Pro-inflammatory cytokine dysregulation is associated with novel avian influenza A (H7N9) virus in primary human macrophages

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Since March 2013, more than 500 laboratory-confirmed human H7N9 influenza A virus infection cases have been recorded, with a case fatality rate of more than 30 %. Clinical research has shown that cytokine and chemokine dysregulation contributes to the pathogenicity of the H7N9 virus. Here, we investigated cytokine profiles in primary human macrophages infected with the novel H7N9 virus, using cytokine antibody arrays. The levels of several pro-inflammatory cytokines, particularly TNF-α, were increased in H7N9-infected macrophages. Induction of the transcriptional and translational levels of the pro-inflammatory cytokines by H7N9 virus seemed to be intermediate between those induced by highly pathogenic avian H5N1 and pandemic human H1N1 viruses, which were detected by ELISA and real-time quantitative PCR, respectively. Additionally, compared with H5N1, the upregulation of pro-inflammatory cytokines caused by H7N9 infection occurred rapidly but mildly. Our results identified the overall profiles of cytokine and chemokine induction by the H7N9 influenza virus in an in vitro cell-culture model, and could provide potential therapeutic targets for the control of severe human H7N9 disease.

INTRODUCTION

In March 2013, three patients in China were found to be infected with a unique avian influenza A (H7N9) virus (Gao et al., 2013b). An outbreak of this disease quickly developed along China’s eastern seaboard, and, as of 23 February 2015, the total number of laboratory-confirmed human infection cases reported to the World Health Organization (WHO) was 571, including 212 deaths (WHO, 2015). While mainland China was the source of nearly all of these cases (552 cases plus several exported cases), several cases were confirmed in Hong Kong (12 cases), Taipei (four cases), Malaysia (one case) and Canada (two cases from China) (WHO, 2015). Most H7N9 patients have a high fever and cough at the onset of the illness, and rapidly develop lower respiratory tract infections, which cause severe symptoms (Gao et al., 2013a; Shen et al., 2014). H7N9 infection causes pneumonia and acute respiratory distress syndrome (ARDS), which result in a case fatality rate of more than 30 % (Chi et al., 2013; Wang et al., 2014; Yu et al., 2013).

Cytokine dysregulation in a host after infection is considered to be an important reason why highly pathogenic avian influenza infections are often fatal (Chi et al., 2013; Mok et al., 2013). Studies have shown that human infection with highly pathogenic avian influenza A (HPAI) (H5N1) can cause hypercytokinaemia, also less formally called a ‘cytokine storm’ (Cheung et al., 2002; Peiris et al., 2009). Consisting of a positive-feedback loop between cytokines and immune cells, hypercytokinaemia is an immune response characterized by highly elevated levels of various cytokines, leading to an overwhelming inflammatory response and tissue destruction (Arilahti et al., 2014; Osterholm, 2005), which can cause serious complications and death. Among H5N1 patients, several pro-inflammatory cytokine levels, such as those of IL-6 and IL-8, have been measured in H7N9-infected patients (Chi et al., 2013; Wang et al., 2014; Zhou et al., 2013). However, the full cytokine expression profile for human H7N9 virus infection and its specific characteristics remain unclear. In this study, primary human monocyte-derived macrophages were used as an in vitro model of infection (Cheung et al., 2002). Cytokine antibody arrays were used to define the full cytokine expression profile. The protein concentrations and transcription levels of IL-6, IL-8, TNF-α, monocyte chemoattractant protein 1 (MCP-1), leptin and RANTES (regulated-on-activation and normally T-cell expressed) were detected by ELISA and real-time quantitative PCR (RT-qPCR), respectively, in samples from H7N9-infected cells at different time points.
Additionally, for each time point, cytokine transcription and protein expression levels induced by the H7N9 virus were compared with those induced by the pandemic H1N1 and HPAI H5N1 viruses.

RESULTS

H7N9, H5N1 and H1N1 influenza A virus can infect primary human monocyte-derived macrophages efficiently

Previous studies have demonstrated that primary human monocyte-derived macrophages are an appropriate in vitro model for investigating the cytokine production induced by influenza virus infection in humans (Cheung et al., 2002). We first isolated monocytes from the whole blood of healthy donors and differentiated the cells into mature macrophages. The differentiated macrophages were infected with influenza A virus subtypes H7N9 (A/Anhui/1/2013), H5N1 (A/Jiangsu/1/2007) and H1N1 (A/Sichuan/1/2009) at an m.o.i. of 2, and three replicate cultures were used in all of the infection groups, including mock infections. The viral titres and survival rates of macrophages at different time points post-infection (p.i.) were determined in triplicate using a TCID50 assay with Madin–Darby canine kidney (MDCK) cells and an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test, respectively (Fig. 1). All three of the influenza viruses replicated efficiently in macrophages. The H5N1 virus reached peak titres at 48 h p.i., while H1N1 and H7N9 viruses reached peak titres at 72 h. The H7N9 virus replicated at a level similar to that observed with H1N1 virus infection in macrophages but at a slightly lower level than that observed with H5N1 (Fig. 1a). Compared with the uninfected control, the survival rates of infected macrophages showed a significant decline at 72 h p.i. The decline induced by H7N9 virus was in between that induced by H1N1 and H5N1 (Fig. 1b).

Several cytokine proteins, especially some pro-inflammatory cytokines, are changed after influenza virus infection

For all three viruses, the three replicate-mixed macrophage supernatants were collected at 24 h p.i., and the cytokines were analysed using protein arrays. Hierarchical clustering was used to analyse the results, and as shown in Fig. 2(a), the relationship of the cytokine protein expression profiles between the H7N9-infected and H5N1-infected macrophages was closer than that between the H7N9-infected and H1N1-infected macrophages, indicating that H7N9 might have characteristics similar to those of H5N1. Several cytokine proteins were highly upregulated or down-regulated when the avian influenza A viruses infected the macrophages (Fig. 2b, Table 1). However, the number of cytokine proteins with concentrations that changed more than twofold was greatest in H5N1-infected cells, whereas the number was smallest in H1N1-infected cells; these results were positively associated with the case fatality rates of these three subtypes of influenza virus. In addition, many of the cytokines that were regulated in infections with all three subtypes were pro-inflammatory, and some, for example TNF-α, exhibited higher fold changes than others.

Variations in the tendencies of typical cytokine proteins at different time points p.i.

Based on the protein array results and considering the original concentrations and fold changes, we chose to measure the
expression levels of MCP-1, IL-6, TNF-α, IL-8, leptin and RANTES at different time points p.i. for each virus. Cytokine protein expression profiles at 3, 6, 12, 24, 48 and 72 h were measured by ELISA in three replicated experiments. As shown in Fig. 3, the H7N9-infected samples had significantly higher concentrations of MCP-1 (at 12 h p.i.), IL-6 (at 12 and 24 h p.i.) and TNF-α (at 6, 12, 24, 48 and 72 h p.i.) than the non-infected samples and H1N1-infected samples, and had lower concentrations of MCP-1, IL-6 and IL-8 (at 48 and 72 h p.i.) and TNF-α (at 12 and 72 h p.i.)

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**Fig. 2.** Cytokine protein expression profiles of primary human macrophages 24 h after infection with three subtypes of influenza A virus, according to a protein array. The samples were mixtures of the three replicate cultures, and the experiment was performed only once. (a) Hierarchical clustering results for the supernatant samples according to the standard values of the protein array. Protein samples with similar relationships clustered more closely together. (b) Highly upregulated or downregulated cytokine proteins following infection, according to the results of the protein array. Signals were normalized and expressed as the ratio to mock-infected cells. FGF-6, Fibroblast growth factor-6; TGF-β3, tumor necrosis factor beta 3; IL-6R, interleukin 6 receptor; TIMP-2, tissue inhibitor of metalloproteinase 2; BLC, B-lymphocyte chemoattractant; BMP-6, bone morphogenetic protein 6; CK β8-1, chemokine-beta-8, CNTF, ciliary neuronotrophic factor; EGF, epidermal growth factor; GDNF, glial-derived neurotrophic factor; Axl, A protein tyrosine kinase (ark); I-TAC, interferon-inducible T cell Alpha chemoattractant.
than the H5N1-infected samples. In particular, compared with H5N1 virus, H7N9 virus induced significantly more IL-6 at 12 and 24 h p.i. In parallel, H5N1 virus induced more MCP-1 (at 12, 24, 48 and 72 h p.i.), IL-6 and IL-8 (at 48 and 72 h p.i.) and TNF-α (at 12, 24, 48 and 72 h p.i.) than H1N1 virus and controls. Overall, the ability of H7N9 virus to induce pro-inflammatory cytokines was between that of H1N1 virus and H5N1 virus.

**Variations in the tendencies of typical cytokine mRNAs at different time points p.i.**

As shown in Fig. 4, the cytokine mRNA expression profiles at 3, 6, 12, 24, 48 and 72 h were measured by RT-qPCR in three replicate experiments. In general, the levels of cytokine mRNAs were upregulated following infection with all three virus subtypes, including leptin and RANTES, although the changes in protein levels of the latter two were not detected using our ELISA kit. The increases in the levels of MCP-1, IL-6 (at 24 h p.i.), IL-8 (at 72 h p.i.), TNF-α (at 12, 24, 48 and 72 h p.i.), leptin (at 24, 48 and 72 h p.i.) and RANTES (at 48 and 72 h p.i.) transcripts following infection with H7N9 were significantly greater than those following infection with H1N1. Additionally, H5N1 increased the levels of MCP-1, IL-6 and IL-8 (at 48 and 72 h p.i.), TNF-α (at 12, 24, 48 and 72 h p.i.) and leptin and RANTES (at 72 h p.i.) transcripts more than H1N1. Compared with H5N1, H7N9 induced more MCP-1, IL-6, TNF-α and leptin transcripts at 24 h p.i. but fewer MCP-1, IL-6, IL-8, TNF-α and leptin transcripts at 48 and/or 72 h p.i., indicating that, for most cytokines, the peak values of the mRNA levels appeared earlier in H7N9 infection. At early time points, the mRNA levels following H7N9 infection were even higher than those observed with H5N1 infection. However, the final mRNA levels with H7N9 infection were lower than those with H5N1 infection. Although the results were less significant, this phenomenon was also observed in the ELISA results, suggesting that the upregulation of pro-inflammatory cytokines occurred more rapidly following H7N9 infection than following H5N1 infection, although the final transcript and protein concentrations were lower following H7N9 infection (Figs 3 and 4).

**DISCUSSION**

A novel recombinant H7N9 avian virus that can cause life-threatening human disease was identified in March 2013, and most of the known patients have died of severe complications, such as pneumonia and ARDS. The cytokine storm caused by cytokine dysregulation after infection is an important cause of serious symptoms and of high mortality. Recent studies have shown that several cytokines are present at higher-than-normal levels in serum samples obtained from H7N9 patients (Chi et al., 2013; Wang et al., 2014; Zhou et al., 2013).

Macrophages play important roles in the body’s immune response. Alveolar macrophages are considered to be the first immune cells to encounter the virus during influenza infections, and in the lower respiratory tract, they function as the major scavenger cells (Tumpey et al., 2005). Compared with human alveolar macrophages, primary human monocyte-derived macrophages from blood can be seen as macrophages that are newly recruited to the lung, and they can release multiple cytokines and chemokines when stimulated. Therefore, instead of human alveolar macrophages, primary human monocyte-derived macrophages could serve as an in vitro model of influenza virus infection. In this study, we used the H7N9 virus to infect macrophages from healthy people, and macrophages infected with a pandemic influenza A (H1N1) virus and with an HPAI (H5N1) virus were used as control groups. We found that both the mRNA and protein levels of relevant cytokines were upregulated following infection with H7N9, to levels between those observed with H1N1 and those observed with H5N1. Previous reports have shown, using serum samples from patients (Peiris et al., 2004) and in vitro cell models...
that the H5N1 virus infection can cause hypercytokinaemia. Additionally, in 2013, Chan et al. (2013) reported that H7N9 virus infection could induce more pro-inflammatory cytokines in human peripheral blood monocyte-derived macrophages. Similarly, we demonstrated that the H7N9 virus could cause cytokine dysregulation at the transcriptional and translational levels, and such changes could cause a cytokine storm and trigger severe tissue damage and death. In addition, we showed that the H7N9-induced H5N1-induced cytokine storms had different characteristics. In general, the H7N9-induced cytokine storm began sooner after infection but resulted in lower final cytokine concentrations than the H5N1-induced cytokine storm, which might be an important reason why H7N9 causes milder clinical symptoms and has a lower case fatality rate than H5N1.

In this study, we chose to analyse the cytokines that were upregulated by influenza viruses. However, our protein array results suggested that a fair number of cytokines were also downregulated after virus infection (Fig. 2b, Table 1). Of particular note was granulocyte–macrophage colony-stimulating factor (GM-CSF), which has been reported to be a resistance factor in influenza virus infection (Huang et al., 2011; Pauksen et al., 2000; Sever-Chroneos et al., 2011). The protein array results showed that infection with all three influenza virus subtypes decreased the concentration of GM-CSF in macrophages (Fig. 2b, Table 1). We suggest that influenza viruses might inhibit the functions of cytokines with antiviral activity, such as GM-CSF, so that they can more easily replicate following infection. Follow-up studies of these antiviral cytokines could suggest new ways in which influenza could be treated.

**METHODS**

**Viruses and cells.** The H1N1 virus (A/Sichuan/1/2009) was isolated from the first known human case of the 2009 influenza pandemic in China (Zhang et al., 2012). The H5N1 virus (A/jiangsu/1/2007) was isolated in 2007 from a respiratory specimen from a patient who died; the patient was from Jiangsu province in China (Wang et al., 2008). The H7N9 virus (A/Anhui/1/2013) was isolated in 2013 from a respiratory specimen from a patient...
who died; this patient was from Anhui province in China (Zhang et al., 2013). Virus stocks were grown in MDCK cells. The infectivity of the virus was assessed by determining TCID_{50} in MDCK cells at different time points. For each time point, gradient dilution was performed for each virus, and each gradient had four replicate cultures. All of the experiments using the H5N1 and H7N9 viruses were conducted in a Biosafety Level 3 facility.

Primary human PBMCs were isolated from the whole blood of healthy donors (obtained from the Jiangsu Engineering Research Center for microRNA Biology and Biotechnology, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, China) by Ficoll-Paque (GE Healthcare Bio-Sciences AB) density-gradient centrifugation and were purified by adherence. The written informed consent of all of the blood donors was obtained when performing this research protocol. The cells were allowed to differentiate for approximately 10 days in RPMI 1640 medium supplemented with 10 % 0.22 mm-filtered autologous serum, 50 U penicillin (Gibco-BRL) ml^{-1}, 50 μg streptomycin (Gibco-BRL) ml^{-1} and 100 ng recombinant human GM-CSF (R&D Systems) ml^{-1}. The macrophages were placed on six-well cell-culture plates.

**Influenza virus infection of macrophages.** Three subtypes of influenza A virus – H1N1, H5N1 and H7N9 – were used to infect differentiated macrophages at an m.o.i. of 2; each virus was used to infect three replicates, and three uninfected cultures were used as mock infections. The virus inoculum was removed after 60 min virus adsorption, and the cells were washed once using Hanks’ balanced salt solution (Gibco-BRL) and incubated in serum-free macrophage medium supplemented with 50 U penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 2 μg N-p-tosyl-l-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) ml^{-1}. At the indicated time points, aliquots of the cell-culture supernatants were collected to analyse cytokine protein expression, and RNA was isolated from cell pellets to analyse cytokine transcript expression. All of the samples were mixtures of the three replicates.

**Protein arrays.** The supernatants of the macrophage cultures were collected at 24 h p.i. The cytokine expression profiles in these supernatants were determined by KangChen Bio-tech in Shanghai, China, using the RayBio Human Cytokine Antibody Array C Series 1000 (8).

**Quantification of cytokine mRNAs by RT-qPCR.** Genomic DNA was removed, and total RNA was isolated using Trizol Reagent (Life Technologies) and reverse transcribed using a PrimeScript RT Reagent kit (Takara Bio). The resulting cDNA was quantified by real-time PCR analysis with an Applied Biosystems 7300 Real-Time PCR System (Life Technologies), using SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio). All of the samples were standardized relative to β-actin.

**Quantification of cytokine proteins by ELISA.** Culture supernatants were collected at the indicated time points p.i., and the
concentrations of cytokine proteins in the culture supernatants were quantified by ELISA (4A Biotech). The detection ranges for the ELISA kits were as follows: MCP-1, 16–1000 pg ml⁻¹; TNF-α, 16–1000 pg ml⁻¹; IL-6, 4–250 pg ml⁻¹; IL-8, 16–1000 pg ml⁻¹; leptin, 32–2000 pg ml⁻¹. All of the samples were diluted to appropriate concentrations before detection.

**Statistical analysis.** The results are expressed as means ± SEM. Two-way ANOVA followed by a Bonferroni post hoc test was used for pairwise comparisons at each time point. P<0.05 was considered statistically significant.

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