to be useful for pulmonary involvement of influenza viruses (H1N1 and H5N1) but may not be of significant benefit for bronchial infection. For influenza virus infections that are resistant to current antiviral agents, interferon therapy offers a

potential benefit.

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Introduction

The influenza A/H1N1pdm virus that emerged in 2009 contained a unique mixture of genes that originated from Eurasian and North American swine. Even though this new virus was associated with a low mortality compared with seasonal influenza, there were concerns that the higher mortality in younger patients (contrasting with a bias towards elderly patients with seasonal influenza) and the potential for this virus to re-assort with avian influenza viruses would result in a high mortality.

This pandemic virus was a reassortant virus with the incorporation of a haemagglutinin gene from the classic swine lineage and a neuraminidase gene from Eurasian-like swine lineage. A younger population (<50 years of age) appeared to be immunologically naïve with respect to previous exposure, but the elderly were partially protected. A vaccine strategy was implemented early in the outbreak, and although effective, still had a lag of 3-4 months from the start of the outbreak. In the early stages of the outbreak, effective treatment relied on existing therapies. Unfortunately, H1N1pdm was resistant to amantadine and rimantidine and there were increased numbers of reports to suggest virus resistance to the neuraminidase inhibitor oseltamivir (Tamiflu).

We planned to investigate the role of interferon (IFN) in the prevention and treatment of H1N1pdm. When cells are infected with influenza virus, they begin to express IFN, which leads to an antiviral response through the activation of effector molecules. The type I IFNs (including IFN α) are produced early in the infection of the cell and function by inhibiting viral replication and protein synthesis. To counteract this host antiviral

mechanism, viruses have developed mechanisms to evade the IFN response. For example, the NS1 protein of influenza virus targets either the post-transcriptional processing of IFN mRNA and/or the IFN-inducible antiviral proteins such as ISG15 and PKR. Evidence of a role for IFN in an anti-influenza virus response has been shown through the use of IFN α/β receptor deficient mice.

IFN α has been shown to effectively control hepatitis B and C in humans, particularly active hepatitis C virus, and to have cured 98% of affected individuals. During the 2003 severe acute respiratory syndrome outbreak in Toronto, IFN alfacon-1, a novel synthetic consensus IFN, showed clinical benefit in patients treated with steroids and IFN compared with those treated with steroids alone. In guinea pigs and ferrets infected with H5N1 and seasonal influenza respectively, IFN treatment resulted in reduced viral titres and improved pulmonary pathology.^{1,2} In the 1980s, intranasal administration of IFN α showed promise in preventing influenza infection, but side-effects prevented the widespread adoption of this route of administration. The Mx1 protein is a downstream protein induced by IFN and thus a suitable target for analysis.

Given that H1N1pdm emerged as a pandemic virus with a low but significant mortality in young patients, together with a potential for increased oseltamivir resistance, there is a need to explore the role of IFN in the management of lower respiratory tract infection. Clinically, there may be little benefit from the use of IFN in uncomplicated nasopharyngeal H1N1pdm infection. We thus explored the utility of IFN treatment in a human ex vivo bronchial/lung culture model.

Methods

This study was conducted from January 2010 to December 2011.

Ex-vivo culture

The ex vivo organ cultures of the lung and bronchial biopsies were obtained from lungs removed at surgery according to previously approved protocols. The bronchial tissues were placed on a sterile sponge to create minimal contact with enough growth medium (F12K + 1% PS) in an air-liquid interface situation and cultured for 24 and 48 hours with influenza viruses as listed below and also with pre-incubation with different concentrations of IFN- α facon-1.

Viruses used

We used an influenza virus isolated from a Mexican patient with H1N1pdm disease in Hong Kong in 2009, (A/Hong Kong/415742/09), a virus from a patient with H5N1 disease in Vietnam (A/VN/3046/04) and Hong Kong (A/Hong Kong/483/97), a human seasonal influenza H1N1 virus (A/Hong Kong/54/98), and a seasonal influenza H3N2 virus (A/Oklahoma/1992/05).

Evaluation of cytokine profile by superarray

Total RNA was extracted from ex vivo tissues using the RNeasy mini Kit (Qiagen). The eluted total RNA (20 μ L) was used for the first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The gene expression of 84 key cytokine genes was then profiled by RT-PCR-based RT² Profiler Interferons and Receptors PCR Array (SABioscience, Frederick, MD, USA) in the ABI 7500 Real-Time PCR System (Applied Biosystems). Fold change of IFNs and IFN receptor expression in experimental samples relative to the control samples (eg mock-infected) was calculated using the $\Delta\Delta$ Ct method. The $\Delta\Delta$ Ct value of each sample was normalised by up to a total of five housekeeping genes (β -2-microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, glyceraldehyde-3-phosphate dehydrogenase, and β -actin). All data were analysed by the RT² Profiler PCR Array Data Analysis Template v3.0 and all gene expression changes >2.5 fold were considered significant.

Detection of IFNAR receptors and Mx1 protein in bronchial and pulmonary tissues

IFNAR1 and IFNAR2 antibodies from Abcam and Lifespan biosciences gave no reaction to formalin fixed tissues. We then applied these to 10 fresh bronchial and lung biopsies that had been excised and embedded in OCT. After blocking with appropriate sera, antibodies were used at 1/50 (2 μ g/mL) concentration for IFNAR1 and 1/50 (5 μ g/mL) for

IFNAR2 for 30 mins at room temperature. Secondary incubation with alkaline phosphatase conjugated streptavidin (Vectorlabs SA-5100) at 1/200 for 30 mins followed by development with Vector Red substrate kit (Vectorlabs SK-5100) at room temperature for 10 mins. The Mx1 protein antibody was obtained from Abcam and also used on frozen tissue.

Infection of mice by pandemic and non-pandemic H1N1

Wild type and knock-in Mx1^{+/+} mice were bred in Toronto and infected with pandemic H1N1. Viral load and interferon gene expression was studied at 3 days post infection.

Results

Analysis of 10 pairs of bronchial and lung biopsies showed that five of these had weak apical expression of IFNAR1 and IFNAR2 in the bronchial epithelium and no significant staining in the pulmonary parenchyma (Fig 1). This staining was only partially membranous and also extracellular. This is consistent with a previous study that reported very low innate expression of IFNAR in the unstimulated epithelium of the respiratory tract. We attempted to determine the IFNAR1 and IFNAR2 expression on the surface of cells in ex vivo cultures after influenza virus infection. Nonetheless, the available IFNAR antibody did not permit staining of fixed tissues. The compulsory 10% formalin fixation of the tissue was due to the safety protocol required as a standard operation protocol. Mx1 protein was detected in the bronchial epithelium and in scanty alveolar macrophages (Fig 1).

To investigate the interaction of influenza with interferon, human lung explant tissues were pretreated with IFN- α facon-1 for 16 hours prior to infection with H5N1 or H1N1 influenza A virus. At different time points post infection, RNA was extracted for cDNA synthesis. Analysis of influenza

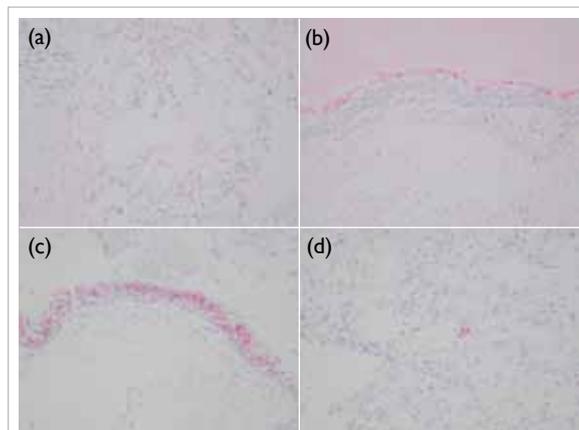


FIG 1. IFNAR1 staining of (a) lung and (b) bronchus from normal tissue (frozen section with Vector Red staining). Mx1 immunohistochemistry of (c) bronchus and (d) lung tissue.

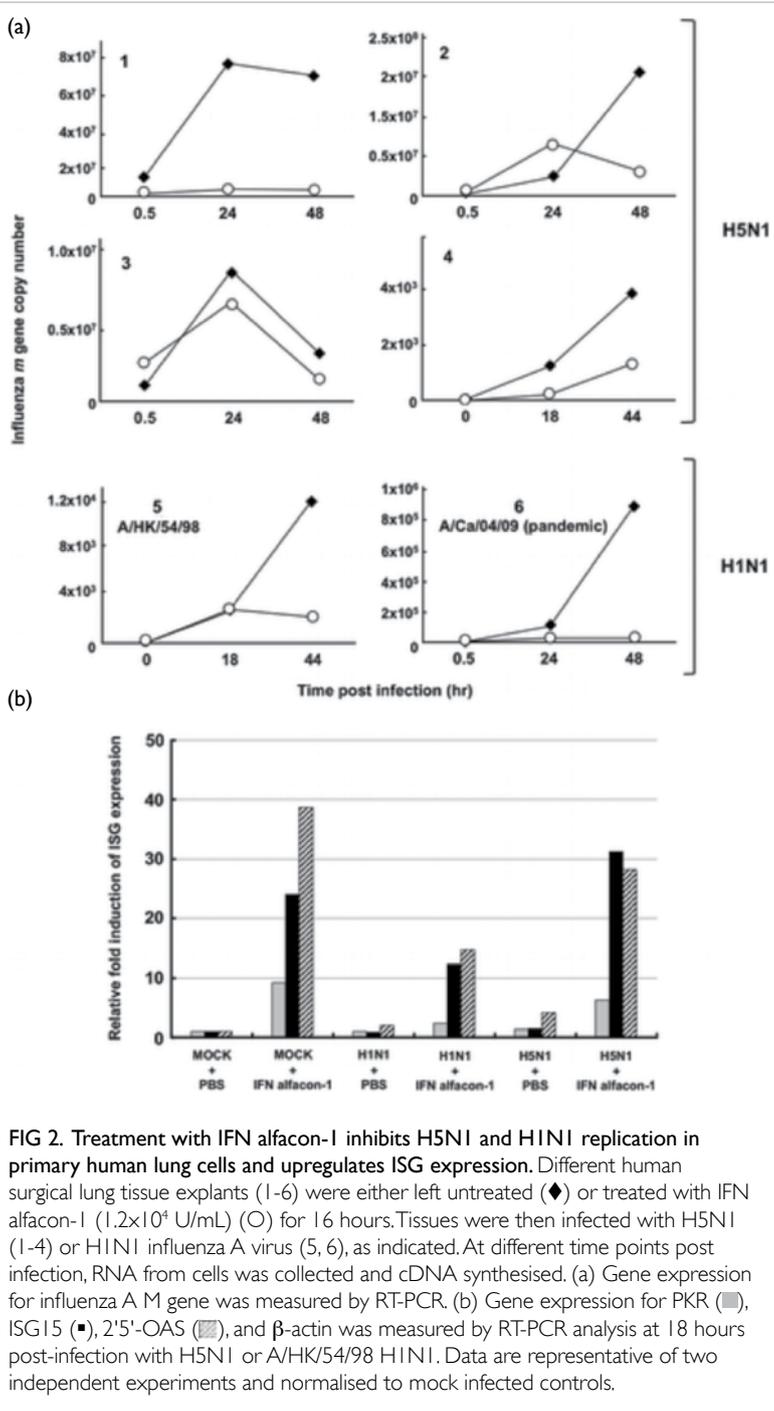


FIG 2. Treatment with IFN alfacon-1 inhibits H5N1 and H1N1 replication in primary human lung cells and upregulates ISG expression. Different human surgical lung tissue explants (1-6) were either left untreated (◆) or treated with IFN alfacon-1 (1.2x10⁴ U/mL) (O) for 16 hours. Tissues were then infected with H5N1 (1-4) or H1N1 influenza A virus (5, 6), as indicated. At different time points post infection, RNA from cells was collected and cDNA synthesised. (a) Gene expression for influenza A M gene was measured by RT-PCR. (b) Gene expression for PKR (■), ISG15 (▣), 2'5'-OAS (▨), and β-actin was measured by RT-PCR analysis at 18 hours post-infection with H5N1 or A/HK/54/98 H1N1. Data are representative of two independent experiments and normalised to mock infected controls.

A matrix (M) gene expression revealed that IFN alfacon-1 pretreatment effectively inhibited H5N1 and H1N1 influenza A replication (Fig 2). Gene expression analysis for 2'5'-OAS, PKR and ISG15, and IFN-stimulated genes (ISGs) associated with an IFN-inducible antiviral response revealed that the expression levels for these ISGs were not highly upregulated in H5N1 or H1N1 virus infected tissues. Notably, IFN alfacon-1 pretreatment of mock infected explants induced a high expression of ISGs. IFN-inducible ISG expression was observed in both H5N1 and H1N1 virus-infected human lung tissues

pre-treated with IFN alfacon-1 (Fig 2).

We further examined the effects of IFN alfacon-1 on pandemic H1N1 influenza A infection when IFN was added post-challenge with virus. Three different human lung explants were infected with H1N1pdm virus, and then 24 hours post-infection treated with 1.2x10⁴ U/mL IFN alfacon-1. At 24 and 48 hours post-treatment, the effects of IFN on viral replication were evaluated by measuring M gene expression and TCID₅₀ values. There was evidence for the protective effects of IFN treatment, even when added post-infection, as assessed by TCID₅₀ and M gene expression (Fig 3). These results were supported by evidence of a reduction in influenza A nucleoprotein expression, visualised in the IFN-treated lung explants (Fig 3).

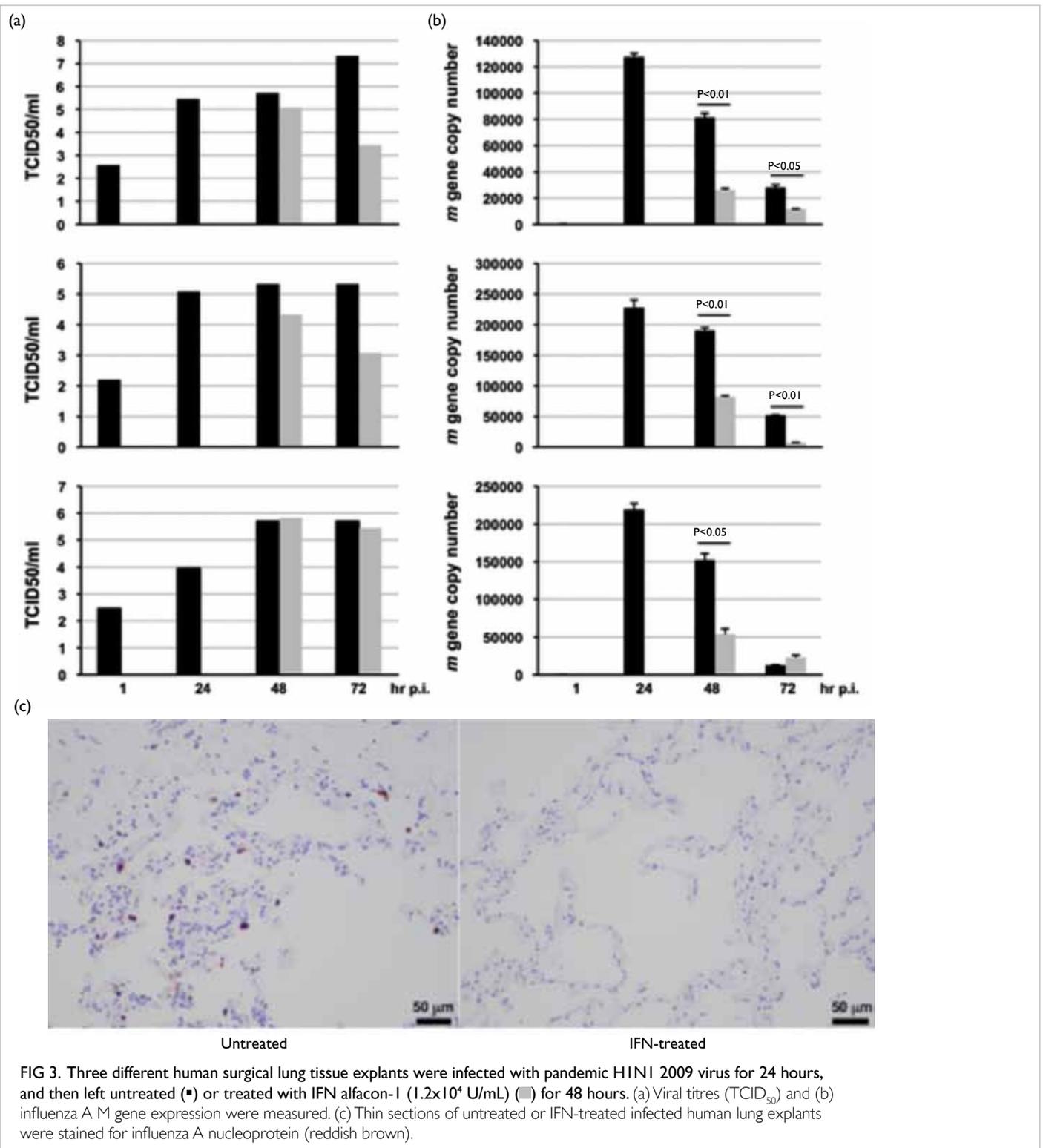
In contrast to the lung data therapeutic treatment, the same IFN post-infection treatment regimen in bronchial tissues did not show a convincing protective effect against H1N1pdm influenza virus infection. In four bronchial biopsies from different patients, we found no protection (2 cases) or a nominal protective effect (2 cases) against H1N1pdm after the IFN post-infection treatment (Fig. 4). In two biopsies where IFN alfacon-1 was added 24 hours after infection, there was no inhibitory effect, whereas for two other biopsies there was an inhibitory effect (Fig 4). Overall, IFN post treatment in bronchial tissue showed no advantage when compared with control treatment during H1N1pdm infection. Similar experiments were not conducted using H5N1 virus as we found no H5N1 replication in human ex vivo bronchus culture (n=6).

In the initial experiments, either H1N1 or H5N1 influenza A virus was used to infect the intact human lung explant tissue. To determine if influenza A infection would inhibit either *ifnar1* or *ifnar2* expression, RNA was collected and analysed at 18 hours post-infection. Infection with both viruses led to a selective reduction in *ifnar1* gene expression when compared with mock-infected control tissues (Fig 5). Notably, infection with the H5N1 influenza A strain led to a greater reduction in *ifnar1* gene expression compared with infection with H1N1 virus. The inhibitory effects of H1N1 and H5N1 infection on *ifnar2* gene expression were not significant.

There was no significant change in weight loss, viral load, or interferon gene production in the wild-type or Mx1^{+/+} mice identified.

Discussion

This study investigated how a newly emerging pandemic virus—H1N1pdm—affected IFN signalling and explored the potential therapeutic potential of exogenous IFN to override any virus-induced inhibitory effects associated with the virally-encoded NS1 protein of influenza virus. Lung biopsies indicated that H5N1 infection was able to inhibit IFNAR and



SOCs protein expression and demonstrated that this may be due to inhibition of the IFN-inducible STAT pathway. Nevertheless, we have provided evidence that IFN treatment overrides the inhibitory effects of influenza virus infection. This IFN-inducible antiviral effect, however, was not reproduced in our ex vivo bronchial culture system. Our immunohistochemistry studies have shown that intrinsic IFNAR1 and IFNAR2

expression is very low in the bronchial epithelium. This anatomical site is not a successful therapeutic target for systemic IFN therapy.

Our ex vivo studies have shown that in the lung explant model, IFN pre-treatment was able to override the inhibitory effects of influenza virus infection. The differential effectiveness of IFN in suppressing both H5N1 and H1N1 subtypes may be

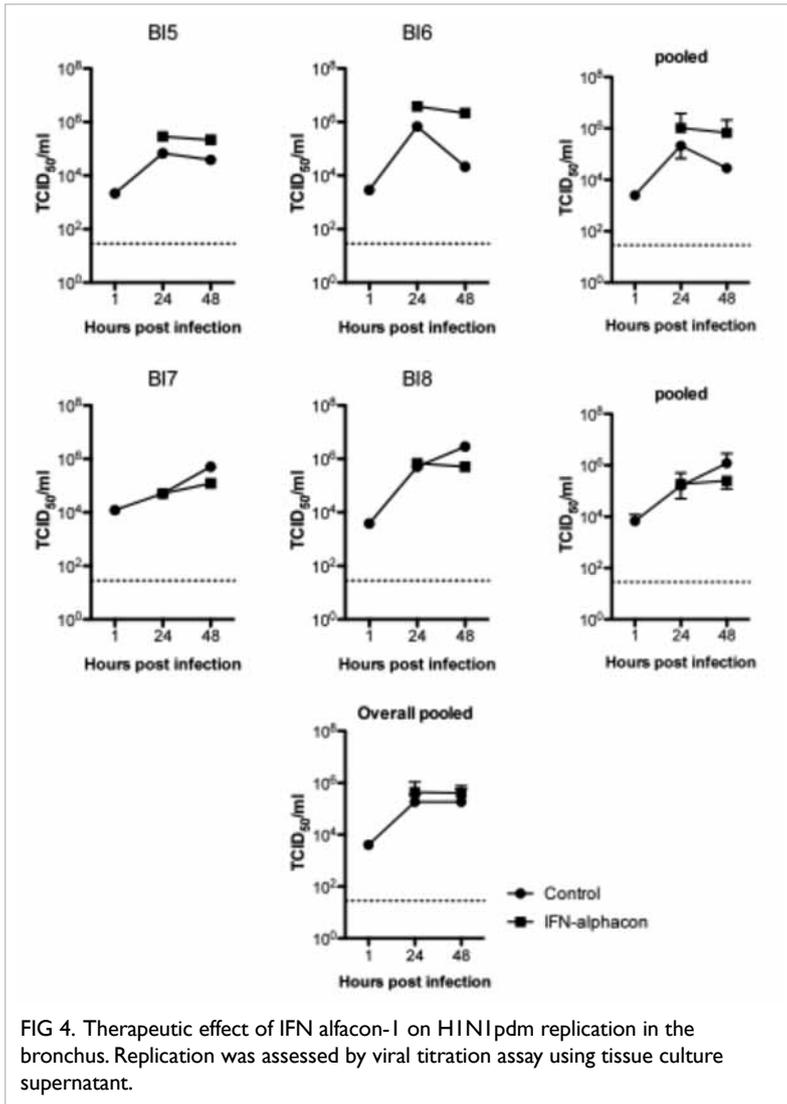


FIG 4. Therapeutic effect of IFN alfacon-1 on H1N1pdm replication in the bronchus. Replication was assessed by viral titration assay using tissue culture supernatant.

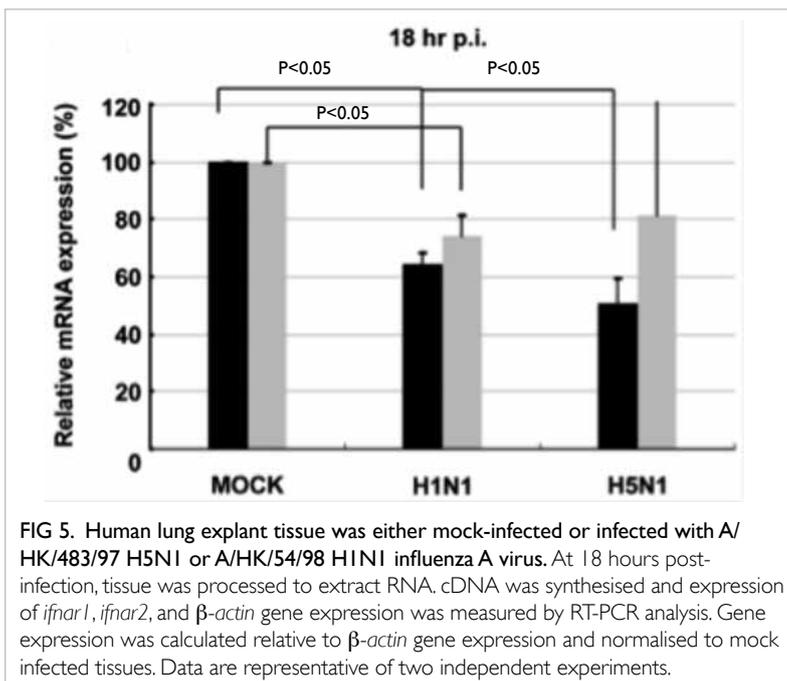


FIG 5. Human lung explant tissue was either mock-infected or infected with A/HK/483/97 H5N1 or A/HK/54/98 H1N1 influenza A virus. At 18 hours post-infection, tissue was processed to extract RNA. cDNA was synthesised and expression of *ifn α 1*, *ifn α 2*, and β -*actin* gene expression was measured by RT-PCR analysis. Gene expression was calculated relative to β -*actin* gene expression and normalised to mock infected tissues. Data are representative of two independent experiments.

due to the differential innate immune responses of the host to these different viruses immediately after infection. Specifically, H5N1 virus is well known to cause hyper-induction of cytokine and chemokines, whereas H1N1 and H1N1pdm do not. The differential regulation of the host sensing receptor by H5N1 and H1N1 virus was discussed in an earlier report in 2009. The differential regulation of the innate immune response between the two subtypes is shown in terms of quantity of the cytokines and chemokines produced and the intensity of the pathways triggered, instead of a qualitative difference. It appears that the innate immune response triggered by seasonal H1N1 and H1N1pdm is actually comparable, and H1N1pdm did not show hyper-induction of cytokines as for H5N1.^{3,4}

Conclusion

We analysed IFN-induced signalling using a focused IFN pathway array. We found that exogenous IFN increased the induction/expression of antiviral genes, yet observed a decrease in the expression level of IFN receptors. This IFN-induction of antiviral proteins, eg Mx, OAS, likely contributed directly to the reduction in viral gene transcription and virus replication observed. Although our data indicated that effectiveness is limited, IFN treatment post-infection with H1N1pdm influenza virus did limit the viral replication in terms of viral titre, M gene, and influenza NP expression.

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