



# Tropism and innate host responses of a novel avian influenza A H7N9 virus: an analysis of ex-vivo and in-vitro cultures of the human respiratory tract

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## Summary

**Background** Since March, 2013, an avian-origin influenza A H7N9 virus has caused severe pneumonia in China. The aim of this study was to investigate the pathogenesis of this new virus in human beings.

**Methods** We obtained ex-vivo cultures of the human bronchus, lung, nasopharynx, and tonsil and in-vitro cultures of primary human alveolar epithelial cells and peripheral blood monocyte-derived macrophages. We compared virus tropism and induction of proinflammatory cytokine responses of two human influenza A H7N9 virus isolates, A/Shanghai/1/2013 and A/Shanghai/2/2013; a highly pathogenic avian influenza H5N1 virus; the highly pathogenic avian influenza H7N7 virus that infected human beings in the Netherlands in 2003; the 2009 pandemic influenza H1N1 virus, and a low pathogenic duck H7N9 virus that was genetically different to the human disease causing A H7N9 viruses.

**Findings** Both human H7N9 viruses replicated efficiently in human bronchus and lung ex-vivo cultures, whereas duck/H7N9 virus failed to replicate in either. Both human A H7N9 viruses infected both ciliated and non-ciliated human bronchial epithelial cells and replicated to higher titres than did H5N1 ( $p < 0.0001$  to  $0.0046$ ) and A/Shanghai/1/2013 replicated to higher titres than did H7N7 ( $p = 0.0002$ – $0.01$ ). Both human A H7N9 viruses predominantly infected type II alveolar epithelial cells and alveolar macrophages in the human lung and replicated to higher titres than did H5N1 ( $p < 0.0001$  to  $0.0078$ ); A/Shanghai/1/2013 replicated to higher titres than did H1N1 ( $p = 0.0052$ – $0.05$ ) and H7N7 ( $p = 0.0031$ – $0.0151$ ). Human H7N9 viruses were less potent inducers of proinflammatory cytokines compared with H5N1 virus.

**Interpretation** Collectively, the results suggest that the novel H7N9 viruses are better adapted to infect and replicate in the human conducting and lower airways than are other avian influenza viruses, including H5N1, and pose an important pandemic threat.

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## Introduction

As of July 7, 2013, 132 laboratory-confirmed human infections with a novel influenza A H7N9 virus have been reported from ten provinces and municipalities in China by the National Health and Family Planning Commission, and one additional case has been reported from Taipei Centres for Disease Control. There have been 43 deaths so far.<sup>1,2</sup> The source of human infection appears to be poultry.<sup>1,3–5</sup> There is so far no evidence of sustained human-to-human transmission within the community. Genetic analysis of these viruses revealed that all eight gene segments were of avian origin; six internal gene segments were derived from avian influenza A H9N2 viruses found in poultry in Asia, and the haemagglutinin and neuraminidase genes were derived from influenza viruses circulating in ducks and wild birds, respectively.<sup>1</sup>

Human infections with other H7 influenza viruses (H7N2, H7N3, and H7N7) have previously been reported in the Netherlands, Canada, USA, and UK<sup>6</sup> in association with outbreaks in poultry and resulted in conjunctivitis

with mild upper respiratory symptoms. However, there was one fatal case in the Netherlands.<sup>6,7</sup>

Cytokine dysregulation contributes to the pathogenesis of human disease caused by highly pathogenic avian influenza A H5N1 as well as the 1918 pandemic H1N1 viruses.<sup>8–12</sup> By comparison with seasonal influenza viruses, some highly pathogenic avian influenza H5N1 viruses induced higher concentrations of pro-inflammatory cytokines from human alveolar epithelial cells and macrophages.<sup>8,9,11–13</sup>

We previously used ex-vivo cultures of human conjunctiva, nasopharynx, tonsil, bronchus and lung to investigate influenza<sup>14,15</sup> and coronavirus tropism<sup>16</sup> and used in-vitro cultures of polarised primary human alveolar epithelial cells and peripheral blood monocyte derived macrophages to compare innate immune responses elicited by different influenza viruses.<sup>8,9,12,13</sup> In this study, we used these ex-vivo and in-vitro models of cultured human tissues and cells to compare the virus tropism and host innate immune responses of the novel

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A H7N9 influenza virus with that of the pandemic H1N1 virus, highly pathogenic avian influenza H5N1 and H7N7 viruses, and a low pathogenic duck H7N9 virus.

**Methods**

**Viruses**

We used avian influenza A H7N9 viruses, A/Shanghai/1/2013 (Sh1/H7N9) and A/Shanghai/2/2013 (Sh2/H7N9); a duck H7N9 virus, A/Duck/Jiangxi/3286/2009 (duck/H7N9), with a different genetic derivation from the two H7N9 viruses as a control; a highly pathogenic avian influenza H7N7 virus isolated from a human patient with fatal disease, A/Netherlands/219/2003 (NL/219/H7N7); a highly pathogenic avian influenza H5N1 virus isolated from a fatal human infection, A/Hong Kong/483/1997 (H5N1); and a 2009 pandemic influenza virus, A/California/07/2009 (H1N1pdm). See appendix for further details. All experiments were done in a biosafety level 3 facility and the use of human tissues had been previously approved by the local institutional review board.

**Ex-vivo cultures**

Fresh bronchus, lung, nasopharynx, and tonsil tissues were obtained from patients aged 51 to 65 years undergoing elective surgery in Hong Kong and were removed as part of clinical care but surplus for routine diagnostic requirements as detailed previously.<sup>14-16</sup> Methods of culture, infection, and analysis are detailed in the appendix.

**In-vitro cultures**

The effect of desialylation of turkey red blood cells on virus haemagglutination was compared (appendix). Sialidase DAS181 (NexBio, San Diego, CA, USA), which removes both  $\alpha$ -2,3-linked and  $\alpha$ -2,6-linked sialic acids, and Sialidase S (Prozyme, Hayward, CA, USA), which removes only  $\alpha$ -2,3-linked sialic acids, were compared with untreated turkey red blood cells as controls.

To assess innate immune responses, primary human pneumocytes and peripheral blood monocyte-derived macrophages were derived and used for infection with influenza virus as previously described.<sup>8,9</sup> They were

See Online for appendix



**Figure 1: Viral replication kinetics of influenza A H7N9 virus in ex-vivo cultures of bronchus (A) and lung (B), infected with 10<sup>6</sup> TCID<sub>50</sub>/mL of influenza viruses at 37°C** Barcharts show the mean virus titre pooled from at least three independent experiments. The horizontal dotted line denotes the limit of detection in the TCID<sub>50</sub> assay; error bars show SEM. Tables show statistical significance between virus titres at each timepoint. Red shows that the reference virus titre (listed at the top of the column) is significantly higher than the comparator (viruses listed on the left) and black shows that it is significantly lower. TCID<sub>50</sub>=tissue culture infective dose. ns=non-significant. \*p<0.0001.

infected with influenza A viruses at a multiplicity of infection of two. The primers and methods used for these assays have been reported previously<sup>8,9,14,16</sup> and detailed methods for quantification of cytokine mRNA protein are available in the appendix.

### Statistical analysis

Experiments with the human ex-vivo cultures and in-vitro cultures of macrophages and pneumocytes were done independently with at least three different donors, each in duplicate. Results shown in figures are the calculated mean and SEM. Mock infected tissues served as negative controls. The differences in  $\log_{10}$  transformed viral titres and quantitative cytokine and chemokine mRNAs between viruses and over time were compared with two-way ANOVA followed by a Bonferroni multiple-comparison test. Differences were deemed significant at  $p < 0.05$ .

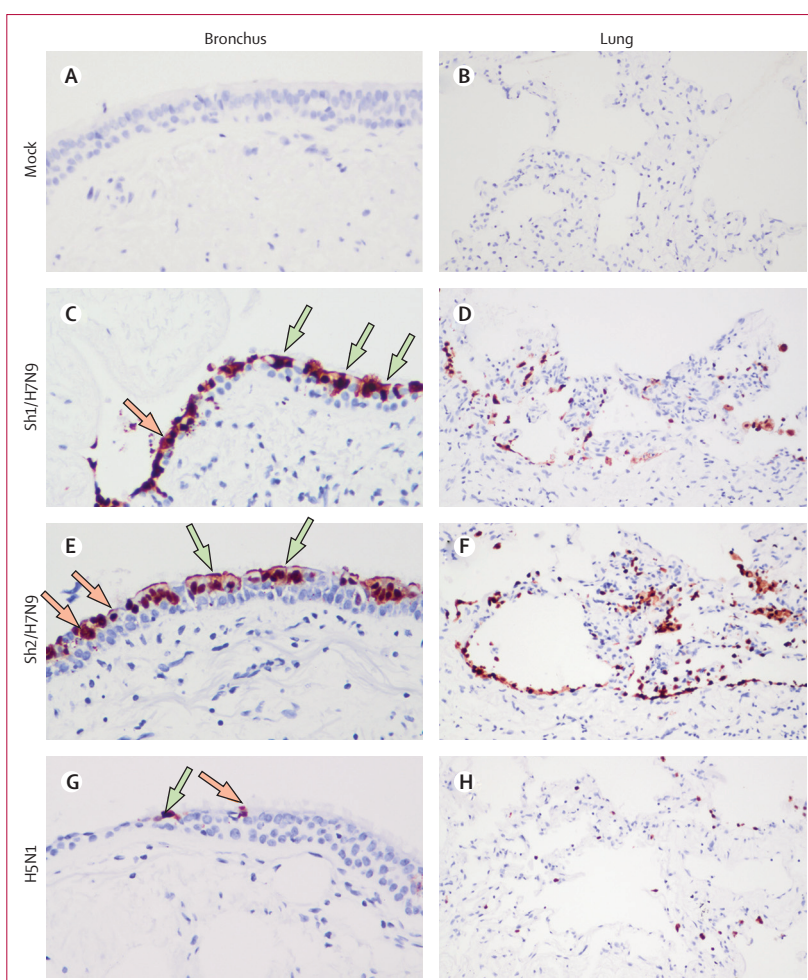
### Role of the funding source

The sponsors had no role in study design, data collection, analysis, or interpretation, or in the writing of the report. JSMP, YG, and JMN had full access to all data in the study and had final responsibility for the decision to submit for publication.

### Results

The human A H7N9 viruses Sh1/H7N9 and Sh2/H7N9 replicated efficiently in ex-vivo cultures of the human bronchus and lung (figure 1). Sh1/H7N9 and Sh2/H7N9 viruses replicated similarly in bronchus, but Sh1/H7N9 replicated to significantly higher titres than Sh2/H7N9 in the lung. Sh1/H7N9 and Sh2/H7N9 virus titres in the bronchus and lung were higher than those observed with H5N1 virus. Sh1/H7N9 and Sh2/H7N9 virus titres in bronchus were similar to that observed with the H1N1pdm virus, but Sh1/H7N9 replicated to higher titres in the lung. Sh1/H7N9 replicated to higher titres than NL/219/H7N7 virus, and NL/219/H7N7 replicated to higher titres than H5N1 virus, in bronchus and lung. Limited experimental replicates of ex-vivo nasopharyngeal (n=2) and tonsil (n=1) cultures restrict statistical analysis, but there was a trend suggesting productive replication of Sh1/H7N9 and Sh2/H7N9 in nasopharynx (appendix). Duck/H7N9 failed to replicate in any of these tissues. Sh2/H7N9 replicated in nasopharyngeal tissues at 33°C (appendix).

Immunohistochemistry showed that Sh1/H7N9 and Sh2/H7N9 viruses extensively infected bronchial epithelium, infecting both ciliated and non-ciliated bronchial epithelial cells (figure 2C, E), the extent of infection being greater than that seen with H5N1 (figure 2G) and other avian viruses (table 1). In the lung, Sh1/H7N9 and Sh2/H7N9 infected mainly type II pneumocytes (figure 3) and alveolar macrophages (figure 3 and appendix). There was no evidence of infection of vascular endothelium in the blood vessels of the lung. The duck/H7N9 virus, which differs in its



**Figure 2: Tissue tropism of influenza A H7N9 virus in ex-vivo cultures of bronchus and lung**

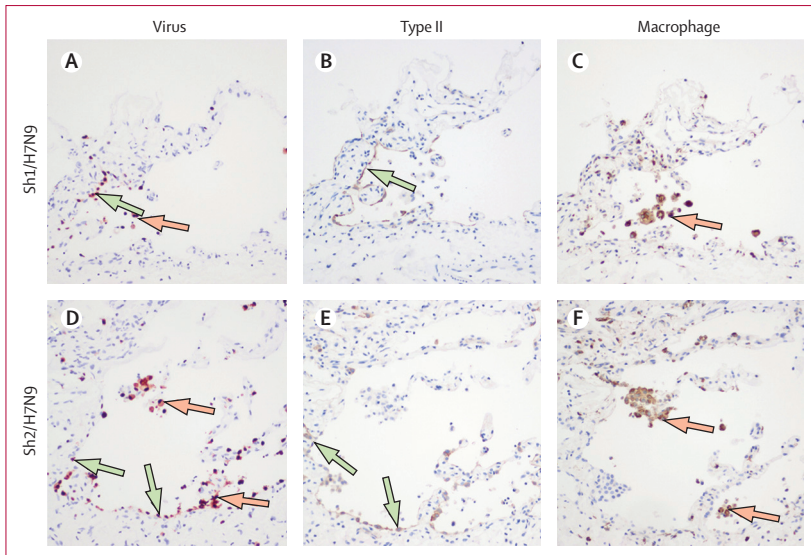
Formalin-fixed paraffin-embedded sections of bronchus (A, C, E, G) and lung (B, D, F, H) after 24 h infection with mock (A, B), Sh1/H7N9 (C, D), Sh2/H7N9 (E, F), and H5N1 (G, H) viruses. Sections were stained with a monoclonal antibody against the influenza nucleoprotein with positive cells identified as a red-brown colour. Ciliated and non-ciliated cells are identified with green and orange arrows, respectively.

	Bronchus	Lung
Sh1/H7N9	++	++
Sh2/H7N9	++	++
NL/219/H7N7	+	+
H5N1	+/-	+
H1N1/pdm	+	++
Duck/H7N9	Sparse	-

Sections were stained with a monoclonal antibody to influenza nucleoprotein and scored in a semiquantitative manner: ++=more than 20% of epithelial cells have positive antigen. +=5–20% of epithelial cells have positive antigen. +/-=less than 5% of epithelial cells have positive antigen. Sparse=only 1–3 positive cells identified per section. -=no positive cells identified.

**Table 1: Tissue tropism of influenza virus infection of human bronchus and lung as assessed by immunohistology**

genetic origin from the viruses causing the current human H7N9 outbreak, failed to infect or replicate in any of the human tissues tested, with the exception of



**Figure 3: Cellular localisation of influenza antigen in human lung tissues infected with influenza A H7N9 virus** Serial sections of ex-vivo cultures of human lungs infected with Sh1/H7N9 (A–C) and Sh2/H7N9 viruses (D–F). Sections were stained with monoclonal antibodies to influenza nucleoprotein (A, D), surfactant protein D (B, E), and CD68 (C, F). Green arrows show type II pneumocytes and orange arrows show alveolar macrophages.

	Control	DAS181 treated	Sialidase S treated
Sh1/H7N9	128	0	128
Sh2/H7N9	64	0	64
H5N1	64	0	0
H1N1pdm	32	0	32

The reciprocal of the haemagglutination titre is denoted. Three independent experiments were done with identical results.

**Table 2: Effect of desialylation on virus haemagglutination of turkey red blood cells**

occasional cells expressing viral antigen seen in the bronchial epithelium (table 1).

Treatment of turkey red blood cells with a sialidase that specifically cleaves the  $\alpha$ -2,3-linked sialic acid with galactose did not have any effect on haemagglutination of either Sh1/H7N9 or Sh2/H7N9, whereas it completely abolished haemagglutination of H5N1 virus (table 2). As a control, DAS181, a sialidase that cleaves both the  $\alpha$ -2,3 and  $\alpha$ -2,6 links of sialic acid, abolished the haemagglutination of human and avian influenza viruses including that of Sh1/H7N9 and Sh2/H7N9.

In human peripheral blood monocyte-derived macrophages, influenza viral matrix (M) gene expression of Sh1/H7N9, Sh2/H7N9, H5N1, and H1N1pdm viruses were generally similar at 3, 8, and 24 h post infection, but Sh2/H7N9 had significantly higher M-gene copy numbers compared with H1N1pdm virus at 8 h after infection and H5N1 had significantly higher M-gene expression than H1N1pdm at 3 h after infection. As expected, H5N1 induced significantly more CCL5, CXCL10, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ; at 3, 8, and 24 h post infection),

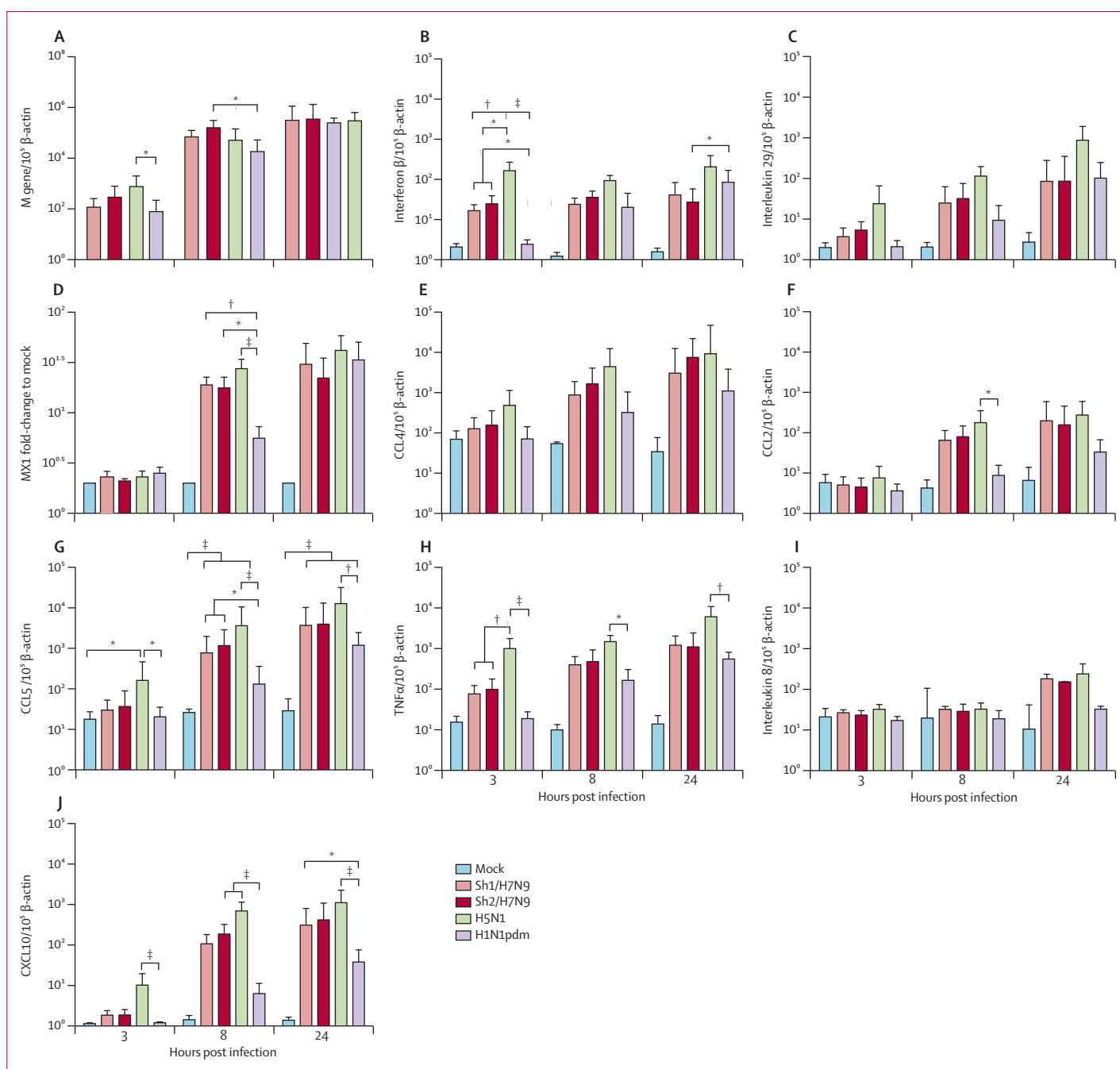
CCL2, MX1 (at 8 h post infection), and interferon  $\beta$  (at 3 h post infection) mRNA compared with H1N1pdm virus (figure 4). Sh1/H7N9 and/or Sh2/H7N9 viruses induced significantly higher concentrations of interferon  $\beta$  (at 3 h post infection), MX1 and CCL5 (at 8 h post infection), and CXCL10 (at 8 h and 24 h post infection) mRNA than did H1N1pdm virus, but less interferon  $\beta$  and TNF $\alpha$  compared with H5N1 at 3 h post infection (figure 4). In parallel, H5N1 virus elicited more CCL5 and TNF $\alpha$  protein secretion compared with H1N1pdm (figure 5). Overall, the induction of proinflammatory cytokines by Sh1/H7N9 or Sh2/H7N9 appeared to be intermediate between those induced by H5N1 and H1N1pdm viruses. Sh1/H7N9 and Sh2/H7N9 viruses both induced significantly higher concentrations of CCL5 and CXCL10 mRNA than did H1N1pdm virus, but less interferon  $\beta$  and TNF $\alpha$  compared with H5N1 (figure 4).

The mRNA expression of genes related to interferon and interferon receptor signalling in human peripheral blood monocyte-derived macrophages after Sh1/H7N9, Sh2/H7N9, H5N1, and H1N1pdm virus infection was investigated by cDNA PCR array and expressed as fold-change over mock infected cells (appendix). Only genes that were three or more fold changes overexpressed or underexpressed relative to H1N1pdm were noted. The interferon stimulated genes *MX1*, *ISG15*, *IFI35*, *IFI44*, *IFIH1*, and *OAS1* were effectively upregulated by Sh1/H7N9 and Sh2/H7N9 infections (data not shown). Expression of *IL20RB*, *IFNAR1*, *IL20RA*, *IL22RA2*, *IL2RB*, *IL9R*, and *IRF4* were overexpressed by either Sh1/H7N9 or Sh2/H7N9 infection compared with H1N1pdm infection. *IL10RB* and *IL4R* were more downregulated by Sh1/H7N9 or Sh2/H7N9 compared with H1N1pdm.

In primary human pneumocytes, replication of Sh1/H7N9 and Sh2/H7N9 viruses (multiplicity of infection of two) were similar, as assessed by viral M-gene quantitation at 1, 6, and 24 h after infection, whereas Sh2/H7N9 had a significantly higher M-gene level compared with H5N1 ( $p=0.05$ ) and H1N1pdm ( $p=0.0346$ ) at 24 h after infection (figure 6A). As expected, H5N1 virus induced significantly more levels of interferon  $\beta$ , interleukin 29, CCL4, CCL5, and CXCL10 mRNA than did H1N1pdm virus infection. Sh1/H7N9 and/or Sh2/H7N9 elicited significantly more interferon  $\beta$ , interleukin 29, CCL5, and CXCL10 mRNA than did H1N1pdm virus infection. However, Sh1/H7N9 and Sh2/H7N9 viruses both elicited less interferon  $\beta$ , interleukin 29, and CCL5 mRNA than did H5N1 virus (figure 6). In virus-infected cell supernatants, H5N1 virus induced significantly more CCL5 protein than did H1N1pdm, Sh1/H7N9, and Sh2/H7N9 viruses and more CXCL10 protein than did H1N1pdm (figure 7).

## Discussion

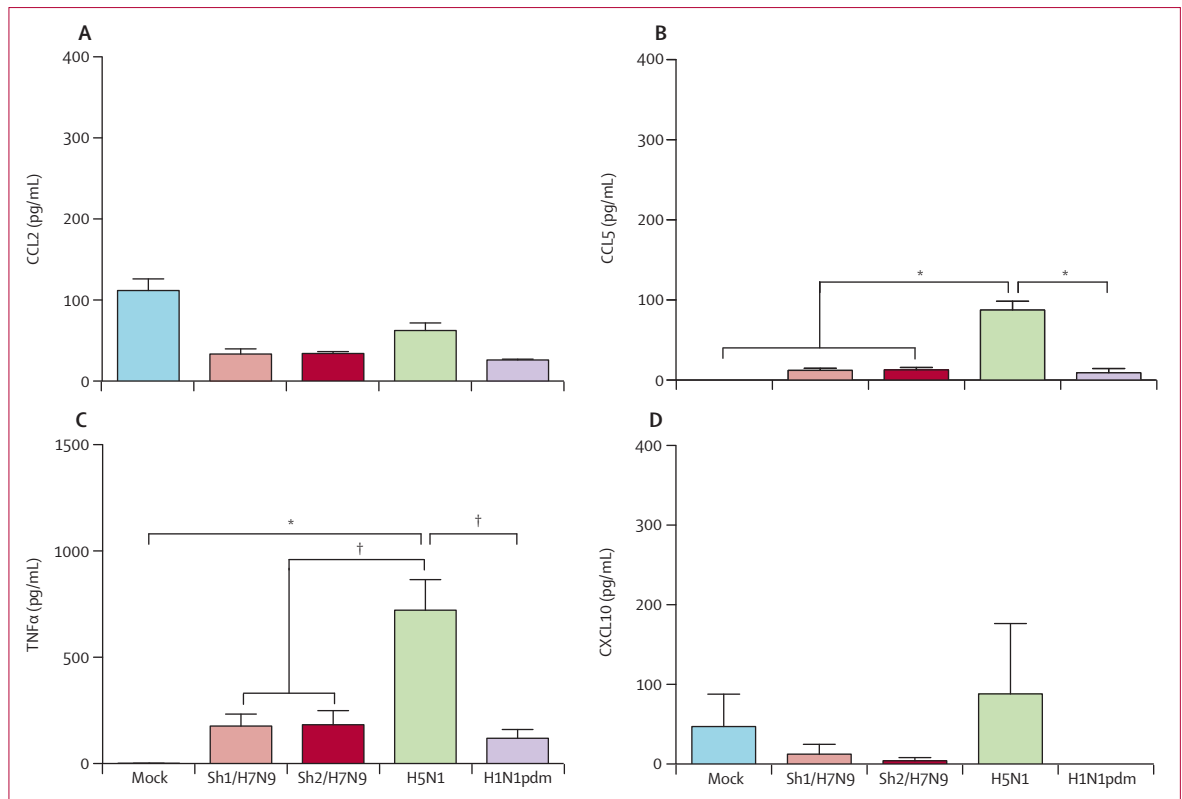
Sh1/H7N9 and Sh2/H7N9 viruses replicated efficiently in ex-vivo cultures of human bronchus and lung with



**Figure 4:** Cytokine and chemokine mRNA expression profile in peripheral blood monocyte-derived macrophages infected with mock, Sh1/H7N9, Sh2/H7N9, H5N1, and H1N1pdm viruses. Expression of the influenza matrix (M) gene (A), interferon  $\beta$  (B), interleukin 29 (C), MX1 (D), CCL4 (E), CCL2 (F), CCL5 (G), tumour necrosis factor  $\alpha$  (TNF $\alpha$ ; H), interleukin 8 (I), and CXCL10 (J) at 3, 8, and 24 h after infection. Graphs show mean mRNA copies expressed per  $10^5$   $\beta$ -actin copies from three independent experiments; error bars show SEM. MX1=myxovirus resistance 1. CCL=chemokine (C-C motif) ligand. CXCL10=chemokine (C-X-C motif) ligand 10. \* $p$ <0.05. † $p$ <0.01. ‡ $p$ <0.005.

peak viral titres similar to and exceeding that seen with H1N1pdm (panel). These findings emphasise the relative ease with which H7N9 viruses can infect human beings and is compatible with epidemiological observations. Peak viral titres in the bronchus and lung were higher than those observed with other avian influenza viruses

that have infected humans such as highly pathogenic avian influenza H5N1 and H7N7. By contrast, the duck/H7N9 virus, a typically avian virus genetically different from Sh1 and Sh2/H7N9 viruses in all eight gene segments, failed to replicate efficiently in any human respiratory tissue.



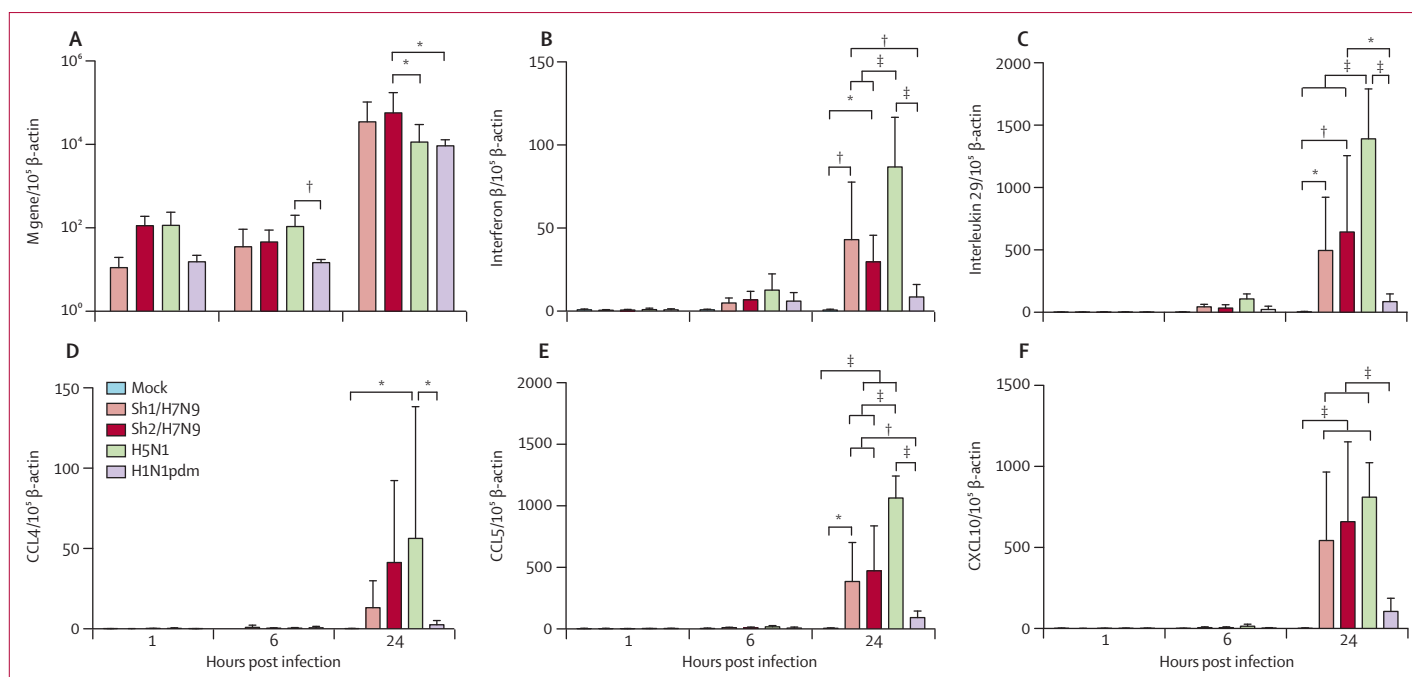
**Figure 5:** Cytokine and chemokine protein expression in cell culture supernatants of peripheral blood monocyte-derived macrophages infected with mock, Sh1/H7N9, Sh2/H7N9, H5N1, and H1N1pdm viruses

Expression of CCL2 (A), CCL5 (B) tumour necrosis factor  $\alpha$  (TNF $\alpha$ ; C), and CXCL10 (D) at 24 h post infection by ELISA (R&D Systems, Minneapolis, MN, USA). Graphs show mean protein from three independent experiments; error bars show SEM. CCL=chemokine (C-C motif) ligand. CXCL10=chemokine (C-X-C motif) ligand 10. \* $p < 0.005$ . † $p < 0.01$ .

Immunohistochemistry of ex-vivo cultures of the human bronchus showed Sh1/H7N9 and Sh2/H7N9 virus infection of both ciliated and non-ciliated epithelial cells. H5N1 only led to limited infection in the human bronchus with infection restricted to ciliated bronchial epithelial cells. In the alveoli, H7N9-infected cells were mainly type II pneumocytes and macrophages. Type II alveolar epithelial cells are important in producing surfactant proteins A and D and are crucial in reconstituting damaged type I alveolar epithelium. Loss of type II pneumocytes would lead to impairment of the repair processes after alveolar damage. The tropism of Sh1/H7N9 and Sh2/H7N9 infection in the alveoli is similar to that seen with H5N1 infection,<sup>17</sup> but the extent of infection of the bronchus and lung is significantly greater than that seen with H5N1. Although individual genetic and environmental variability might result in diverse phenotypes, that three separate donors yielded very similar trends is interesting (data not shown). Future work needs to address whether genetic (eg, *IFITM3*) or other host factors play a part in susceptibility to infection of ex-vivo human respiratory cultures.

Sh1/H7N9 and Sh2/H7N9 viruses differ in aminoacid residues Gln226Leu, Ser138Ala, and Gly186Val (H7/H3

numbering) in haemagglutinin, which are expected to enhance binding of Sh2/H7N9 to  $\alpha$ -2,6 glycans found in the human upper airways.<sup>4</sup> However, Sh1/H7N9 and Sh2/H7N9 infect human bronchus with similar efficiency. A sialidase specific for cleaving the  $\alpha$ -2,3 glycosidic abolished the haemagglutination of turkey red blood cells by H5N1 virus, but did not affect haemagglutination by Sh1/H7N9 or Sh2/H7N9 viruses suggesting that both Sh1/H7N9 and Sh2/H7N9 predominantly bind  $\alpha$ -2,6 glycans, irrespective of differences in aminoacid residues Gln226Leu, Ser138Ala, and Gly186Val of haemagglutinin. Both Sh1/H7N9 and Sh2/H7N9 have lost a glycosylation site (Thy160Ala) in the 150-loop of haemagglutinin that is known to result in increased affinity for binding  $\alpha$ -2,6-linked glycans in H5N1 viruses.<sup>4</sup> By contrast, others have reported that a recombinant Sh2/H7N9-like virus haemagglutinin bound poorly to human trachea and a Gly228Ser aminoacid change was needed to confer improved binding to the human upper airways.<sup>18</sup> Recombinant viral haemagglutinin might not accurately reflect haemagglutinin binding of native virions where multivalent trimeric haemagglutinin is present together with neuraminidase and fails to take into account any



**Figure 6: Cytokine and chemokine mRNA expression profile in human pneumocytes infected with mock, Sh1/H7N9, Sh2/H7N9, H5N1, and H1N1pdm viruses**  
Expression of influenza matrix (M) gene (A), interferon  $\beta$  (B), interleukin 29 (C), CCL4 (D), CCL5 (E), and CXCL10 (F) at 1, 6, and 24 h after infection. Graphs show mean mRNA copies expressed per  $10^5$   $\beta$ -actin copies from three independent experiments; error bars show SEM. CCL=chemokine (C-C motif) ligand. CXCL10=chemokine (C-X-C motif) ligand 10. \* $p<0.05$ . † $p<0.01$ . ‡ $p<0.005$ .

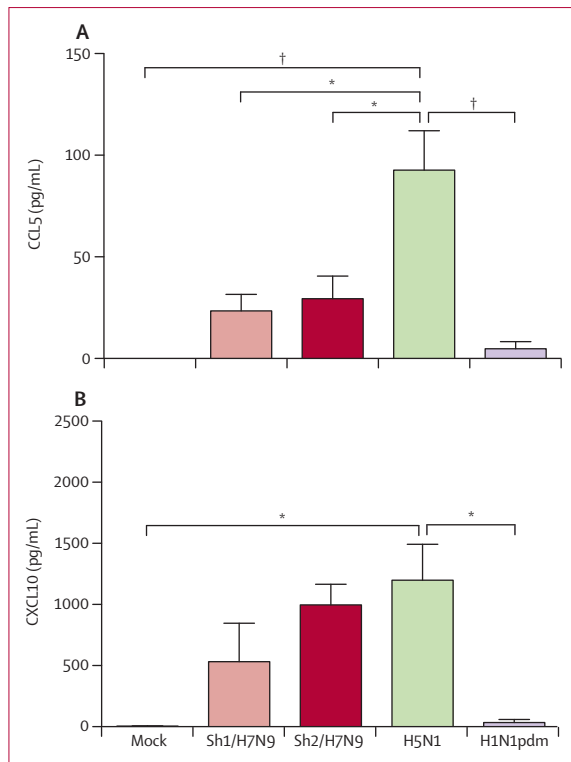
role that the virus neuraminidase (via the catalytic site or the second neuraminidase binding site) might have in virus binding. On the other hand, others have used labelled H7N9 to define virus binding to human respiratory tract tissues, indicating that both Sh1/H7N9 and Sh2/H7N9-like viruses bind very efficiently to the human bronchus (T Kuiken, personal communication).

Recent mass spectrometric data have shown that the bronchus contains sialylated  $\alpha$ -2,3 glycans,<sup>19</sup> explaining its infection by avian viruses (H5N1 and H7N7) whose glycan array profiles indicate predominant  $\alpha$ -2,3 receptor binding. Receptor binding and structural analysis of Sh2/H7N9-like virus haemagglutinin shows efficient binding of both  $\alpha$ -2,3 and  $\alpha$ -2,6 receptors.<sup>20</sup> The functional changes in receptor binding and PB2 Glu627Lys are some of the key functional changes identified by Herfst and colleagues<sup>21</sup> in 2012 to be important in adapting an avian H5N1 virus towards transmission in mammals. Since A/Hong Kong/483/97 (H5N1) virus also has lysine at position 627 in PB2, the efficient replication of H7N9 viruses in human ex-vivo cultures of the bronchus is unlikely to be explained solely on this basis.

Autopsy data for the lung would provide useful data to understand the pathogenesis of human A H7N9 disease. However, autopsy data usually only provide information about lung pathology at late stages of the illness and thus fail to provide insights into virus tropism in the early stages of infection. Thus data from experimental infection of ex-vivo cultures of the respiratory tract provide unique insights into virus tropism and

pathogenesis. Reported patients with H7N9 disease have been older than those with human infections with avian H5N1 or H9N2 viruses, and severe disease appears to be more common with increasing age.<sup>3</sup> The biological basis for this unusual epidemiological pattern needs to be understood because it differs from that seen in individuals infected with H5N1. Whether milder cases in young people are unrecognised, thereby skewing the case-fatality ratio as well as the age distribution, is unclear. Paediatric lung and bronchial tissues differ from adult tissues in the balance of sialylated  $\alpha$ -2,3 and  $\alpha$ -2,6 glycans,<sup>19</sup> and this factor might contribute to the increased disease severity with increasing age. However, using similar techniques we found that Sh1/H7N9 and Sh2/H7N9 viruses efficiently infected ex-vivo lung from a 1-year-old child (data not shown). Chronic lung disease increases numbers of type II pneumocytes (the target cells for influenza viruses) and might contribute to an increased disease severity compared with younger, healthier individuals.<sup>22</sup>

Dysregulation of proinflammatory innate immune responses by highly pathogenic avian influenza H5N1 virus contributed to the unusual severity of human H5N1 disease and investigation of whether Sh1 or Sh2/H7N9 virus infections lead to exaggerated proinflammatory innate immune responses was relevant. Since ex-vivo cultures are heterogeneous in cell type and susceptibility to virus and the infecting dose cannot be precisely controlled, we chose to compare innate immune responses to H7N9 infection with that of highly pathogenic



**Figure 7: Cytokine and chemokine protein expression in cell culture supernatants of pneumocytes infected with mock, Sh1/H7N9, Sh2/H7N9, H5N1, and H1N1pdm viruses**

Expression of CCL5 (A) and CXCL10 (B) at 24 h post infection by ELISA (R&D Systems, Minneapolis, MN, USA). Graphs show mean protein from three independent experiments; error bars show SEM. CCL=chemokine (C-C motif) ligand. CXCL10=chemokine (C-X-C motif) ligand 10. \* $p < 0.05$ . † $p < 0.01$ .

avian influenza H5N1 and H1N1pdm viruses in well defined primary human macrophages and alveolar epithelial cells. By comparison with H5N1 virus (a high cytokine-inducing virus) and H1N1pdm virus (a low cytokine-inducing virus), Sh1/H7N9 and Sh2/H7N9 appear to have an intermediate phenotype in their intrinsic capacity to induce proinflammatory cytokines such as CCL5 and TNF $\alpha$ . The expression of interferon stimulated genes (eg, *MX1*, *ISG15*) did not appear to be impaired. Lungs and serum of mice experimentally infected with Sh2/H7N9 also had lower concentrations of proinflammatory chemokines and cytokines compared with those of H5N1-infected mice.<sup>23</sup> In a patient with mild disease and one with severe disease in whom cytokine concentrations were studied, there was no difference in proinflammatory cytokines in serum. However, the patient who died had high serum concentrations of the anti-inflammatory cytokine serum interleukin 10.<sup>5</sup> Raised plasma or lung cytokine concentration might be secondary to more severe pathology in severely ill patients,<sup>24</sup> or secondary to increased replication competence of a virus rather than being the intrinsic cause of such pathology. Therefore, the experiments we have described in which similar virus infecting doses of different viruses are

#### Panel: Research in context

##### Systematic review

We searched PubMed on May 31, 2013 with the terms "H7N9" and "virus tropism" or "innate immunity" or "autopsy" and found none pertaining to the novel influenza A H7N9 virus. No date or language restrictions were applied. There were papers pertaining to other H7 influenza viruses and one pertaining to the pathogenesis of this virus. Those relevant to the data presented in this Article have been cited in the bibliography. An understanding of the tropism of a novel influenza virus for the human respiratory tract is important to assess the zoonotic and pandemic risk posed by these viruses.

##### Interpretation

The novel avian origin human influenza A H7N9 viruses replicated more efficiently than did other avian influenza viruses and similarly to the pandemic 2009 H1N1 virus in ex-vivo cultures of the human respiratory tract. Collectively, the results suggest that the novel H7N9 viruses pose a significant zoonotic and pandemic threat.

compared for their capacity for cytokine induction provides important data to define the intrinsic viral capacity for proinflammatory cytokine induction, which might be of relevance for pathogenesis and therapy. From our findings, Sh2/H7N9-like viruses appear to be intrinsically more potent inducers of the proinflammatory cytokine responses than are H1N1pdm viruses, but less so than H5N1 viruses. Thus, proinflammatory cytokine responses might contribute in part to the severity of human H7N9 disease, but the unusual tissue tropism of this virus for human bronchus and lung and attendant direct viral cytopathology probably has a more important role in pathogenesis, as was seen with the novel Middle East respiratory syndrome coronavirus.<sup>16</sup>

Our findings suggest that Sh2/H7N9-like avian viruses are exceptionally fit to infect mammalian species including human beings. Ferrets experimentally infected with Sh2/H7N9 showed efficient virus replication in the respiratory tract with inefficient ferret-to-ferret transmission by airborne droplets.<sup>25</sup> Although no sustained human-to-human transmission has been noted to date, the efficient replication of these H7N9 viruses in the human respiratory tract and the detection (albeit inefficient) of ferret-to-ferret transmission by the airborne route by these viruses without previous adaptation increases the pandemic concern associated with these viruses.

##### Contributors

MCWC contributed to study design and coordination, analysis and interpretation of results, experiments, and writing of the report. RWYC contributed to study design and coordination, analysis and interpretation of results, and writing of the report. LLYC and CKPM did experiments and contributed to analysis and interpretation of results. KPYH, JHMF, and KPT did experiments and contributed to analysis of results. LLMP developed quantitative PCR assays. YG contributed to study design and



critical review of the report. JMN contributed to study design, analysis and interpretation of results, and writing of the report. JSMP contributed to study design and overall coordination, analysis and interpretation of results, and writing of the report.

#### Conflicts of interest

We declare that we have no conflicts of interest.

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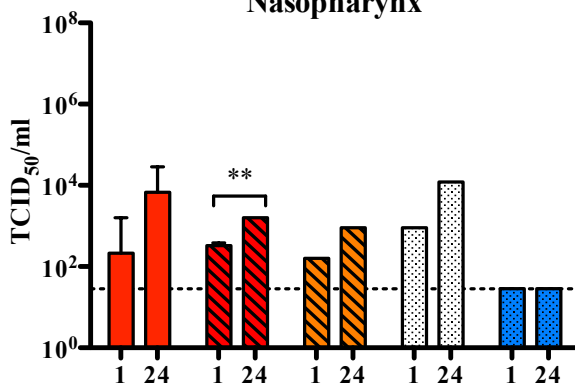
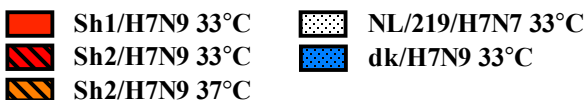
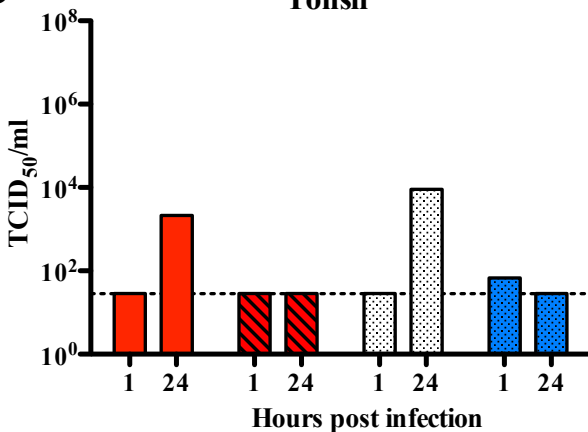
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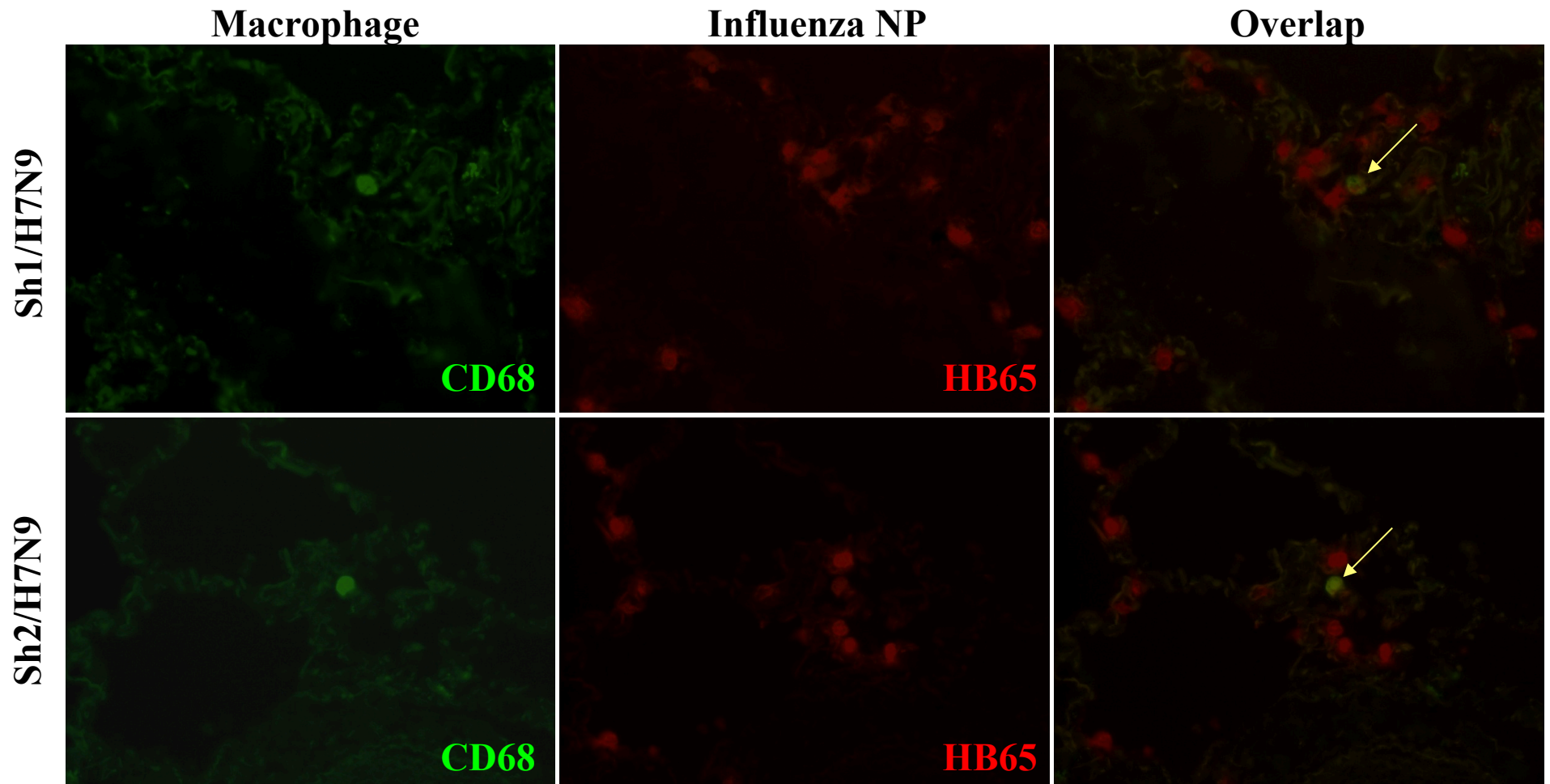
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## Supplementary appendix

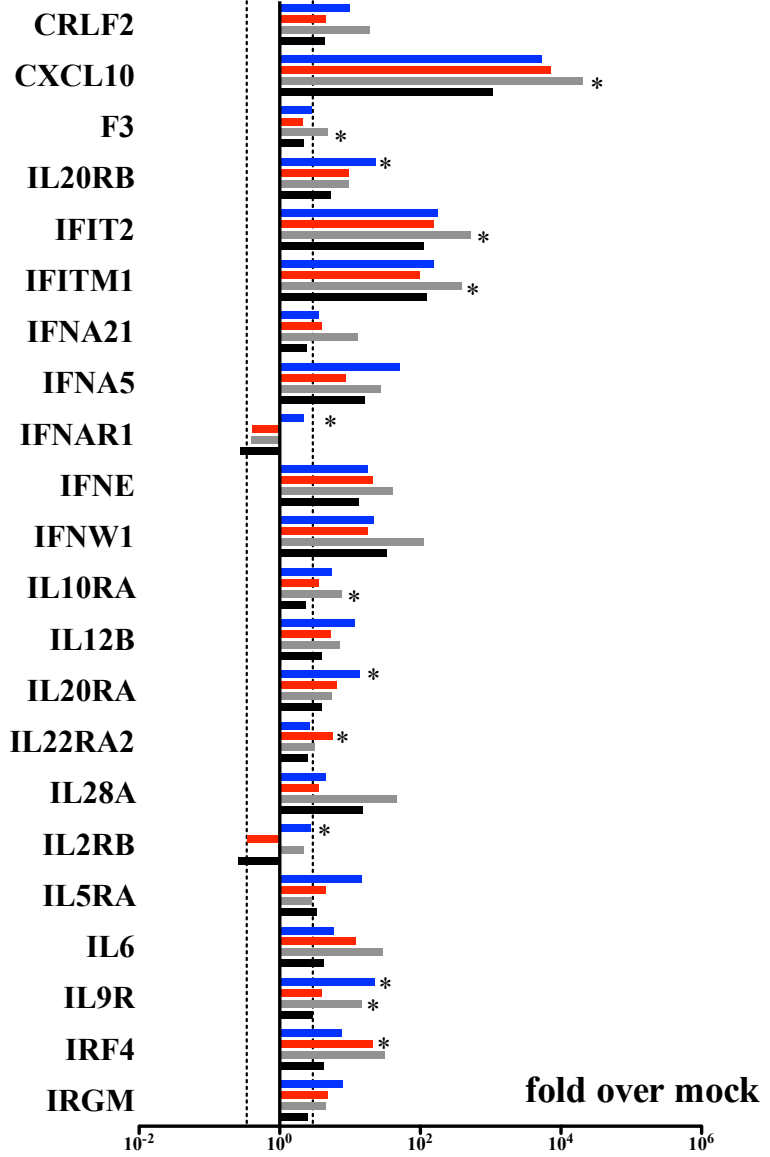
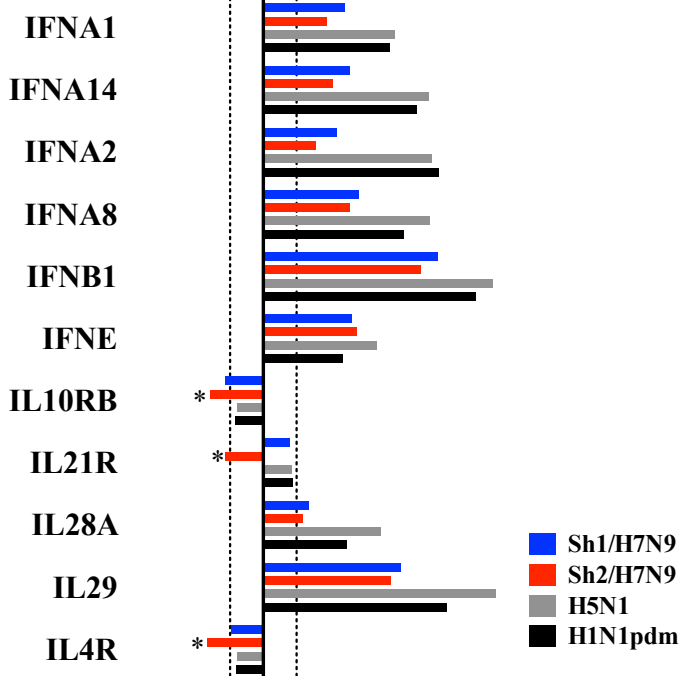
This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Chan MCW, Chan RWY, Chan LLY, et al. Tropism and innate host responses of a novel avian influenza A H7N9 virus: an analysis of ex-vivo and in-vitro cultures of the human respiratory tract. *Lancet Respir Med* 2013; published online July 25. [http://dx.doi.org/10.1016/S2213-2600\(13\)70138-3](http://dx.doi.org/10.1016/S2213-2600(13)70138-3).

**A****Nasopharynx****B****Tonsil****Supplementary Figure 1<sub>1</sub>**



Supplementary Figure 2

**A****B**

## Supplementary Figure legends

### Supplementary Figure 1

Viral replication kinetics of influenza H7N9 viruses in *ex vivo* cultures of nasopharynx (A) and tonsil (B), infected with  $10^6$  TCID<sub>50</sub>/ml of influenza viruses at 37°C or 33°C, as indicated. The bar chart shows the mean and the SE of mean of the virus titer. Significance is denoted as  $**p < 0.01$ .

### Supplementary Figure 2

Double labeling of lung tissue sections infected with influenza Sh1/H7N9 and Sh2/H7N9 viruses and stained with the antibody to CD68 (macrophage) and HB65 (influenza NP), showing the co-localization of influenza positive cells in alveolar macrophages (yellow arrow). Magnification  $\times 400$ .

### Supplementary Figure 3

Fold regulation of selected genes related to IFN and IFN receptor signaling in human peripheral blood monocyte derived macrophages following influenza Sh1/H7N9, Sh2/H7N9, H5N1 and H1N1pdm virus infection, compared with mock infected cells are shown. mRNA was isolated in triplicate from cells infected at an MOI of two, collected at 24 hpi and examined by real-time RT-PCR array analysis. A total of 84 genes were examined. Only genes which exhibited greater than three-fold change in expression over (A) or under (B) that expressed by H1N1pdm-infected cells are shown. Asterisk signifies genes where statistical significance is achieved ( $p < 0.05$ ) when compared to H1N1pdm. The graph shows the mean fold change from three independent experiments.

## **Material and methods**

### **Viruses**

The viruses used in this study were the avian-origin influenza H7N9 viruses A/Shanghai/1/2013 (Sh1/H7N9) and A/Shanghai/2/2013 (Sh2/H7N9); A/Duck/Jiangxi/3286/2009 (duck/H7N9) which is a duck virus that is of different genetic derivation from the human H7N9 viruses currently causing zoonotic infection; a highly pathogenic avian influenza (HPAI) avian influenza H7N7 virus A/Netherland/219/2003 (NL/219/H7N7) isolated from a fatal human infection; HPAI H5N1 virus isolated from a fatal human infection A/Hong Kong/483/1997 (H5N1); and the 2009 pandemic influenza virus A/California/07/2009 (H1N1pdm). The viruses were passaged in Madin-Darby canine kidney (MDCK) cells and virus stock was aliquoted and titrated to determine tissue culture infection dose 50% (TCID<sub>50</sub>) in MDCK cells. The experiments were carried out in a Bio-safety level 3 (BSL-3) facility in the Centre of Influenza Research, School of Public Health, The University of Hong Kong.

### ***In vitro* and *ex vivo* cell cultures**

Primary human *in vitro* cultures of pneumocytes, peripheral blood monocyte derived macrophages and *ex vivo* cultures of human respiratory tract were derived and cultured as previously described<sup>1-5</sup> with modifications.

### **Human alveolar epithelial cells isolation**

Primary human pneumocytes were isolated using human non-malignant lung tissue obtained from patients undergoing lung resection in the Division of Cardiothoracic Surgery, Department of Surgery, Queen Mary Hospital, Hong Kong SAR, under a study approved by the Institutional Review Board of the University of Hong Kong and Hospital Authority Hong Kong West Cluster. After removing visible bronchi, the lung tissue was minced into pieces of > 0.5 mm thickness using a tissue chopper and washed with balanced salt solution (BSS) containing Hanks' balanced salt solution (Gibco, USA) with 0.7 mM sodium bicarbonate (Gibco, USA) at pH 7.4 three times to partially remove macrophages and blood cells. The tissue was digested using a combination of 0.5% trypsin (Gibco, USA) and 4 U/ml elastase (Worthington Biochemical Corporation, USA) for 40 min at 37°C in a shaking water-bath. The digestion was stopped by adding DMEM/F12 medium (Gibco, USA) with 40% FBS in and DNase I (350 U/ml) (Sigma, USA). Cell clumps were dispersed by repeatedly pipetting

the cell suspension for 10 min. A disposable cell strainer (gauze size of 50  $\mu\text{m}$ ) (BD Bioscience, USA) was used to separate large undigested tissue fragments. The single cell suspension in the flow-through was centrifuged and resuspended in a 1:1 mixture of DMEM/F12 medium and small airway basal medium (SABM) (Lonza, USA) supplemented with 0.5 ng/ml epidermal growth factor (hEGF), 500 ng/ml epinephrine, 10  $\mu\text{g/ml}$  transferrin, 5  $\mu\text{g/ml}$  insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 0.5  $\mu\text{g/ml}$  hydrocortisone, 30  $\mu\text{g/ml}$  bovine pituitary extract and 0.5 mg/ml BSA together with 5% FBS and 350 U/ml DNase I. The cell suspension was plated on a tissue culture grade plastic flask (Corning, USA) and incubated in a 37°C water-jacketed incubator with 5% CO<sub>2</sub> supply for 90 min. The non-adherent cells were layered on a discontinuous cold Percoll density gradient (densities 1.089 and 1.040 g/ml) and centrifuged at 25 $\times$ g for 20 min without brake. The cell layer at the interface of the two gradients was collected and washed four times with BSS to remove the Percoll. The cell suspension was incubated with magnetic beads coated with anti-CD14 antibodies at room temperature (RT) for 20 min under constant mixing. After the removal of the beads using a magnet (MACS CD14 MicroBeads), cell viability was assessed by trypan-blue exclusion. The purified pneumocyte suspension was resuspended in small airway growth medium (Lonza, USA) supplemented with 1% FBS, 100 U/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin and plated at a cell density of  $3\times 10^5$  cells/cm<sup>2</sup>. The cells were maintained in a humidified atmosphere (5% CO<sub>2</sub>, 37°C) under liquid-covered conditions and growth medium was changed daily starting from 60 h after plating the cells. When the cell layer approached 75% confluence, the pneumocytes were trypsinised and detached into Hank's buffered saline solution

### **Isolation and culture of primary human peripheral blood monocyte derived macrophages**

Peripheral blood leucocytes were separated from buffy coats of healthy blood donors from the Hong Kong Red Cross Blood Transfusion Service by centrifugation on a Ficoll-Paque density gradient and monocytes were purified by adherence on plastic petri dishes as described previously<sup>3</sup>. Monocytes were seeded onto tissue culture plates in RPMI 1640 medium supplemented with 5% heat-inactivated autologous plasma. The purity of the monocyte preparations was confirmed by immunostaining for the surface receptor CD14 (BD Biosciences, USA). Cells were allowed to differentiate for 14 days *in vitro* before use.



Culture medium was changed to macrophage serum free medium SFM (Gibco, USA) a week before infection.

### ***Ex vivo* culture of human respiratory tract**

Fresh biopsies of human bronchus, lung parenchyma, tonsil and/or nasopharynx obtained from patients undergoing surgical resection of lung tissue at Department of Cardiothoracic Surgery or Ear Nose & Throat surgery, The University of Hong Kong, Queen Mary Hospital as part of clinical care but surplus for routine diagnostic requirements were used in this study. All the studies were approved by the Institutional Review Board of the University of Hong Kong and Hospital Authority Hong Kong West Cluster. For the *ex vivo* cultures of human lung and bronchus, the age range of the donors from Hong Kong providing bronchus and lung tissues was from age of 51 to 65 years. The lung tissue fragments were placed into culture medium (F-12K nutrient mixture with L-glutamine, and antibiotics) in 24-well tissue culture plates incubated at 37°C. The tonsil, nasopharyngeal and bronchial biopsies or tissue fragments were placed into culture medium (F-12K nutrient mixture with L-glutamine, and antibiotics) incubated at 37°C or 33°C as indicated. The tonsil, nasopharyngeal and bronchial tissues were incubated with a sterile surgical pathology sponge to establish an air-liquid interface condition in 24-well culture plates.

### **Infection of human pneumocytes and peripheral blood monocyte derived macrophages *in vitro***

Human pneumocytes and macrophages cultures were infected with influenza A viruses at a MOI of two for the analysis of cytokine and chemokine expression. MEM medium (Gibco, USA) with 100 U/ml penicillin and 100 µg/ml streptomycin was used as inoculum in the mock infected cells. The cell cultures were incubated with the virus inoculum for one hour in a water-jacketed 37°C incubator with 5% CO<sub>2</sub>. The cells were rinsed three times with warm PBS and replenished with the appropriate growth medium. The infected cells were harvested for mRNA collection at 3, 8 and 24 hpi for macrophages and 1, 6 and 24 hpi for pneumocytes. Influenza viral matrix (M) gene was quantified using qPCR as described<sup>1,2</sup>.

### **Influenza virus infection of *ex vivo* cultures**

Fragments of normal human bronchus, lung parenchyma, tonsil or nasopharynx were cut into multiple 2-3 mm fragments and were infected with the respective influenza A viruses within

two hours of collection. The viruses were used at a titer of  $1 \times 10^6$  TCID<sub>50</sub>/ml (a similar titer to that used by Shinya *et al*<sup>6</sup>) for infecting the *ex vivo* cultures. The biopsy or tissue fragments were incubated at 37°C on the cell culture plates or culture sponge to establish an air-liquid interface for 24 hour for lung and bronchus tissue respectively, at which time the tissues were fixed in 10% neutral buffered formalin and processed for paraffin embedding and immunohistochemistry (see below) using a mouse anti-influenza nucleoprotein antibody (HB65, EVL Laboratories, The Netherlands). To determine productive viral replication in bronchus, lung parenchyma, tonsil or nasopharynx, tissue fragments were cultured in 1 ml of F12K with 100 units/ml penicillin and 100 µg/ml streptomycin at 33°C, and infected with influenza A viruses at a titer of  $1 \times 10^6$  TCID<sub>50</sub>/ml. After one hour, infection the unattached virus was removed by washing and viral yield in the cell free supernatant was assessed at 1, 24, 48 and 72 hpi by titration in quadruplicate in MDCK cells. The increasing virus titers in the cull supernatants provided evidence of productive virus replication.

#### **Viral titration by TCID<sub>50</sub> assay**

A confluent 96-well tissue culture plate of MDCK cells was prepared one day before the virus titration (TCID<sub>50</sub>) assay. Cells were washed once with PBS and replenished with serum-free MEM medium supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin and 2 µg/ml of TPCK (tosylsulfonyl phenylalanylchloromethyl ketone) treated trypsin. Serial dilutions of virus supernatant, from 0.5 log to 7 log, were performed before adding the virus dilutions onto the plates in quadruplicate. The plates were observed for cytopathic effect daily. The end-point of viral dilution leading to CPE in 50% of inoculated wells was estimated using the Karber method<sup>7</sup>.

#### **Haemagglutination (HA) of turkey red blood cells and the effect of desialylation**

Turkey red blood cells (TRBC) were purchased from Lampire, USA. They were washed and reconstituted to 1% in cold PBS. Desialylation was carried out by incubating the cells with either 500U/ml of sialidase, DAS181 (NexBio,USA) or 20mU/ml of Sialidase S (Prozyme, USA) at 37°C for 2h. The treated 1% TRBC were then washed with cold PBS by centrifugation at 2000 rpm until the supernatant was clear and then resuspended in PBS to 0.5% for haemagglutination assay.

Serial two-fold dilutions of virus using PBS were performed in a U-bottom 96 well plate in triplicate. PBS served as negative control of the HA assay. Equal volumes of control (untreated) or sialidase pre-treated 0.5% TRBC were added to the diluted viruses. After 20 min of incubation at room temperature, the plates were read. A red button formed in the negative control. HA titer of each treatment was recorded as a reciprocal of the highest titre that gave haemagglutination.

### **Immunohistochemical staining**

Immunohistochemical staining of the respiratory tract tissue was carried out for the influenza nucleoprotein as follows. The tissue sections were incubated with 0.05% Pronase (Roche, Switzerland) in 0.1% CaCl<sub>2</sub> pH 7.8 at 37 °C for 2 min, blocked with 3% H<sub>2</sub>O<sub>2</sub> in TBS for 10 min followed by treatment with an avidin/biotin blocking kit (Vector Lab, USA). After blocking with 10% normal rabbit serum for 10 min at RT, the sections were incubated with 1/100 (15 µg/ml) HB65 (EVL anti-influenza NP, subtype A) antibody for 1 h at room temperature (RT) followed by biotinylated rabbit anti-mouse (Dako Cytomation, USA) diluted 1/100 for 30 min at RT. After incubation with strep-ABC complex (Dako Cytomation, USA) diluted 1/100 for 30 min at RT, the sections were developed with 0.5 mg/ml DAB (Sigma) in 0.02% H<sub>2</sub>O<sub>2</sub> for 20 min and then microwaved in 10 mM citrate buffer pH 6.0 for 20 min to expose the second antigen. The sections were then incubated with FITC-conjugated CD68 (Dako Cytomation, USA) at 1/10 for 1 hour at RT and then counterstained with 1.5 µM propidium iodide (Sigma, USA) for 4 min and then mounted with fluorescent mounting medium (Dako Cytomation, USA). Antibodies to CD68 for macrophages and surfactant protein D (Abcam, UK) for type II pneumocytes were used for consecutive slide labeling.

### **Quantification of cytokine and chemokine mRNA profile by RT-qPCR**

Primary human pneumocytes and peripheral blood-derived macrophages were infected with influenza A viruses at a multiplicity of infection (MOI) of two. mRNA from infected cells was extracted using RNeasy Mini kit (Qiagen, Germany) and treated with DNase. cDNA was synthesized with Oligo-dT primers and Superscript III reverse transcriptase (Invitrogen, USA) and quantified by real-time PCR amplification using ABI 7500 PCR system (Applied Biosystem, USA). The gene expression profiles of the influenza viral matrix 1 gene; cytokines TNF- $\alpha$ , interleukin (IL)-29, interferon  $\beta$  (IFN $\beta$ ); chemokines CXCL10, RANTES, MCP-1, MIP-1 $\beta$  and interferon stimulated genes Mx1 were quantified and normalized using

the housekeeping gene  $\beta$ -actin mRNA at the time points stated above. The primers and methods used for these assays have been reported previously<sup>1, 4, 8</sup>. RT<sup>2</sup> Profiler PCR array (PAHS-064Z, SABioscience, Qiagen, Germany) is used for the detection of mRNA level of human interferons and receptors. Master-mix composed of Power SYBR Green PCR Master Mix (Applied Biosystem, USA) and cDNA prepared from 500 ng total RNA extracted from macrophages at 24hpi was subjected to real-time PCR amplification for 40 cycles using ABI 7500 PCR system (Applied Biosystem, USA). Gene expression and statistical analysis were calculated according to the manufacturer's instructions.

### **Quantification of cytokine and chemokine proteins in infected culture supernatants by ELISA**

Protein expression levels of CXCL10, IL-8, MCP-1, MIP-1 $\beta$ , TNF $\alpha$ , RANTES, in the virus-free cell supernatant collected from influenza viruses infected alveolar epithelial cells and macrophages were quantitatively determined by ELISA assay as recommended by the manufacturer (R&D Systems, USA). In brief, cells infected with different strains of influenza viruses and cell culture supernatants were collected at 24 hpi. 100  $\mu$ l of supernatant from each sample was processed according to the manufacturer's protocol.

### **Statistical analysis**

Experiments with the human *ex vivo* cultures of bronchus and lung and *in vitro* cultures of macrophages and pneumocytes were performed independently with at least three different donors, each in duplicate. Results shown in Figures are the calculated mean and standard error of mean. Mock infected tissues served as negative controls. The differences in log<sub>10</sub> transformed viral titres, quantitative cytokine and chemokine mRNAs between viruses and over time was compared using two-way analysis of variance followed by a *Bonferroni* multiple-comparison test. Differences were considered significant at a  $p < 0.05$ .

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