Highly pathogenic H5N1 avian influenza viruses have caused infection in humans, with a high mortality rate, since 1997. While the pathogenesis of this infection is not completely understood, hypercytokinaemia and alveolar macrophages are thought to play a role. To gain further insight into the cytokine-mediated pathogenesis of this infection in humans, we measured various cytokines produced by primary human macrophages infected with H5N1, pandemic H1N1 or seasonal influenza viruses. We found that many cytokines were produced at higher levels on infection with the H5N1 strains tested compared with seasonal influenza viruses. Interestingly, the extent of cytokine induction varied among the H5N1 strains and did not correlate with replicative ability in macrophages. Further, a pandemic H1N1 virus induced higher levels of several cytokines compared with seasonal viruses and some H5N1 strains. Our results demonstrate that high cytokine induction is not a universal feature of all H5N1 viruses.

Alveolar macrophages are central players in both innate and adaptive immune responses to respiratory infection. In studies in vitro, human primary macrophages infected with H5N1 viruses produce higher levels of cytokines compared with those infected with seasonal viruses (Cheung et al., 2002; Guan et al., 2004; Lee et al., 2009; Mok et al., 2009; Woo et al., 2010). However, the H5N1 viruses examined in these studies were isolated before 2005, and the number of cytokines studied was limited.

In 2009, a novel swine-origin H1N1 influenza virus (2009 pandemic H1N1 virus; pdm H1N1) caused a pandemic (Khan et al., 2009). During the first phase of this pandemic, 9% of patients were hospitalized with pneumonia, respiratory failure or acute respiratory distress syndrome.
(Dawood et al., 2009; Louie et al., 2009). The pdm H1N1 virus induced cytokine production and gene expression in dendritic cells and primary human macrophages at levels similar to or below those induced by seasonal viruses (Osterlund et al., 2010; Woo et al., 2010).

To gain further insight into the cytokine-mediated pathogenesis of influenza virus infection in humans and to assess the possible relationship between hypercytokinaemia and the pathogenicity of influenza viruses in humans, we measured 48 different cytokines in culture supernatants of primary human macrophages infected with H5N1 viruses of various clades or subclades, a pdm H1N1 virus and seasonal influenza viruses.

First, we prepared primary human macrophages from PBMCs, which were separated from theuffy coat of five different donors, and incubated them with granulocyte-macrophage colony stimulating factor (GM-CSF) (5 ng ml⁻¹). Our research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science, University of Tokyo (approval numbers 18-15-0129 and 19-24-0430). Macrophage differentiation was confirmed by using FACS analysis for CD68 high and CD14 low (Peng et al., 1999; Shimizu et al., 2007; Taylor et al., 2005). The phenotype of the human macrophages derived from the peripheral blood monocytes used in this study is similar to that of human alveolar macrophages (Shimizu et al., 2007; Taylor et al., 2005), suggesting that this is a useful model with which to study the dynamics of influenza virus infection in the human lung.

To evaluate the susceptibility of primary human macrophages to avian H5N1 and human influenza viruses, we examined whether α-2,3- and α-2,6-linked sialic acids, which are avian and human virus receptors, respectively, were present on the cells by using lectins specific for these sialooligosaccharides: *Sambucus nigra* agglutinin (SNA) for α-2,6-linked and *Maackia amurensis* agglutinin II (MMAI) (Vector laboratories) for α-2,3-linked sialic acids. Cells were incubated with biotinylated lectins and then conjugated with streptavidin–Alexa-Fluor 488 (Invitrogen). FACS analysis showed a right-shift compared with controls without lectins, indicating that both α-2,6- and α-2,3-linked sialic acids were expressed on the primary human macrophages, as they were on Madin–Darby canine kidney (MDCK) cells, but not chicken embryo fibroblasts (CEF), which predominantly express MAAII-positive oligosaccharides (Fig. 1a).

Since our receptor analysis indicated the replicative potential of both avian and human viruses in human macrophages, we infected the cells with H5N1, seasonal influenza or pdm H1N1 viruses at an m.o.i. of 2 and measured the virus output in infected-cell supernatants by means of a plaque assay with MDCK cells. Virus replication was assessed in primary human macrophages derived from five different donors, and the means ± SD for macrophages from the five individuals are presented in Fig. 1(b). At 12 h post-infection (p.i.), we found no significant difference in titres among the virus strains. However, at 36 h p.i., some H5N1 strains, such as A/Vietnam/UT3028II/2003 clone2 (clade 1; VN3028Icl2/03) and A/Indonesia/UT3006/2005 (clade 2.1.3; IDN3006/05), grew significantly better than did the seasonal viruses A/Kawasaki/UTK-4/2009 (H1N1; UTK4) and A/Kawasaki/UTK-20/2008 (H3N2; UTK20), whereas other H5N1 strains, such as A/Hong Kong/483/97 (clade 0; HK483/97), A/chicken/Miyazaki/K11/2007 (clade 2.2; CkMiyazaki/07) and A/Vietnam/UT31203A/2007 (clade 2.3.4; VN31203/07), as well as the pdm H1N1 virus, A/California/04/2009 (CA04), replicated less efficiently than did the seasonal viruses. Thus, although all of the viruses tested could infect human macrophages, levels of replication differed even among the H5N1 viruses.

We then measured 48 different cytokines from the culture supernatants of primary human macrophages upon virus infection at an m.o.i. of 2 by using Bio-Plex human 27-plex and 21-plex panels (Bio-Rad). To aid in the visualization of the cytokine arrays, we generated a heat-map by analysing the means of data individually obtained with macrophages from the different individuals (Fig. 2). The individual macrophage data are shown in Supplementary Fig. S1, available in JGV Online.

We observed marked upregulation of two cytokines, tumour necrosis factor (TNF)-α and monokine-induced by interferon (IFN)-γ (MIG), throughout the time-course of the experiment (6–36 h p.i.) with all influenza viruses tested (Fig. 2a). Similarly, the cytokines shown in Fig. 2(b) were upregulated to various degrees upon infection with most of the test strains at the late phase (24 and 36 h p.i.). Of these, the anti-inflammatory cytokine interleukin (IL)-1 receptor antagonist (ra) was produced at the highest level. Upregulation of the IL-1 family (IL-1α, IL-1β and IL-18) and of macrophage migration inhibitory factor (MIF) was also notable. By contrast, changes in the levels of the cytokines shown in Fig. 2(c) were limited. Interestingly, stem-cell growth factor (SCGF)-β was strongly downregulated by many of the influenza viruses tested at 36 h p.i., especially the seasonal viruses (Fig. 2d).

Several of the cytokines shown in Fig. 2(e) were upregulated upon infection with most of the H5N1 strains tested compared with the seasonal influenza viruses (UTK4/H1N1 and UTK20/H3N2), although the levels of expression varied among the cytokines (Fig. 2e); three proinflammatory cytokines, including chemokines [IFN-γ-inducible protein (IP)-10, regulated upon activation normal T-cell expressed and secreted (RANTES) and IL-6] and stem-cell factor (SCF), were upregulated strongly by infection with all of the viruses, but more so with the H5N1 strains, especially during the late phase of infection. However, the remaining 17 cytokines shown in Fig. 2(e), for example, IL-12, IL-16 and IL-9, which activate cell-mediated immunity, were induced markedly by some H5N1 virus strains, such as HK483/97 (clade 0) and CkMiyazaki/07 (clade 2.2), induced somewhat by H5N1 strain VN3028Icl2/03 (clade 1) and upregulated only slightly or not by other H5N1 strains, IDN3006/05 (clade 2.1.3) and VN31203/07 (clade 2.3.4), or by seasonal viruses. Therefore,
the former H5N1 viruses can be defined as high-cytokine inducers and the latter as low-cytokine inducers. Notably, the chemokines macrophage inflammatory protein (MIP)-1α and -1β (Fig. 2f) were also upregulated by all of the viruses, but more so by the high-inducer H5N1 strains, whereas these chemokines were downregulated by seasonal viruses during the late phase of infection. Another chemokine, monocyte chemotactic protein (MCP)-3, was upregulated by the high-inducers, but not by the seasonal strains.

The 2009 pandemic H1N1 virus, CA04, induced several cytokines at higher levels than those induced by the seasonal viruses; in particular, the expression of IL-6, TRAIL, MCP-1, IL-9 and MCP-3 was enhanced. Interestingly, these levels of expression were even higher than those induced by low-cytokine-inducing H5N1 viruses (Fig. 2e, f). However, since we did not confirm that this cytokine production profile is common to all pandemic H1N1 viruses, further studies may be needed to establish whether this profile is a universal trait.

In this study, we examined the cytokine profiles of influenza viruses by using the means of data obtained in independently with macrophage preparations from five individuals. The results are expressed as means ± so. *, P<0.01, significant difference compared with seasonal virus (UTK4/H1N1 and UTK20/H3N2); one-way ANOVA.
Cytokine production profile of influenza viruses

(a) TNF-α
   MIG

(b) IL-1ra
   IL-1α
   MIF
   IL-1β
   IL-18
   IL-15
   GM-CSF
   IL-2
   FGF basic

(c) VEGF
   Eotaxin
   IL-4
   IL-7
   IL-8
   GROα
   IL-10
   PDGF-bb
   HGF
   IL-5
   IL-13
   M-CSF

(d) SCGF-β

(e) IP-10
   RANTES
   IL-6
   SCF
   TRAIL
   IL-12(p40)
   IL-12(p70)
   IL-16
   MCP-1
   IL-9
   IL-17
   LIF
   β-NGF
   IFN-α2
   IL-3
   CTACK
   IL-2Rα
   TNF-β
   SDF-1α
   G-CSF
   IFN-γ

(f) MIP-1α
   MIP-1β
   MCP-3

6 h  12 h  24 h  36 h

log2(Ratio)
different individuals (Supplementary Fig. S1). This approach was essential to obtain general cytokine responses upon virus infection, since the levels of cytokine production upon influenza virus infection, especially H5N1 virus infection, varied among the donors (although the overall trend was similar). For example, IL-6 was induced strongly in two donors, but weakly in another, even for the cytokine high-inducer H5N1 strains. Similarly, the induction levels of other cytokines, such as IL-1ra and MIP-1α, with cytokine high-inducer H5N1 strains varied among blood donors. Induction levels of TNF-α also varied among the donors upon seasonal UTK20/H3N2 virus infection. These variations may contribute to the different clinical progression and disease outcomes observed in patients infected with seasonal, pdm H1N1 or H5N1 viruses.

Since the clinical presentation of patients infected with an H5N1 virus resembles that of septic shock, with a cytokine storm caused by the lipopolysaccharide (LPS) of Gram-negative bacteria (Bozza et al., 2007; Kellum et al., 2007), we also examined cytokine production by human macrophages upon LPS treatment, a well-known stimulus for cytokine production.
production by these cells. We found that the range and kinetics of cytokine production upon LPS treatment (10 ng for 60 min at 37 °C) were substantially different from those seen on influenza virus infection. LPS-treated human macrophages produced several proinflammatory cytokines at high levels (Fig. 2), as was observed with H5N1 virus infection. However, IL-8 and GROα were induced specifically by LPS, which explains the neutrophil activation in septic shock. A broader range of cytokines was upregulated in H5N1 virus infection than upon LPS treatment, indicating that H5N1 viruses may activate the host immune system in a more complicated manner than occurs during septic shock.

The cytokine production profiles with the influenza viruses obtained here (Fig. 2) did not correlate with the viral replication levels in human macrophages (Fig. 1b). These data suggest that cytokine upregulation upon H5N1 virus infection may be regulated by factors other than virus replicative ability in these cells. To address this issue, we measured cytokine induction in macrophages incubated with VN3028IIccl2/03 virus that was inactivated by 0.1 % (final) β-propiolactone (Logrippo & Hartman, 1955; Smith & Srb, 1951) for 16 h at 4 °C. We confirmed that cells treated with β-propiolactone alone, at the concentration in the inactivated virus preparation, did not affect the cytokine response (not shown). We found that almost all cytokines were produced at the same or only slightly higher levels than those of the mock infection (Fig. 3), indicating that virus replication is required for cytokine induction in human macrophages.

Here, we found that many cytokines were produced at high levels upon infection with H5N1 strains (although the levels of expression varied among strains) in comparison with infection with seasonal influenza viruses, confirming, at least in part, the hypercytokinaemia that is often observed in patients infected with H5N1 viruses. The patients from whom HK483/97, VN3028IICl2/03 and IDN3006/05 were isolated died, whereas the patient infected with VN31203/07 recovered (Le et al., 2010). Notably, the IDN3006/05 strain that killed its victim was a cytokine-low inducer. However, since the cytokine levels of the patient infected with this virus were not available, we cannot speculate as to whether the patient’s death involved cytokine-independent pathogenesis. Nonetheless, we would argue that clinical outcomes of H5N1 virus infection in humans may be affected not only by hypercytokinaemia, but also by other, as-yet unknown factors. We also found that a pandemic H1N1 2009 virus induced higher levels of several cytokines than those induced by seasonal viruses and even some H5N1 strains. These results suggest that, although hypercytokinaemia may play a part in the severe disease progression and ultimate death of patients with H5N1 virus infection, induction of high levels of cytokines may not be a feature common to all H5N1 viruses.

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**References**


