



<b>Title</b>	<b>Interferon dysregulation and virus-induced cell death in avian influenza H5N1 virus infections.</b>
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# Interferon dysregulation and virus-induced cell death in avian influenza H5N1 virus infections

## Key Messages

1. Hyper-induction of cytokines and chemokines was found in human blood macrophages infected with the avian influenza H5N1 and H9N2/G1 viruses, as compared to those infected with human influenza H1N1 virus.
2. IRF3 played a significant role in the hyperinduction of cytokines including IFN- $\beta$ , IFN- $\lambda$ 1, IFN- $\alpha$  subtypes, MCP-1, and TNF- $\alpha$ , and also played a part in subsequent cytokine-induced cell signalling cascades.
3. Compared with H1N1 viruses, avian influenza viruses including H5N1/97 and its precursors triggered a caspase-mediated but delayed apoptotic response in human macrophages.
4. Therapies that can minimise immunopathology-associated dysregulation of innate immunity without impairing effective host defence may be valuable adjuncts to antiviral therapy.

## Introduction

A novel influenza A H1N1 virus of swine origin caused an influenza pandemic in 2009,<sup>1</sup> but avian influenza H5N1 virus remains the major concern to humans. Since the first documented cases of avian influenza H5N1/97 virus in Hong Kong, these viruses have continued to evolve and cause outbreaks in poultry in European and Asian countries.<sup>2</sup> Sporadic cases of human infections have high mortality rates of >60%.<sup>1</sup> Understanding its pathogenesis enhances the development of novel therapy.

Influenza A viruses are known to replicate in epithelial cells and leukocytes, resulting in induction of cytokines and chemokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IP-10, RANTES, MCP-1, and interferons (IFN- $\alpha$  and - $\beta$ ). Using our H5N1/97-macrophage model, H5N1/97 induced dramatically higher levels of cytokine expression, compared to those infected with 'seasonal flu' human H1N1 or H3N2 viruses. Subsequently, the TNF induction and its cytotoxicity were mediated, at least in part, by mitogen-activated protein kinases, including p38 and ERK.<sup>3</sup>

We further investigated the underlying mechanisms of interferon dysregulation and virus-induced cell death in influenza-infected cells by examining the activation of different types of transcription factors. The expressions of IFN- $\beta$ , IFN- $\lambda$ 1 and TNF- $\alpha$  were regulated by IRF-3 following virus infections. Moreover, a delayed onset of caspase-mediated apoptotic pathways was found in H5N1-infected human macrophages, compared to those infected by non-avian viruses. These findings may be useful for the design of novel immunotherapeutic regimens to abrogate or lessen the cytokine dysregulation in those highly pathogenic avian viruses.

## Methods

This study was conducted from 1 October 2006 to 30 September 2008. Peripheral blood monocytes were isolated from buffy coats of healthy donors (from the Hong Kong Red Cross Blood Transfusion Service) by Ficoll-Paque (Pharmacia Biotech) density gradient centrifugation and purified by adherence.<sup>3</sup> The purity of the monocyte preparations was examined by immunostaining with anti-CD14 antibodies (BD Biosciences). The cells were allowed to differentiate for 14 days in RPMI 1640 medium supplemented with 5% heat-inactivated autologous plasma. Macrophages were seeded onto 24-well (1.5x10<sup>5</sup> cells/well) tissue culture plates for studies of mRNA expression. Differentiated macrophages were incubated in serum-free macrophage SFM medium (GIBCO BRL) supplemented with 0.6  $\mu$ g/mL penicillin and 60  $\mu$ g/mL streptomycin (Sigma-Aldrich) one day before virus challenge.

H5N1 influenza viruses A/HK/483/97 (H5N1/97) and A/Vietnam/3212/04 (H5N1/04) isolated from H5N1-infected patients were cultured in Madin Darby canine kidney cells. Precursors of H5N1/97, A/Quail/Hong Kong/G1/97 (H9N2/G1) virus, and seasonal human influenza H1N1 virus A/HK/54/98 (H1N1) were

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isolated from other infected patients. These viruses were then purified by pre-adsorption to and elution from Turkey red blood cells.<sup>3</sup> Virus stock was aliquoted and stored at -80°C until use.

Differentiated macrophages were infected at a multiplicity of infection of two for 30 to 45 minutes. Then, the virus inoculum was removed and the cells were washed once and incubated in macrophage SFM medium supplemented with 0.6 µg/mL penicillin, 60 µg/mL streptomycin, and 2 µg/mL N-p-tosyl-L-phenylalaninechloromethyl ketone-treated trypsin (Sigma-Aldrich). The culture supernatant was collected for cytokine analysis and the total cellular RNA was extracted for gene expression analysis at indicated times. Duplicate wells of monolayer cells were fixed and analysed by immunofluorescent staining for influenza virus-specific nucleoprotein (DAKO Imagen) at 8 hours post-infection.

DNase-treated total RNA, isolated by RNeasy Mini Kit (Qiagen), was reverse-transcribed by using poly(dT) primers and Superscript III reverse transcriptase (Invitrogen) and quantified by real-time PCR analysis with a LightCycler (Roche Diagnostics). The methods used for quantifying cytokine and β-actin mRNA have been described.<sup>4</sup>

Macrophages on coverslips were infected with influenza virus for 3 hours. Cells were fixed with 4% paraformaldehyde and permeabilised with 0.2% Triton X-100 dissolved in PBS. Cells were washed with PBS, stained with anti-IRF3 antibodies, washed and stained with fluorescein-conjugated anti-rabbit Ig (ZyMed). Nuclei of the cells were counter-stained with 1 µg/mL of DAPI (Sigma-Aldrich) and mounted in 50% glycerol in PBS.

Whole cell extracts were prepared by lysing macrophages with cold whole cell lysis buffer (50 mM KCl, 1% NP-40, 25 mM HEPES [pH 7.4], 1 mM DTT, protease inhibitor cocktail [Roche Diagnostics] and phosphatase inhibitor cocktail [Calbiochem]) on ice for 10 minutes. The cell lysates were collected with a cell scraper and the whole cell extract was harvested by centrifugation at 13 000 rpm for 10 minutes at 4°C. The protein content was determined

with a Bio-Rad Protein Assay (Bio-Rad Laboratories) using BSA as a standard.

Thirty micrograms of whole cell lysate or 15 µg of cytoplasmic protein was heat denatured in a sample buffer (62.5 mM Tris [pH 6.8], 35% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue), separated by using 8% SDS-PAGE and transferred to polyvinylidene difluoride or nitrocellulose membranes. The membranes were immersed with 5% skim milk/PBS for 1.5 hours and immunoblotted with primary antibodies. Anti-actin antibodies were used as loading controls for the cytoplasmic fraction. The immunoblots were then incubated with HRP-coupled goat anti-rabbit or anti-mouse IgG antibodies, and the signals were visualised by ECL plus solution (GE Healthcare).

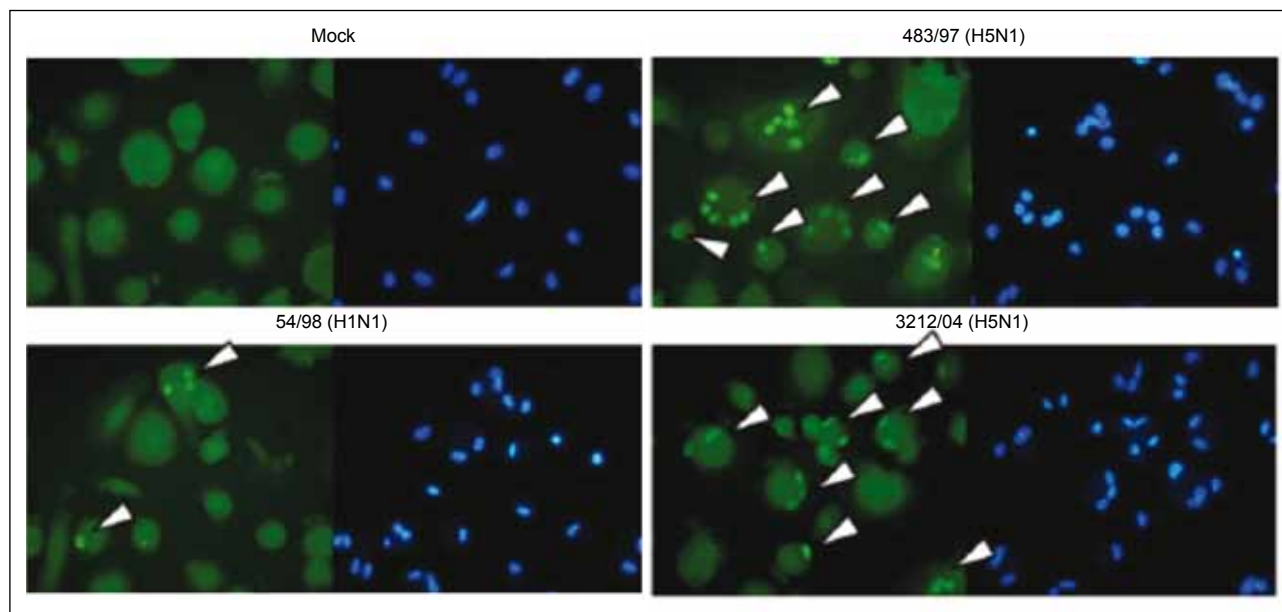
## Results

A hyper-induction of IFN-β and IFN-α was found in human blood macrophages infected with the avian influenza H5N1 and H9N2/G1 viruses, as compared with those infected with human influenza H1N1 virus. There was a differential induction of IFN-α subtypes including IFNα1, 2 and 8 in H9N2/G1-infected cells by using genechip analysis, as compared with those induced by H1N1 (Table). The results were validated by Q-PCR and ELISA assay (data not shown).

There was hyperinduction of cytokines and chemokines, including IFN-β, IFN-λ1, TNF-α, IP-10, MCP-1, MIP-1α, and RANTES in H5N1-infected macrophages.<sup>4</sup> To investigate the mechanisms of cytokine induction, IRF3 activation in virus-infected macrophages was examined by immunofluorescent staining. A differential IRF3 activation was found in macrophages infected with H5N1 viruses, compared to those infected with H1N1 virus (Fig 1). By using the IRF3-specific siRNA oligos, the mRNA levels of IFN-β and IFN-λ1 inducible by the H5N1 virus were abrogated.<sup>4</sup> The IRF3 was the key transcription factor for IFN gene regulation. However, the up-regulation of TNF-α in H5N1-infected macrophages was moderately suppressed by IRF3 siRNA, indicating that IRF3 only contributed, in

**Table. Differential induction of IFN-α subtypes in influenza virus infections**

IFN-α subtype	3 hours post-infection			8 hours post-infection		
	Mock	H1N1	H9N2	Mock	H1N1	H9N2
IFN-α 1	1	16.11	214.40	1	37.25	175.9
IFN-α 2	1	2.53	25.74	1	2.78	18.56
IFN-α 4	1	1.40	10.14	1	1.30	3.67
IFN-α 7	1	1.51	8.11	1	2.12	5.72
IFN-α 8	1	1.93	25.03	1	2.97	26.35
IFN-α 10	1	1.30	2.64	1	1.71	5.39
IFN-α 13	1	5.80	70.92	1	5.46	25.55
IFN-α 14	1	1.76	13.12	1	2.31	7.04
IFN-α 16	1	1.36	9.49	1	2.31	7.79
IFN-α 17	1	1.14	2.99	1	1.22	2.65
IFN-α 21	1	1.39	3.96	1	2.18	6.07



**Fig 1. H5N1 viruses differentially activate IRF3 translocation as compared to H1N1 virus**

Differentiated primary human macrophages were infected with 54/98 (H1N1), 483/97 (H5N1), or 3212/04 (H5N1) at a multiplicity of infection of two. Mock infected cells served as controls. Immunofluorescence staining assay of IRF3 translocation. At 3 hours post-infection, the cells were fixed with 4% paraformaldehyde. Permeabilised cells were stained with anti-IRF3 Ab and FITC-conjugated secondary Ab. Cell nuclei were counter-stained with DAPI. Cells with IRF3 translocation were indicated with arrowheads. (Figures were adopted from *J Immunol* 2009;182:1088-98 with permission, approval pending)

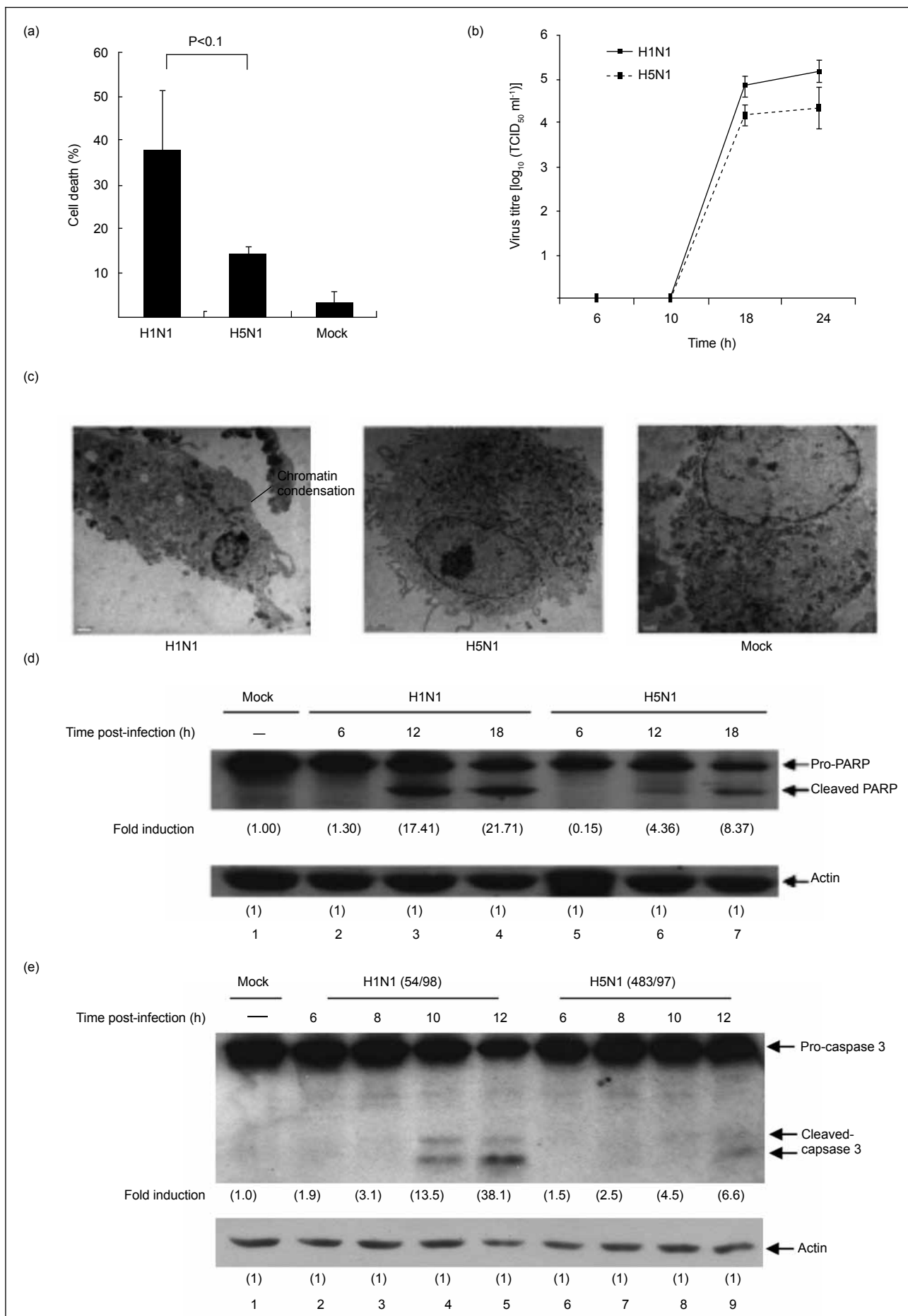
part, to the H5N1-induced TNF- $\alpha$  expression. Other H5N1-induced cytokines including IP-10, RANTES, MCP-1, and MIP-1 $\alpha$  were inhibited by the IRF3-specific siRNA.<sup>4</sup> As the expression of these cytokines may involve de novo protein synthesis, the detailed regulatory mechanism needs to be investigated. Interestingly, IRF3 showed similar regulatory roles on cytokine expression induced by H1N1 virus, although the potency of H1N1 was less than that of H5N1 virus (data not shown). Additional results delineating the role of IRF-3 have been reported.<sup>4</sup>

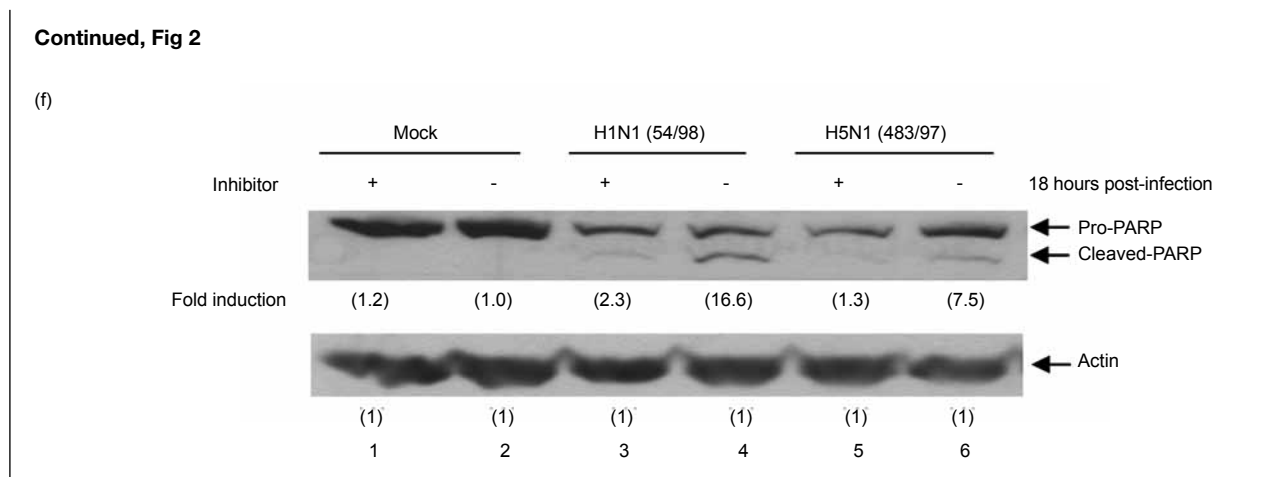
In addition to the mechanism of cytokine induction, the onset of cell death in human macrophages infected with the H5N1/97 virus and its precursors was delayed. Primary macrophages were mock-treated or infected with H5N1/97 or H1N1 influenza viruses at a multiplicity of infection of two for 30 minutes and harvested at indicated time points for analysis. At 18 hours post-infection, infected cells were examined by staining with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and cell death was determined by nuclear condensation or nuclear fragmentation. Less dead cells were found after H5N1/97 than H1N1 virus infection (Fig. 2a). There was no significant difference of viral replication in the macrophages infected by H1N1 or H5N1/97 (Fig. 2b). Using transmission electron microscopy, the cellular morphology of H5N1/97-infected macrophages was comparable to that of the mock-treated cells at 12 hours post-infection (Fig 2c). In contrast, the characteristics of apoptotic cells including nuclear condensation and chromatin adherence to nuclear membrane were noted in the H1N1-infected cells (Fig 2c). Our results demonstrated

a differential onset of cell death in macrophages infected with the H5N1/97 compared to those infected with the H1N1 virus.

The activity of caspase-activated poly [ADP-ribose] polymerase (PARP, Pharmingen) was further examined at 6, 12, and 18 hours post-infection, using Western analysis (Fig 2d). Over the time course, the levels of the cleaved-PARP fragment in the H5N1/97-infected cells were lower than those in the H1N1-infected cells (Fig 2d). The activity of caspase 3 was then measured by Western analysis. In contrast to a strongly cleaved caspase 3 fragment found in the H1N1-infected cells at 10 hours, caspase 3 was barely activated in the H5N1/97-infected cells at 12 hours (Fig 2e). By treating virus-infected cells with a specific caspase inhibitor, Z-IETD-FMK, the level of the cleaved-PARP fragment in H5N1/97- or H1N1-infected macrophages was significantly reduced by 82% and 86%, respectively, as compared to the corresponding untreated samples (Fig 2f). Hence, the delayed activation of the caspase cascade in H5N1/97 infected cells appears to determine the differential onset of cell death in H5N1/97 and H1N1 infection. More importantly, the characteristic delayed onset of apoptosis in H5N1/97 infection was also found in its precursor viruses including H9N2/G1, A/Teal/HK/W312/97 (H6N1), and A/Goose/Guangdong/1/96 (H5N1/437.6). All these results have already been reported.<sup>5</sup>

H5N1 virus differentially activates p38 mitogen-activated protein kinases to mediate the hyperinduction of proinflammatory cytokines including TNF- $\alpha$ .<sup>3</sup> H5N1





### Fig 2. H5N1/97-infected macrophages show less cell death and apoptosis

(a) Primary human blood macrophages ( $0.5 \times 10^6$ ) were fixed with 4% paraformaldehyde, stained with DAPI at 18 hours post-infection with H5N1 (483/97) or H1N1 (54/98) virus at a multiplicity of infection of two. Values presented as mean  $\pm$  SD of cells from three different donors and statistically analysed by the two-tailed, paired t-test. (b) Primary macrophages ( $1 \times 10^6$ ) were infected with H1N1 (54/98) or H5N1 (483/97). The culture supernatants were collected at 6, 10, 18, and 24 hours post-infection. The viral titres (TCID<sub>50</sub>) of the samples were measured by titration in Madin Darby canine kidney cells. The results shown are representative of experiments performed on cells from three different donors. (c) Primary macrophages ( $2 \times 10^6$ ) were infected with influenza virus H5N1 (483/97) or H1N1 (54/98) at a multiplicity of infection of two. At 12 hours post-infection, the cells were collected for ultrastructural examination by transmission electron microscopy. H1N1-infected macrophages (left), H5N1-infected macrophages (middle), mock-treated macrophages (right) are shown with magnification  $\times 28\,000$ . (d) Primary macrophages ( $1 \times 10^6$ ) were harvested at 6, 12, and 18 hours post-infection with H5N1 (483/97) or H1N1 (54/98) virus. The activated level of PARP was assayed by Western analysis using a monoclonal anti-PARP antibody. The numbers in the brackets are density values of the activated PARP relative to that of the actin. (e) Delayed activation of caspase 3 in the H5N1-infected human macrophages. Primary macrophages ( $1 \times 10^6$ ) were harvested at 6, 8, 10, and 12 hours post-infection with H5N1 (483/97) or H1N1 (54/98) virus. The activation of caspase 3 was assayed by Western analysis using polyclonal anti-caspase 3 antibodies. (f) Primary human macrophages ( $1 \times 10^6$ ) were pretreated with or without 50  $\mu$ M of caspase inhibitor, Z-IETD-FMK, for 1 hour at 37°C and infected with H5N1 (483/97) or H1N1 (54/98) virus, or without infection. The total proteins were harvested at 18 hours post-infection and assayed by Western analysis using anti-PARP antibody. Equal loading of protein samples was determined using anti-actin antibodies. The density of the protein band was determined by using Bio-Rad Quantity One imaging software. The numbers in the brackets are density values of the activated-caspase 3, or activated-PARP relative to that of the actin. (Figures were adopted from J Gen Virol 2007;88:1275-80 with permission, approval pending)

virus differentially activated IRF3 leading to activation of IFNs and chemokines. Moreover, H5N1 and H9N2/G1 viruses triggered a delayed and caspase-mediated apoptotic response in human macrophages as compared with the human influenza viruses. Hence, further investigation of the pathogenic properties of the H5N1 virus including delayed onset of apoptosis and hyperinduction of proinflammatory cytokines may contribute to the understanding of how these novel viruses cause the fatal disease in humans. Furthermore, therapies that minimise immunopathology-associated dysregulation of innate immunity without impairing effective host defence may be valuable adjuncts to antiviral therapy.

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