Avian influenza virus A H7N9 infects multiple mononuclear cell types in peripheral blood and induces dysregulated cytokine responses and apoptosis in infected monocytes

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Abstract
Most patients with avian influenza A H7N9 virus (H7N9) infection suffer from severe illness, accompanied by dysregulated cytokine/chemokine response, delayed viral clearance and impaired neutralizing antibody response. Here, we evaluated the role of peripheral blood mononuclear cells (PBMCs) in the pathogenesis of H7N9 infection using an ex vivo infection model. H7N9 infected a significantly higher percentage of PBMCs (23.9 %) than those of avian influenza A H5N1 virus (H5N1) (12.3 %) and pandemic H1N1 virus (pH1N1) (5.5 %) (P<0.01). H7N9 infected significantly more B and T lymphocytes than H5N1. When compared with pH1N1, H7N9-infected PBMCs had significantly higher mRNA levels of proinflammatory cytokines and type I interferons (IFNs) at 6 h post-infection (p.i.), but significantly lower levels of IFN-γ and IP-10 at 12 h p.i. Among the PBMCs, CD14+ monocytes were most permissive to H7N9 infection. The percentage of infected CD14+ monocytes was significantly higher for H7N9 than that of pH1N1, but not significantly different from that of H5N1. H7N9-infected monocytes showed higher expression of MIP-1α, MIP-1β and RANTES than that of pH1N1 at 6 h p.i. H7N9-infected monocytes died rapidly via apoptosis. Furthermore, pH1N1- but not H7N9-infected monocytes showed increased expression of the monocyte activation and differentiation markers. Unlike pH1N1, H7N9 showed similar PBMC/monocyte cytokine/chemokine expression profile, monocyte cell death and expression of activation/differentiation markers to H5N1. Besides proinflammatory cytokine activation leading to a cytokine storm, impaired IFN-γ production, rapid monocytc death and lack of monocyte differentiation may affect the ability of H7N9-infected innate immune cells to recruit protective adaptive immunity.

INTRODUCTION
Since 2013, avian influenza A H7N9 virus (H7N9) has been the most common avian influenza virus subtype infecting humans, with a mortality rate of 40 % [1–4]. Clinically, H7N9 infection typically causes rapidly progressive pneumonia with pulmonary or extrapulmonary complications [5, 6]. Ongoing virological surveillance has revealed that H7N9 continues to circulate in farmed poultry [7–9].

Several postulations have been put forward to explain the high virulence of H7N9 infection in humans. First, there is a lack of pre-existing immunity against H7N9 among the general population and even among poultry workers [10–12]. Second, H7N9 infection triggers an exaggerated cytokine and chemokine response [3, 6, 13, 14]. Immunomodulatory treatment with celecoxib, a non-steroidal anti-inflammatory drug, alleviated the lung pathology and improved the survival of H7N9-infected mice [13]. Dysregulated cytokine and chemokine response is a key feature of severe influenza virus infection [6, 13, 15, 16]. Third, there is impaired humoral immune response against H7N9 infection. The convalescent neutralizing antibody titres after natural H7N9 infection in humans were much lower than those of other seasonal influenza virus infections [17]. In a mouse model, the neutralizing antibody after H7N9 infection was much lower than that of influenza A H5N1 virus (H5N1) or 2009 pandemic influenza A H1N1 virus (pH1N1) infection, and the poor antibody response may be related to the internal genes of H7N9 [18]. The relatively low titre of neutralizing antibody in infected...
mammalian hosts certainly impaired viral clearance and increased the severity of this disease.

Innate immune cells play a central role in modulating inflammatory and immune responses during influenza virus infection. Influenza virus can be in direct contact with peripheral blood immune cells in the pulmonary circulation during respiratory tract infection and in the systemic circulation during viraemia [16, 19, 20]. The effect of direct infection of H7N9 on peripheral blood immune cells has been studied previously. H7N9-infected monocyte-derived dendritic cells impair the innate immune response by dampened IFN-α production [21]. H7N9 infection of monocyte-differentiated macrophages induced a rapid proinflammatory cytokine expression, especially TNF-α, although its magnitude was milder than that of H5N1 [22]. However, there have been no reports of H7N9 infection of human monocytes before their differentiation into macrophages and myeloid dendritic cells.

In the current study, we further evaluated the role of infected PBMCs in the pathogenesis of H7N9 infection. We demonstrated that H7N9 can effectively infect PBMCs, especially CD14+ monocytes, and to a lesser extent B and T lymphocytes. The cytokine and chemokine response of H7N9-infected PBMCs is similar to that of H5N1. Importantly, we showed that H7N9 infection of monocytes induced apoptosis, which can affect antigen presentation and hence affect the production of antibodies and perhaps other adaptive immune responses.

RESULTS

H7N9 effectively infected human PBMCs and induced early proinflammatory cytokine responses

We first determined the susceptibility of human immune cells to H7N9, and compared our results with those for H5N1 and pH1N1. Freshly isolated PBMCs were infected with A/Anhui/01/2013 (H7N9), A/VNM/1194/2004 (H5N1) or A/HK/415742/2009 (pH1N1) at an m.o.i. of 2. The number of infected cells was determined by enumerating the number of cells with viral nucleoprotein (NP) expression using flow cytometry assay. The mean percentage of NP positive cells was significantly higher for PBMCs infected with H7N9 (23.9%) than those infected with H5N1 (12.3%) or pH1N1 (5.5%) (P<0.01) (Fig. 1a). Quantitative real-time reverse-transcriptase PCR (RT-PCR) showed an approximately 10-fold increase in viral M gene mRNA in H7N9-infected cells at 6 h post-infection (p.i.) and 12 h p.i. (Fig. 1b), and the transcription then gradually declined with time. However, TCID_{50} assay did not detect any significant increase in viral load in the culture supernatant when compared with the viral titre at 1 h p.i. (Fig. 1c). Overall, all three viruses showed similar increases in viral gene expression without increases in viable virus particle production.

To study the innate immune response of PBMCs to H7N9 infection, quantitative RT-PCR assay was used to study host gene expression. At 6 h p.i., H7N9-infected cells had significantly higher mRNA levels of the proinflammatory cytokines IL-6 and TNF-α, and antiviral cytokines IFN-α and IFN-β, than those of pH1N1-infected cells (Fig. 1d). However, at 12 h p.i., there was no difference in the levels of IL-6, TNF-α, IFN-α and IFN-β between H7N9 and pH1N1. Both H7N9 and H5N1 elicited a low level and transient expression of type II IFN antiviral cytokine/chemokine IFN-γ and IFN-γ-inducible protein 10 (IP-10) at 6 h p.i., which subsided at 12 h p.i. In contrast, pH1N1 induced significantly higher levels of IFN-γ and IP-10 at 12 h p.i., which remained elevated until 24 h p.i. and 72 h p.i., respectively (Fig. 1d, bottom panel). The level and expression pattern of H7N9-infected PBMCs were similar to those of H5N1. Taken together, our data suggested that H7N9 induced a rapid surge in proinflammatory cytokine, but lower level of IFN-γ and IP-10 response, in human PBMCs.

Many PBMC types are susceptible to H7N9, but monocytes are especially susceptible

To determine the different cell types in PBMCs that are preferentially targeted by H7N9, we employed a multi-coloured flow cytometry technique. The cell type most permissive to H7N9 infection was CD14+ monocytes, which was significantly higher than that of pH1N1-infected cells (mean percentage of infected cells, 33.9 versus 7%, P<0.01) (Fig. 2a). However, there was no significant difference in the proportion of monocytes infected by H7N9 or H5N1. H7N9 infected significantly higher proportions of B cells and T cells than H5N1 [21.8 versus 10.0% for B cells (P<0.05), 13.6 versus 3.4% for T cells (P<0.01)] and pH1N1 [21.8 versus 10% for B cells (P<0.010), 13.6% versus 2.1% for T cells (P<0.01)]. These findings indicated that different immune cell types in PBMCs are susceptible to H7N9, especially CD14+ monocytes.

The number of CD14+ monocytes was reduced by >80% in infection by both H7N9 and H5N1 at 12 h p.i. when compared to mock-infected cells. On the contrary, there was no reduction in the number of CD14+ monocytes at 12 h p.i. or 24 h p.i. for pH1N1 (Fig. 2b, c). However, the proportion of T cells (CD3+CD4+, CD3+CD8+) and B cells (CD19+) showed no significant reduction.

H7N9 infection of CD14+ monocyte induced cytokine responses

Since the monocyte was the most susceptible cell type in H7N9 infection, we specifically investigated the effect of H7N9 infection on monocytes. CD14+ monocytes were purified from PBMCs using CD14 magnetic beads. Purified CD14+ monocytes were then infected with H7N9, H5N1 or pH1N1. Twelve hours post-virus inoculation, immunofluorescent staining showed abundant viral NP-positive cells in H7N9- and H5N1-inoculated monocytes. In contrast, only a few NP-positive cells were found in pH1N1-inoculated monocytes (Fig. 3a). The infectivity was further quantified by flow cytometry assay. At 12 h p.i., up to 70% of monocytes were positive for viral NP in H7N9 and H5N1 infection, while only 15% of monocytes were infected by pH1N1.
These findings further indicated that human monocytes were highly susceptible to H7N9 and H5N1.

The cytokine/chemokine expression profiles in infected purified monocytes were determined using quantitative real-time RT-PCR. At the early stage of infection (6 h p.i.), the induction of IL-6 and TNF-α mRNA expression of H7N9- and H5N1-infected monocytes was higher than that of pH1N1-infected monocytes, but there was no significant difference for IL-1β (Fig. 4a). H7N9-infected monocytes had significantly higher levels of IFN-α and IFN-β than pH1N1 at 6 h p.i., but there was no significant difference for IFN-γ (Fig. 4b). The peak levels of the chemokines MIP-1α, MIP-1β, RANTES, MCP-1 and IP-10 occurred at 6 h p.i. for H7N9-infected monocytes, while the peak level of the chemokine IL-8 occurred at 24 h p.i. (Fig. 4c). A similar pattern of gene expression was found for H5N1 virus. However, when compared to pH1N1, H7N9 induced significantly higher levels of proinflammatory cytokine/chemokine (IL-6, TNF-α, MIP-1α, MIP-1β and RANTES) gene expression, but lower levels of MCP-1, IL-8 and IP10. Hence, the induction of cytokine/chemokine genes was similar between H7N9 and H5N1, but different from pH1N1.

**H7N9 infection of monocytes did not induce monocyte differentiation, but induced monocytes to undergo rapid apoptotic cell death**

Fig. 2(c) shows a decrease in CD14+ cells for both H7N9- and H5N1-infected PBMCs. To determine whether the decrease in CD14+ cells was due to the death of monocytes or to the reduction of CD14 expression on monocytes, we first infected purified CD14+ monocytes with different
viruses and determined the changes in CD14 levels after virus infection. Both H7N9- and H5N1-infected purified monocytes lost their CD14 expression as early as 12 h p.i., while pH1N1-infected monocytes continued to express CD14 (Fig. 5a). Since the loss of CD14 expression may be associated with cell differentiation, we measured the expression of the cell activation and differentiation-associated markers HLA-DR, CD11b, CD11c, CD86 and CD83. MHC class II molecule HLA-DR expression on infected monocytes was not significantly different from mock-infected controls, and was significantly lower than that of pH1N1-infected monocytes (Fig. 5a). CD11b and CD11c were expressed at significantly lower levels than mock-infected controls (Fig. 5b). There was no increased expression of co-stimulatory molecule CD86 and dendritic cell maturation marker CD83 up to 72 h p.i. (Fig. 5c). These markers were not significantly different between H7N9- and H5N1-infected monocytes. These findings indicated that only pH1N1 infection could induce the monocytes to differentiate into dendritic cells, but not under H7N9 or H5N1 infection.

To determine whether the depletion of CD14+ monocytes was due to cell death, purified monocytes were infected with different viruses. We found that H7N9 and H5N1 infection led to a reduction in cell viability to 43.9 and 30.8 % at 48 h p.i., and 14.9 and 9.05 % at 72 h p.i., respectively (Fig. 6a), whereas the pH1N1-infected monocytes remained viable (92.4 %) (Fig. 6a).

To determine whether the cell death noted in H7N9 and H5N1 infection was caused by the activation of apoptotic pathways, we assayed for activated caspase 3 and performed TUNEL staining. A significantly higher proportion of cells infected with H7N9 (66.5 %) and H5N1 (63.9 %) showed
caspase 3 activation than that of pH1N1 (12.0%) at 12 h p.i. (P<0.01) (Fig. 6b).

TUNEL assay further showed that at 12 h p.i., 20.9 and 21.9% of monocytes under H7N9 and H5N1 infection, respectively, were TUNEL-positive, indicating activation of apoptotic cell death (no significant difference; P=0.781). pH1N1-infected monocytes had a lower proportion of TUNEL-positive cells (11.8%; P=0.029) compared with H7N9. Therefore, the decrease in CD14+ cells among the infected monocytes was related to both the loss of CD14 expression and cell death.

**DISCUSSION**

Avian influenza viruses A H7N9 and H5N1 induce much higher mortality than human pandemic or seasonal influenza viruses [2]. Previous studies have attempted to explain the difference in clinical severity in terms of pre-existing immunity, cytokine and chemokine response, antibody
production, receptor specificity, tissue tropism and viral replication [23]. In the current study, we infected PBMCs or purified monocytes with H7N9, pH1N1 and H5N1, and compared their efficiency regarding PBMC infection, cytokine/chemokine response, cell differentiation and cell death. We found that the effect of H7N9 infection on PBMCs was very different from that of pH1N1, but resembled that of H5N1 in terms of monocytic infection. H7N9 infected a higher proportion of CD14+ monocytes, B cells and T cells than pH1N1. H7N9 induced significantly higher levels of gene expression of proinflammatory cytokines and type I IFNs during the early stage of PBMC infection (6 h p.i.), but significantly lower levels of type II IFN (IFN-γ) and IP-10 than pH1N1 at a later stage of infection (12 h p.i.). Since monocytes were most permissive to H7N9 infection, we concentrated on the consequences of monocyte infection using purified monocytes. pH1N1 infection did not affect the number of purified monocytes or the expression of monocyte activation and differentiation markers, which is in concordance with findings from previous studies [24–26]. However, H7N9- and H5N1-infected monocytes were not able to undergo activation and differentiation, and died rapidly from apoptosis. Therefore, the ex vivo PBMC findings in our study correlate with the clinical severity associated with these viruses.

When comparing H7N9 and H5N1, there was no significant difference in the levels of cytokines/chemokines for infected PBMCs or purified monocytes. This is similar to the response seen in human lung epithelial cells in a previous study comparing H7N9 and H5N1, in which there was only a marginal difference between the levels of IL-6 and IL-8, and no difference for TNF-α [27]. However, studies using human monocyte-derived macrophages and bronchial epithelial cells showed that H5N1 infection induced higher levels of TNF-α, IL-6, IL-8 and MCP-1 than did H7N9 infection [14, 22, 28]. Therefore, the difference in the cytokine/chemokine response depends on the cell type.

Monocytes are the precursors of monocyte-derived macrophages and dendritic cells, which present antigens to T and B lymphocytes. Activated CD4+ T lymphocytes will activate naïve B lymphocytes to produce antibodies. Activated T cells also secrete IFN-γ, which activates monocytes and macrophages. Macrophages are important in the control of virus replication. In mice infected 1918 pandemic influenza A H1N1 virus infection, those with macrophage depletion had higher lung viral titres than those with no macrophage depletion [29]. In our study, the rapid monocyte cell death and lack of monocyte activation/differentiation in H7N9- and H5N1-infected monocytes may have affected antigen

![Fig. 3. H7N9, H5N1 and pH1N1 infection of purified CD14+ monocytes. Purified CD14+ monocytes were infected with different viruses at an m.o.i. of 2. (a) Representative images of immunofluorescent staining of viral NP protein expressed in infected monocytes at 12 h p.i. Green, NP; blue, DAPI counterstaining of cell nuclei. Original magnification, ×100. (b) Quantification of monocytes expressing NP antigen at 12 h p.i. Left, representative histogram of FITC-conjugated mouse anti-NP antibody-stained monocytes gated on forward- and side-scatter plots. Right, percentage of NP-positive monocytes as determined by flow cytometry assay. n=6. ** P<0.01 when compared with H7N9.](https://www.microbiologyresearch.org/article-pdf/10.1099/mic.0.003486)
Fig. 4. Cytokine and chemokine gene expression profile of CD14+ monocytes infected with H7N9, H5N1 and pH1N1. Purified CD14+ monocytes from six healthy donors were infected with different influenza virus subtypes at an m.o.i. of 2. Cells collected at indicated time points were lysed for total RNA extraction. The levels of gene expression were determined using quantitative real-time RT-PCR for each specific primer pair. GAPDH mRNA was also amplified in parallel for RNA normalization. A ΔΔCt method was applied to calculate the fold change against uninfected control cells. Error bars indicate SEM. * P<0.05, ** P<0.01 when compared with H7N9-infected cells. (a) IL-6, TNF-α and IL-1β; (b) IFN-α, IFN-β and IFN-γ; (c) MIP-1α, MIP-1β, RANTES, MCP-1, IL-8 and IP-10.
presentation to T and B lymphocytes, leading to a decrease in production of IFN-γ by T cells and altered macrophage function. On the other hand, pH1N1-infected monocytes remained viable and able to express activation/expression markers, which correlated with a high level of expression of IFN-γ. Previous studies have shown that seasonal influenza
A H3N2 virus induced apoptotic cell death in monocytes [30, 31]. However, we cannot compare our results directly to those for H3N2 because of the different methodologies used. For purified monocytes, the levels of IFN-γ were not significantly different between H7N9 and pH1N1. This is not unexpected because IFN-γ is mainly secreted by T cells or NK cells, but not by monocytes.

IP-10, a chemokine that is secreted in response to IFN-γ, was highly expressed in PBMCs infected with pH1N1, but not in those infected with H7N9 or H5N1. IP-10 is important in attracting different immune cells, including monocytes/macrophages, dendritic cells, NK cells and T cells. Higher blood levels of IP-10 can be found in patients with severe pH1N1 and H7N9 infection when compared to those with mild infection [16, 32]. Anti-IP-10 has been shown to ameliorate acute lung injury in mice infected with pH1N1 [33].

Although the B- and T-lymphocyte response to influenza virus infection has been studied in detail [34], there is a paucity of information on the consequences of direct infection of B and T lymphocytes by influenza virus. In the current study, we found that B and T lymphocytes from PBMCs were more susceptible to H7N9 infection than those of pH1N1 or H5N1. However, infection of B and T lymphocytes did not seem to cause cell death, as there was no difference in the number of B and T lymphocytes between H7N9 and pH1N1 or H5N1 infection. Previous studies showed that direct infection of lymphocytes occurs in vivo. In a mouse model, lung-resident B lymphocytes were shown to be infected with H1N1 virus [35]. Mice with infected B lymphocytes had lower antibody titres in the bronchoalveolar fluid than those inoculated with inactivated influenza A virus. Therefore, direct infection of B lymphocytes can affect antibody production. In a patient infected with H5N1, influenza NP and haemagglutinin were present in T lymphocytes of the hilar lymph nodes [36]. Fan et al. showed in a mouse model that influenza virus can infect T lymphocytes in the spleen and thymus [37]. Direct infection of human T lymphocytes (Jurkat) resulted in Itk/PLC-γ signalling, which suggests that direct infection of T lymphocytes may lead to T-lymphocyte activation.

**Fig. 6.** Monocyte viability and apoptosis after infection with H7N9, H5N1 and pH1N1. Purified monocytes from six healthy donors were infected with different viruses at an m.o.i. of 2. (a) Cell viability was determined at the indicated times post-infection by Zombie dye labelling and flow cytometry assay. (b) Flow cytometry assay for activated caspase 3 at the indicated times post-infection. (c) Representative histograms of TUNEL-labelled cells at 12 h p.i. (left); percentage of TUNEL-positive cells at 12 h p.i. (right). Error bars indicate SEM. * P<0.05, ** P<0.01 when compared with H7N9-infected cells.
We and others have previously shown that the neutralizing antibody response is impaired in both humans and mice with H7N9 infection [17, 18]. The results from the current study provide several possible explanations. First, rapid monocyte cell death and the lack of monocyte differentiation would lead to a reduction in monocyte-derived dendritic cells or macrophages, which are professional antigen-presenting cells. Previous studies also showed that CD14+ monocytes from H7N9-infected patients had impaired antigen-presenting capacity [38]. Second, there was a higher proportion of B and T cells infected by H7N9, when compared with pH1N1 and H5N1. Third, the levels of IFN-γ mRNA expression were much lower in H7N9-infected than in pH1N1-infected PBMCs. A previous study showed that the level of neutralizing IgG2 titre was markedly reduced in mice lacking IFN-γ [39].

CD14 plays an important role in the innate immune response of monocytes during influenza virus infection. Previous studies have shown that blocking of CD14 reduced the cytokine response of monocytes during influenza virus infection due to impaired TLR signalling [40, 41]. Binding of CD14 by eritoran diminished the TLR4-MD2 signalling, which protected mice from influenza virus infection [42]. In our current study, CD14 expression was reduced in purified monocytes infected with H7N9 or H5N1 at 12 h p.i., at which the levels of most cytokine/chemokine levels declined.

There are several limitations to this study. First, although we have demonstrated that the apoptotic pathway was activated in H7N9- and H5N1-infected monocytes, we need to further study the involvement of other cell death mechanisms. Influenza virus infection can trigger the activation of inflammasomes, which has been associated with necroptosis and pyroptosis in bone-marrow-derived macrophages [43, 44]. Mice with impaired apoptosis and necroptosis are more susceptible to influenza A virus infection than wild-type mice [45]. However, excessive necroptosis has been associated with increases in mortality [46]. Pyroptosis, a form of programmed lytic cell death mediated by caspase 1, has been shown to be a major cause of cell death for CD4+ T lymphocytes during HIV infection [47]. Second, further in vivo studies are required to determine whether there are fewer monocyte-derived dendritic cells or macrophages in H7N9- or H5N1-infected animals. Third, we did not examine the effect of H7N9 and H5N1 on apoptosis in purified B and T cells. Further studies are required to determine whether B cells or T cells undergo apoptosis after H7N9 or H5N1 infection.

In conclusion, our findings indicate that H7N9 has the same capacity to infect human PBMCs and monocytes as H5N1 virus, although infectious virions were not produced. The induction of dysregulated cytokine response and apoptosis of monocytes may make important contributions to severe lung pathology and suboptimal adaptive antibody responses, due to poor antigen presentation.

**METHODS**

**Viruses**

Influenza A virus strains H7N9 (A/Anhui/1/2013), H5N1 (A/VNM/1194/2004) and pH1N1 (A/HK/415742/2009) were used in this study. The titres of the virus stocks were 10^8.048 p.f.u. ml^-1, 10^8.706 p.f.u. ml^-1 and 10^7.628 p.f.u. ml^-1 for H7N9, H5N1 and pH1N1, respectively. These viruses were propagated in 10-day-old specific pathogen-free chicken embryos. Infected allantoic fluid was harvested at 36 h p.i. (for H5N1) or 72 h p.i. (for H7N9 and pH1N1). Viral titres were determined using plaque assay or TCID₅₀ assay in Madin–Darby canine kidney (MDCK) cells as we described previously [48]. The virus stocks were aliquoted and stored at −80 °C until use.

**Isolation of human PBMCs and CD14+ monocytes**

Fresh buffy coats from healthy blood donors were obtained from the Hong Kong Red Cross Blood Transfusion Service. The research protocol was approved by the Institutional Review Board of the University of Hong Kong (UW-16-106). PBMCs were isolated from the buffy coat by gradient centrifugation in Ficoll-Paque PLUS solution (GE Healthcare, Little Chalfont, UK) as described previously [49]. Briefly, buffy coat blood was first diluted 1:1 with 1% FBS-PBS, gently layered on Ficoll-Paque PLUS solution, and centrifuged at 750 g for 20 min at 25 °C. The PBMC layer was collected and washed once with 1% FBS-PBS, and the red blood cells were lysed in ammonium chloride buffer (150 mM NH₄Cl, 10 mM NaHCO₃ and 1 mM EDTA) for 5 min at room temperature. The isolated PBMCs were then washed twice with 1% FBS-PBS and re-suspended in serum-free RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA).

CD14+ monocyte purification was carried out using the positive selection method with CD14 magnetic microbeads according to the manufacturer’s instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, isolated PBMCs were washed and re-suspended in cold isolation buffer (PBS, pH 7.2, 0.5% FBS and 2 mM EDTA). CD14 microbeads were added and incubated for 15 min at 4 °C. The cells were washed once and loaded onto an MACS column placed in the magnetic field. The column was washed three times before the elution of the labelled CD14+ monocytes. Purity and viability were determined by flow cytometry and Trypan blue assay, respectively.

**Influenza virus infection**

Infection of PBMC with influenza viruses was performed in 15 ml Falcon conical centrifuge tubes (Fisher Scientific). PBMCs or monocytes (2×10⁶ cells) were inoculated with H7N9, H5N1 or pH1N1 at an m.o.i. of 2 in serum-free RPMI-1640 medium. After 1 h of virus adsorption at 37 °C, the cells were centrifuged at 300 g for 5 min and were then washed with PBS to remove the viral inoculum. After washing, the cells were seeded into the culture plate and were further incubated at 37 °C and 5% CO₂ in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-
streptomycin, 1 % GlutaMAX, 1 mM sodium pyruvate, 1 % non-essential amino acid, and 50 μM 2-mercaptoethanol. All medium supplements were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The culture medium for purified CD14+ monocytes also contained IL-4 (10 ng ml⁻¹) and GM-CSF (10 ng ml⁻¹), both obtained from R&D systems (Minneapolis, MN, USA). At 6, 12, 24, 48 and 72 h p.i., the cells and culture supernatant were collected for further analysis. Mock-infected cells were cultured in parallel as control. All experiments involving live viruses were carried out in a biosafety level 3 laboratory.

**Determination of infectious virus titre in culture supernatant**

Culture supernatants of the virus-infected cells were collected at different times post-infection and stored immediately at −80 °C until testing. Culture supernatants in 10-fold serial dilutions were inoculated onto MDCK cells in 96-well plates. After 1 h incubation at 37 °C, the inocula were removed and the cells were washed and further incubated at 37 °C and 5 % CO₂ with minimum essential medium (MEM) containing 1 % penicillin/streptomycin and 2 μg ml⁻¹ l-1-tosylamide-2-phe- nylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, Missouri, USA). Cytopathic effects were evaluated 72 h after incubation. The viral titre was determined using TCID₅₀ assay [48].

**Immunofluorescent staining of viral NP protein**

The infected and mock-infected cells were harvested at the indicated times post-infection and immediately fixed on slides in chilled acetone and methanol (1:1) for 20 min at −20 °C. They were subsequently stained with mouse anti-influenza NP antibody [50], followed by FITC-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA). Mounting and DNA staining were performed with VECTASHIELD DAPI mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were captured with a Nikon 80i fluorescent microscope.

**Staining of PBMC or monocytes for flow cytometry assay**

Infected and mock-infected cells were collected at the indicated time points post-infection. At the indicated time points post-infection (up to 72 h), the cells in suspension were collected first. The attached cells were washed three times with PBS, incubated with 1 ml of ice-cold 10 mM EDTA on ice for 20 min, and were then detached by pipetting. All cells (the suspended cells and the attached cells) were then combined and centrifuged. The cells were then re-suspended in PBS for further staining with different antibodies. The following antibodies were used for analysis of cell types, and monocyte activation and differentiation: APC-CD3, PE/Cy7-CD4, PE/Cy7-CD8, PE/Cy7-CD14 or APC/Cy7-CD14, APC-CD19, Brilliant Violet 421-CD11b, PE/Cy7-CD11c, APC-CD83, Brilliant Violet 605-CD86, PE-HLA-DR and corresponding isotype control antibodies (Biolegend, San Diego, CA, USA). Zombie violet and zom- bie aqua fixable viability kit (Biolegend, San Diego, CA, USA) and Alexa Fluor 647 anti-active caspase 3 antibody (BD Biosciences, San Jose, CA, USA) were used for analysis of cell viability and caspase 3 activation. For double staining of viral NP and cell markers, the cells were first stained with cell marker antibodies and fixed with 4 % paraformaldehyde. The cell membranes were then permeabilized with 0.1 % Triton X-100 for 5 min and subsequently stained with FITC-conjugated mouse anti-influenza A virus NP antibody (Abcam, Cambridge, UK). Different cell populations in the PBMCs were first gated on an FSC-H/FSC-A scatter plot for singlets and then debris gated out on an SSC/FSC scatter plot; different cell types that were infected and expressing viral NP protein were identified by double-labelling of cell surface CD markers and viral NP protein. Apoptotic cells were detected with an APO-BrdU TUNEL assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. All samples were ana- lysed using a BD LSRFortessa cell analyser (BD Bioscience,

Table 1. Sequences for primers

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<th>Gene name</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
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<td>IFN-γ</td>
<td>CTAATATTCGCTAGTACGTGTA</td>
<td>AGAGTTGACAGCCATCTGGA</td>
</tr>
<tr>
<td>IL-1B</td>
<td>CAGAAGATCCATCGAGCTGCC</td>
<td>AGATTTGAGCTGAGTCGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>GGCAGACGAGCAGACGACAAC</td>
<td>ATCTAGGAGGCCATGCTAC</td>
</tr>
<tr>
<td>IL-8</td>
<td>CCACCGGGAAGGACACATCTC</td>
<td>GGATGTTGAAAAATGGT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>GATCTCAGTGCAAGGAGCTG</td>
<td>TTCAATGCTGTCAGGAGGTC</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CATCTCAGTCACTGCTGAACA</td>
<td>GGCTCCTGTGCTTCAATTGAGT</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>GTCTGTTGCTAGTCCAGTGTA</td>
<td>TAAGCTGCTGTGAGGCGGTT</td>
</tr>
<tr>
<td>RANTES</td>
<td>AGCAAGAGGATCACGCTCAGT</td>
<td>ATGCAGGAAGGAGCTCAGT</td>
</tr>
</tbody>
</table>
San Jose, CA, USA) and data analysed using Flowjo software (TreeStar).

Determination of cytokine and chemokine gene expression by quantitative real-time RT-PCR

Infected or mock-infected PBMCs or monocytes collected at different times post-infection were lysed in RLT lysis buffer for total cellular RNA extraction using a Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). The extracted RNA was reverse transcribed with oligo-dT primer and a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). Quantitative real-time PCR was performed on a LightCycler480 system (Roche, Basel, Switzerland) using SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and the gene-specific primers listed in Table 1. The expression of Kong (UW-16 approved by the Institutional Review Board of the University of Hong Kong Red Cross Blood Transfusion Service. The research protocol was

Ethical statement

PrimeScript RT reagent kit (Takara Bio, Shiga, Japan).

Determination of cytokine and chemokine gene

expression by quantitative real-time RT-PCR

The viral load of influenza A virus was determined by quantitative RT-PCR for the influenza A virus M gene. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified in parallel for normalization of the amount of RNA. A ΔΔCt method was applied for evaluation and comparison of the differential gene expression between samples.

Quantiative RT-PCR for the influenza A virus M gene

The viral load of influenza A virus was determined by quantitative RT-PCR of the M gene, and was expressed as log M gene copies per 10^3 copies of GAPDH. The pCRII-TOPO vectors containing M gene or GAPDH were used as standards. The detection limit of the influenza A virus M gene quantitative RT-PCR was 100 copies per reaction.

Statistics

All statistical analysis was computed using PRISM 6.0 (GraphPad Software, CA, USA). Statistical analysis between the groups was performed by Student’s t-test. P-values of <0.05 were considered to be statistically significant.

Funding information

This work was supported in part by the Larry Chi-Kin Yung, Professor Richard Yu and Carol Yu, Providence Foundation Ltd (in memory of the late Lui Hac Minh), Cheer Master Investments and the Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, the Ministry of Education of China.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Fresh buffy coats from healthy blood donors were obtained from Hong Kong Red Cross Blood Transfusion Service. The research protocol was approved by the Institutional Review Board of the University of Hong Kong (IUW-16–106).

Reference


