

# Induction of Proinflammatory Cytokines in Primary Human Macrophages by Influenza A Virus (H5N1) Is Selectively Regulated by IFN Regulatory Factor 3 and p38 MAPK<sup>1</sup>

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The hyperinduction of proinflammatory cytokines and chemokines such as TNF- $\alpha$ , IFN- $\beta$ , and CCL2/MCP-1 in primary human macrophages and respiratory epithelial cells by the highly pathogenic avian influenza H5N1 is believed to contribute to the unusual severity of human H5N1 disease. Here we show that TNF- $\alpha$ , IFN- $\beta$ , and IFN- $\lambda$ 1 are the key mediators directly induced by the H5N1 virus in primary human macrophages. In comparison with human influenza (H1N1), the H5N1 virus more strongly activated IFN regulatory factor 3 (IRF3). IRF3 knockdown and p38 kinase inhibition separately and in combination led to a substantial reduction of IFN- $\beta$ , IFN- $\lambda$ 1, and MCP-1 but only to a partial reduction of TNF- $\alpha$ . IRF3 translocation was independent of p38 kinase activity, indicating that IRF3 and p38 kinase are distinct pathways leading to cytokine production by H5N1 virus. We conclude that IRF3 and p38 kinase separately and predominantly contribute to H5N1-mediated induction of IFN- $\beta$ , IFN- $\lambda$ 1, and MCP-1 but only partly control TNF- $\alpha$  induction. A more precise identification of the differences in the regulation of TNF- $\alpha$  and IFN- $\beta$  could provide novel targets for the design of therapeutic strategies for severe human H5N1 influenza and also for treating other causes of acute respiratory distress syndrome. *The Journal of Immunology*, 2009, 182: 1088–1098.

Influenza H5N1 viruses continue to transmit zoonotically, causing severe human disease, and pose a pandemic threat. The mechanisms underlying its pathogenesis remain uncertain. Increased viral replication competence, capacity for dissemination beyond the respiratory tract, and unusual tissue tropism have all been proposed to be relevant to pathogenesis (1). In addition to these mechanisms, there is in vitro and in vivo evidence that cytokine dysregulation contributes to the pathogenesis of human H5N1 disease (2–5).

Macrophages are present in most tissues including the lung and are central players in both innate and adaptive immune responses against viruses and other intracellular pathogens. Alveolar epithelial cells and macrophages have been identified as targets of H5N1 virus infection within the human lung in autopsy studies and in ex vivo cultures (6, 7). Humans and mice infected with H5N1 viruses have markedly elevated numbers of macrophages in the lung (4,

8). When compared with human influenza virus (e.g., H1N1)-infected macrophages in vitro, H5N1 virus-infected human primary macrophages produce higher levels of cytokines and chemokines such as TNF- $\alpha$ , IFN- $\beta$ , IL-6, IL-1 $\beta$ , CXCL10/IFN- $\gamma$ -inducible protein 10 (IP-10),<sup>3</sup> CCL5/RANTES, CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , and CCL4/MIP-1 $\beta$  (2, 8, 9). Similarly, H5N1 viruses hyperinduce chemokines such as IP-10 and MCP-1 from primary respiratory epithelial cells in vitro (3). H5N1 virus genotypes differ in their capacity to induce cytokines in vitro (9), but the precise viral and genetic determinants underlying this high cytokine inducing phenotype are not well understood. Patients with H5N1 disease have higher serum levels of IP-10 and MCP-1 than patients infected with seasonal influenza (4, 5). The serum concentrations of these mediators were higher in patients with fatal H5N1 disease than in those who survive and are correlated with viral load in the respiratory tract. TNF- $\alpha$  is associated with the pathogenesis of acute respiratory distress syndrome, and chemokines such as IP-10 and MCP-1 are chemoattractants of monocytes and macrophages that may amplify the inflammatory response. Lungs of mice infected with some isolates of H5N1 virus such as A/Thailand/SP/83/2004 have markedly elevated levels of macrophage tropic chemokines (MCP-1, MIP-1 $\alpha$ ) compared with those infected with H1N1 virus (8). Thus, these findings support the hypothesis that differential host responses might contribute to the pathogenesis of human H5N1 disease. It is relevant that the pathogenesis of the 1918 Spanish influenza pandemic is also believed to be associated with cytokine dysregulation (10, 11) and provides an interesting parallel with human H5N1 disease. Mice with disrupted *CCR2* or

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<sup>3</sup> Abbreviations used in this paper: IP-10, IFN- $\gamma$ -inducible protein 10; CHX, cycloheximide; IRF3, IFN regulatory factor 3; M, matrix; MFI, median fluorescence intensity; MOI, multiplicity of infection; *RIG-I*, retinoic acid-inducible gene 1; siRNA, small interfering RNA; TBK-1, TANK-binding kinase 1; USF-2, upstream transcription factor 2; SB203580, p38 kinase inhibitor; MDCK, Madin-Darby canine kidney; COX2, cyclooxygenase 2.

*TLR-3* genes infected with mouse-adapted influenza viruses had improved survival despite increased viral load in the lung (12, 13). Thus, a better understanding of the mechanisms underlying the cytokine hyperinduction mediated by H5N1 virus could contribute to the development of novel therapeutic options for managing H5N1 disease and a potential H5N1 pandemic. Furthermore, these studies may provide insights into other cytokine-driven lung pathologies including acute respiratory distress syndrome.

Cytokines and chemokines are activated in a complex autocrine and paracrine cascade. In the differentially activated cytokine response induced by H5N1 virus, it is not clear which of these mediators are primarily induced by the virus and which ones result from autocrine or paracrine feedback. Additionally, the cell-signaling pathways responsible for such cytokine activation are not fully understood. The p38 kinase is a member of the MAPK superfamily, which plays important roles in signal transduction in a wide range of biological processes such as cytokine synthesis, cell survival, and apoptosis. Treatment with p38 kinase inhibitor (SB203580) shows a suppressed production of inflammatory mediators such as TNF, IL-1, IL-6, CXCL8/IL-8, and GM-CSF (14). p38 kinase is essential for virus-induced cytokine production by bronchial epithelial cells including IL-6, IL-8, and GM-CSF (15–17). We have shown that p38 kinase is involved in the hyperinduction of TNF- $\alpha$  expression in H5N1-infected macrophages (18), although the mechanisms underlying this hyperinduction of cytokine by H5N1 influenza virus have not been elucidated. Recent studies have reported hyperactivation of phospho-p38 kinase with increased cytokine concentrations in plasma samples of patients infected with severe seasonal influenza A virus (19). However, there is a lack of information concerning the role of p38 kinase on the production of other cytokines and chemokines mediated by H5N1 virus infection.

IFN regulatory factor 3 (IRF3) is a transcription factor of IFN- $\beta$ . Upon viral infection, I $\kappa$ B kinase  $\epsilon$  and TANK-binding kinase 1 (TBK-1) phosphorylate IRF3, leading to activation and homodimerization of the molecule. Dimerized IRF3 complexes with transcriptional coactivators p300, and CREB accumulates in the nucleus (20, 21), initiating the transcription of IFN- $\beta$  (22). Influenza virus nonstructural 1 (*NS1*) gene or its products help the virus evade the IFN antiviral systems by blocking IRF3 activation and the subsequent transcription of IFN- $\beta$  promoter (23). Its role in the differential hyperinduction of cytokines mediated by H5N1 virus remains to be defined.

In this study, we have examined the involvement of IRF3 and p38 kinase in the induction of cytokines and chemokines in human macrophages infected with human seasonal influenza H1N1 and highly pathogenic influenza A virus H5N1. Because monocyte cell lines might not reflect physiological reality, we have conducted these studies on primary human monocyte-derived macrophages, even though this is a more challenging model for experimentation.

## Materials and Methods

### Reagents and Abs

SB203580 was purchased from Calbiochem, cycloheximide (CHX) was from Sigma-Aldrich, and rabbit polyclonal Abs against human IRF3 and upstream transcription factor 2 (USF-2) were from Santa Cruz Biotechnology. Mouse mAb against human actin was purchased from Chemicon, and HRP- and FITC-conjugated secondary Abs were products of Zymed.

### Cells and viruses

PBMCs were separated 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 (2). The purity of the monocyte preparations was confirmed by immunostaining for CD14 (BD Biosciences). The cells were allowed to differentiate for 14 days

in RPMI 1640 supplemented with 5% heat-inactivated autologous plasma. Macrophages were seeded onto 24-well ( $1.5 \times 10^5$  cells/well) tissue culture plates for studies of mRNA expression. Differentiated macrophages were incubated in serum-free macrophage medium (Life Technologies) supplemented with 0.6  $\mu$ g/ml penicillin and 60  $\mu$ g/ml streptomycin (Sigma-Aldrich) 1 day before virus challenge.

H5N1 influenza viruses A/HK/483/97 (483/97) and A/Vietnam/3212/04 (3212/04) were isolated in Madin-Darby canine kidney (MDCK) cells from patients with H5N1 disease. The genetic sequences of the hemagglutinin genes of these two viruses indicate that they are highly pathogenic avian influenza viruses belonging to clade 0 and clade 1 H5N1 viruses respectively. The other 7 gene segments of the two viruses have different origins and they are designated as two different genotypes (24). A/HK/54/98 (H1N1; 54/98) was a seasonal human influenza virus isolated in MDCK cells. These viruses were cultured in MDCK cells and purified by preadsorption to and elution from turkey RBC (2) to avoid carryover of cellular mediators which may confound the experimental observations. Virus stock was aliquoted and stored at  $-80^\circ\text{C}$  until use.

### Virus infection and drug treatment

Differentiated macrophages were infected at a multiplicity of infection (MOI) of 2. After 45 min of virus adsorption, the virus inoculum was removed, and the cells were washed once and incubated in serum-free macrophage medium supplemented with 0.6  $\mu$ g/ml penicillin, 60  $\mu$ g/ml streptomycin, and 2  $\mu$ g/ml *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Sigma-Aldrich). At the times indicated, aliquots of culture supernatant were collected for cytokine analysis, and RNA was extracted from cells for analysis of cytokine gene expression. At 8 h after infection, replicate cell monolayers were fixed and analyzed by immunofluorescent staining specific for influenza virus nucleoprotein (DAKO Image). When CHX or SB203580 were used in these experiments, the drug was applied 45 min before infection, and the same concentrations were maintained throughout the infection process.

### Small interfering RNA (siRNA)-mediated gene silencing

siRNA against human IRF3 and control siRNA were obtained from Ambion and Qiagen, respectively. Human macrophages were transiently transfected by using the Human Macrophage Nucleofector Kit (Amaxa Biosystems). In brief, macrophages were detached with 5 mM EDTA and a cell scraper, and  $2 \times 10^6$  cells were transiently transfected with 3  $\mu$ g of siRNA using 100  $\mu$ l of Macrophage Nucleofector Solution according to the manufacturer's instructions. Four days after transfection, cells were infected with influenza virus at a MOI of 2. Two other different siRNAs targeting IRF3 were used in three independent experiments. The three siRNAs had different target sites and their sequences were as follows: IRF3 1 sense sequence, GGAGGAUUUCGGAUUCUUCt; IRF3 1 antisense sequence, GAAGAUUCGGAAAUCCUCctg; IRF3 2 sense sequence, GCUC UGCCCUCAACCGCAA; IRF3 2 antisense sequence, UUGCGG UUGAGGGCAGAGC; IRF3 3 sense sequence, UGGAUGAGUUAC UGGGUA; IRF3 3 antisense sequence, UUACCCAGUAACUCA UCCA.

The efficiency of transfection was measured with FITC-conjugated siRNA. Flow cytometric analysis was used to measure the percentage of FITC-positive cells, which were regarded as transfected cells.

### Quantification of mRNA by real-time quantitative RT-PCR

DNase-treated total RNA was isolated by means of an RNeasy Mini kit (Qiagen) and reverse transcribed by using polydeoxythymidylate 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  $\beta$ -actin mRNA have been described previously (2).

### Immunofluorescent staining assay

Macrophages were seeded on coverslips and infected with influenza virus for 3 h. Immunofluorescence staining procedures were modified from a previously published method (25). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 dissolved in PBS. Cells were washed with PBS and first stained with rabbit anti-IRF3 Ab. After a washing with PBS, the cells were subsequently stained with fluorescein-conjugated anti-rabbit IgG Ab (Zymed). Cell nuclei were stained with 1  $\mu$ g/ml 4',6'-diamidino-2-phenylindole (Sigma-Aldrich) and mounted in 50% glycerol in PBS.

### Preparation of cytoplasmic, nuclear proteins, and whole-cell extract

Nuclear and cytosolic fractions of macrophages were prepared using a modification of a previously published method (26). In brief, the macrophages were washed in PBS and lysed with cold buffer A (50 mM HEPES (pH 7.4), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitor mixture (Roche Diagnostics), phosphatase inhibitor mixture set II (Calbiochem), and 0.5% Nonidet P-40) on ice for 20 min. The cell lysates were removed with a cell scraper, and the cytoplasmic portion of the lysate was harvested by centrifugation for 5 min at 4°C. The nuclear pellet was resuspended in 75  $\mu$ l of ice-cold buffer C (10% glycerol, 50 mM HEPES (pH 7.4), 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitor mixture (Roche Diagnostics) and phosphatase inhibitor mixture from Calbiochem), and the nuclei were disrupted on ice with agitation for 15 min. The nuclear protein extract was clarified by centrifugation at 12,000  $\times$  g for 10 min at 4°C.

Whole-cell extracts were prepared by lysing macrophages with cold whole-cell lysis buffer (50 mM KCl, 1% Nonidet P-40, 25 mM HEPES (pH 7.4), 1 mM DTT, protease inhibitor mixture (Roche Diagnostics), and phosphatase inhibitor mixture from Calbiochem) on ice for 10 min. The cell lysates were collected with a cell scraper, and the whole-cell extract was harvested by centrifugation at 13,000 rpm for 10 min at 4°C. The protein content was determined with a Bio-Rad Protein Assay (Bio-Rad Laboratories) using BSA as a standard.

### Western blot analysis

For Western blot analysis, 30  $\mu$ g of whole-cell lysate, 15  $\mu$ g of cytoplasmic protein, and 1  $\mu$ g of nuclear protein were heat denatured in sample buffer (62.5 mM Tris (pH 6.8), 35% glycerol, 2% SDS, 5% 2-ME, 0.05% bromophenol blue). The proteins were separated by electrophoresis on 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in PBS containing 5% (weight-to-volume ratio) skim milk for 1.5 h and immunoblotted with Abs to USF-2, as a marker for the nuclear fraction, actin (Chemicon International), as marker for the cytoplasmic fraction, and IRF3 (Santa Cruz Biotechnology). Bound Abs were visualized by incubation with HRP-coupled goat anti-rabbit or anti-mouse IgG Abs (ZyMed) and ECL plus solution (GE Healthcare).

### Quantification of cytokines by ELISA

Culture supernatants were collected at various time points postinfection and were irradiated with UV light (CL-100 Ultra Violet Cross-linker) for 15 min to inactivate infectious agents (2). The concentrations of cytokines and chemokines in culture supernatants were quantified by ELISA assays (R&D Systems). The detection limits for the ELISA development kits are as follows: IFN- $\beta$ , 32–2000 pg/ml; TNF- $\alpha$ , 16–1000 pg/ml; MCP-1, 16–1000 pg/ml; and IP-10, 31–2000 pg/ml.

### Phosphoepitope staining for flow cytometric analysis

The procedures of intracellular epitopes staining were modified from the protocol described by Krutzik and Nolan (27). Human macrophages were detached with cold 10 mM EDTA after incubation at 4°C for 60 min. Cell suspensions were infected with mock or influenza viruses and cultured in an ultralow attachment plate (Corning) for various times. Infected human macrophages were fixed with CytoFix and CytoPerm buffer (BD Biosciences) for 10 min and pelleted. After replacement with 4% paraformaldehyde, the cells were permeabilized with Perm III buffer (BD Biosciences) and incubated at 4°C for 30 min;  $4 \times 10^5$  cells were washed twice in staining buffer and incubated with 100  $\mu$ l of diluted anti-TBK-1 phosphorylated at Ser<sup>172</sup> Ab (1:10, v/v; BD Biosciences) at room temperature for 1 h. The cells were washed with 2 ml of staining buffer and resuspended in 200  $\mu$ l of staining buffer for flow cytometric analysis. Analysis was performed on a FACSCalibur cytometer (BD Biosciences) equipped with a 488 nm laser and emission filter for PE. Comparison was made on the median fluorescence intensity (MFI) of the samples. The percentage increase of the MFI was calculated by dividing the difference between the MFI of infected and uninfected samples by that of the uninfected sample and multiplied by 100 at the corresponding time point.

## Results

### Kinetics of cytokine and chemokine induction by influenza viruses

Because macrophages are a major source of cytokine production during infection, we compared the gene expression of IFN- $\beta$ , TNF- $\alpha$ , IFN- $\lambda$ 1, MCP-1, IL-1 $\beta$ , IP-10, MIP-1 $\alpha$ , and RANTES in

influenza virus (54/98 H1N1, 483/97 H5N1, 3212/04 H5N1)-infected primary human macrophages by quantitative real-time PCR.

The induction of TNF- $\alpha$  mRNA by different H1N1 human isolates and two H5N1 human isolates was measured 6 h postinfection in differentiated primary human macrophages. All three low-pathogenic human H1N1 viruses induced much less TNF- $\alpha$  mRNA expression than the two highly pathogenic H5N1 human isolates (Fig. 1A). We then investigated the induction of cytokines and chemokines by 54/98 (H1N1), 483/97 (H5N1), and 3212/04 (H5N1) viruses in the following experiments.

The cytokine and chemokine mRNA induced in primary human macrophages after infection with H5N1 viruses (483/97 and 3212/04) or H1N1 (54/98) virus is shown. IFN- $\beta$ , TNF- $\alpha$  and IFN- $\lambda$ 1 mRNA began to increase as early as 3 h postinfection of 483/97 and 3212/04, whereas MCP-1, IL-1 $\beta$ , IP-10, MIP-1 $\alpha$ , and RANTES did not increase until 6 h later (Fig. 1B). The mRNA level of influenza A matrix (M) gene expression was measured. 54/98 (H1N1) replicated at a level comparable with those of 483/97 and 3212/04. These results suggest that the up-regulation of cytokine expression seems not to be related to the replication level. Fig. 1 shows the mean of three independent experiments, each from a different donor. UV-irradiated viruses did not induce cytokine or chemokine expression (Fig. 1C) suggesting that the induction of cytokines and chemokines after infection requires replicating virus.

### Effect of protein synthesis inhibition on cytokine and chemokine gene expression

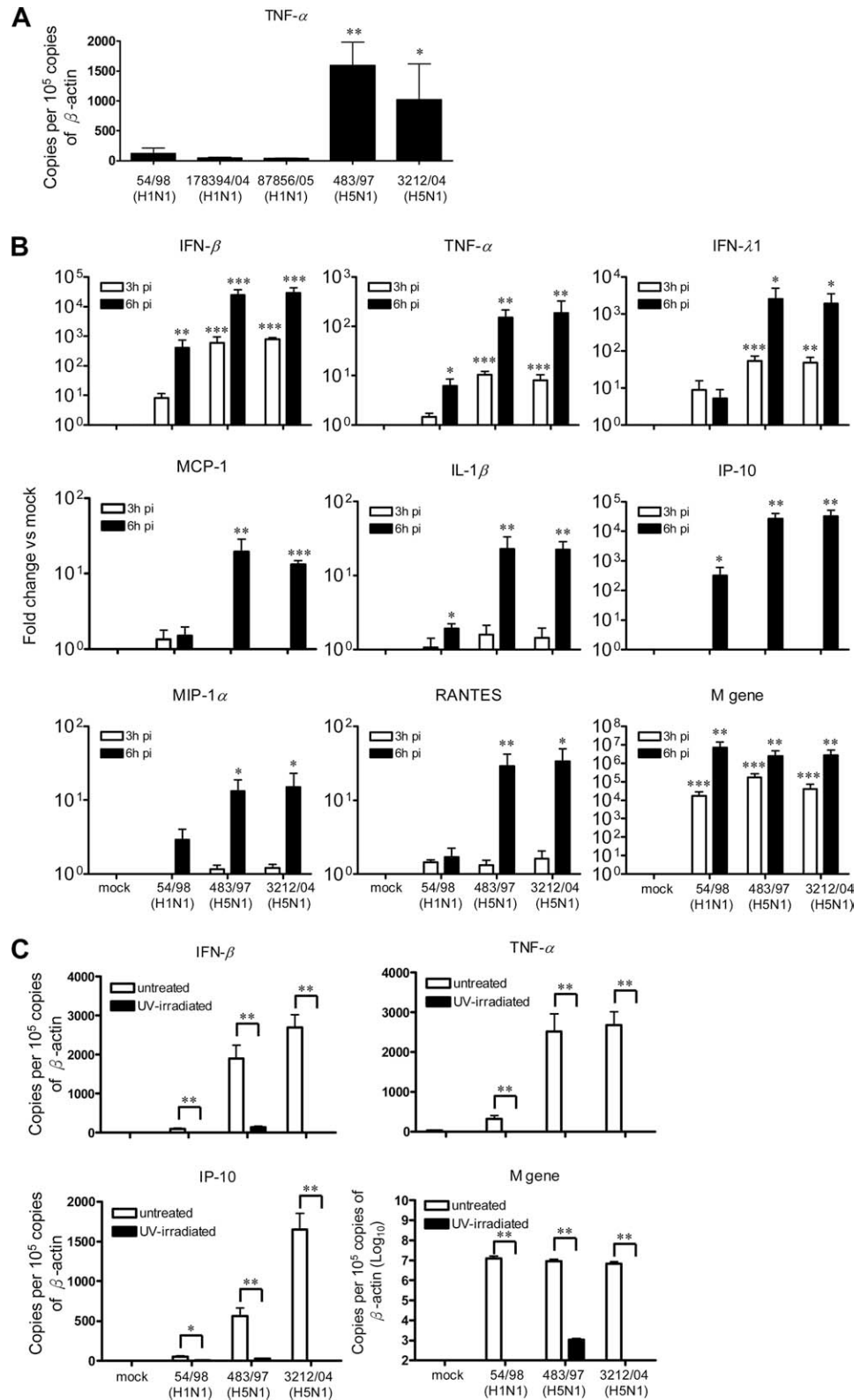
To determine which of these cytokines and chemokines are induced directly by virus replication rather than via the autocrine/paracrine feedback effects from other cytokines, a protein synthesis inhibitor, CHX, was used to treat the macrophages throughout the process of influenza virus infection. In fact, the mRNA levels of some cytokines (e.g., TNF- $\alpha$ ) in mock infected cells were enhanced after CHX treatment, and this might be related to the removal of a negative feedback mechanism of cytokine induction. However, the up-regulation of IFN- $\beta$  (Fig. 2A), TNF- $\alpha$  (Fig. 2B), and IFN- $\lambda$ 1 (Fig. 2C) in CHX-treated cells was further enhanced by virus infection, demonstrating that the induction of these cytokines was not inhibited by CHX treatment.

In contrast, the increase of other cytokines and chemokines (including MCP-1, as in Fig. 2D, and IP-10, as in Fig. 2E) mRNA levels vs mock was greatly suppressed by treatment with CHX, suggesting that de novo protein synthesis is required for the induction of these genes. The effect of CHX pretreatment on IL-1 $\beta$  expression was variable (Fig. 2F). Because TNF- $\alpha$  is one of the most abundant cytokines secreted by the infected macrophages, the concentrations of TNF- $\alpha$  were measured by ELISA to examine the inhibitory effect of CHX treatment. In two independent experiments, TNF- $\alpha$  was detected in supernatants of infected macrophages at indicated time points with 483/97 (H5N1) and 3212/04 (H5N1) viruses but not in those with 54/98 (H1N1) (Fig. 2G), whereas in the presence of 30 or 100  $\mu$ M CHX, there was no detectable TNF- $\alpha$  in culture supernatants after 483/97 or 3212/04 infection.

### Direct activation of IRF3 by H5N1 viruses

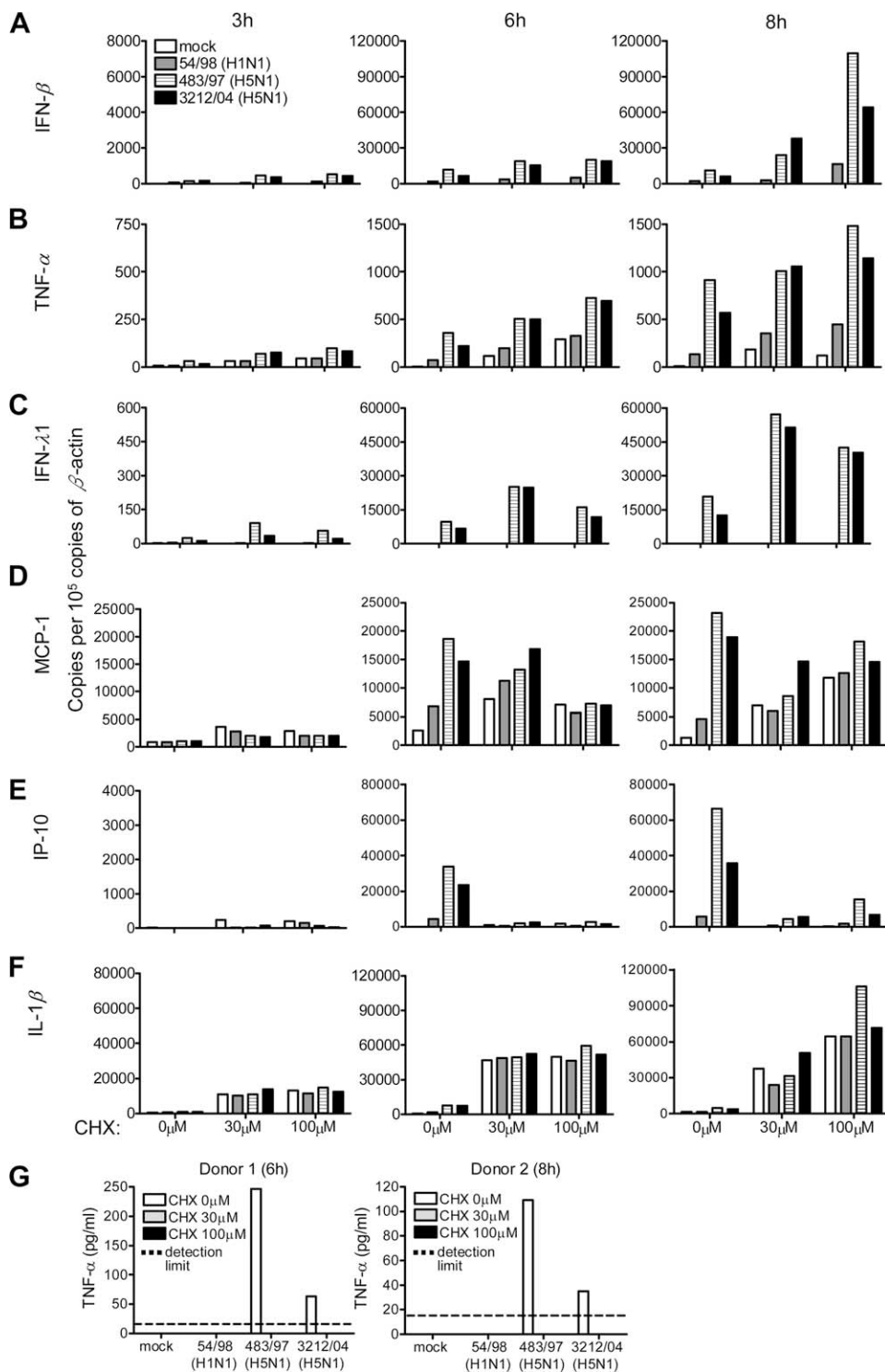
Because IFN- $\beta$  was differentially up-regulated by 483/97 (H5N1) and 3212/04 (H5N1) viruses compared with 54/98 (H1N1) virus, the activation of IRF3 was examined. IRF3 is an essential transcription factor for the expression of IFN- $\beta$  (28). It is constitutively expressed in the cytoplasm of resting cells. Following viral infection, dsRNA transfection, or the presence of stress inducers, the kinases TBK-1 and I $\kappa$ B kinase  $\epsilon$  are activated leading to the

**FIGURE 1.** Influenza virus-mediated cytokines and chemokines exhibit different expression kinetics and are dependent on virus replication. Differentiated primary human macrophages were infected with influenza viruses as indicated at a MOI of 2. *A*, Cell lysates were collected at 3 h postinfection (pi), and the expression of TNF- $\alpha$  mRNA was measured by using real-time PCR. Results are expressed as mean  $\pm$  SD. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; compared with 54/98-infected cells. *B*, Differentiated primary human macrophages were infected with 54/98 (H1N1), 483/97 (H5N1), or 3212/04 (H5N1) at a MOI of 2. Mock infected cells served as controls. Cell lysates were collected at 3 and 6 h postinfection and analyzed by real-time PCR for mRNA expression of cytokines, chemokines, and influenza A *M* gene as indicated. Undetectable cytokines or chemokines in mock samples were assigned one copy for calculation of fold change. Results are presented as means  $\pm$  SEM of three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; compared with mock infected cells at corresponding time points. By comparing with 54/98 (H1N1), statistical significance was observed in the expression of IFN- $\beta$  and TNF- $\alpha$  at 3 h postinfection with 483/97 and 3212/04 ( $p < 0.05$ ) whereas at 6 h postinfection with 483/97 and 3212/04, statistical significance was observed in the expression of IFN- $\beta$ , TNF- $\alpha$ , MCP-1, IL-1 $\beta$ , and RANTES ( $p < 0.05$ ). *C*, Viruses with or without UV irradiation were used to infect primary human macrophages at a MOI of 2. RNA was extracted 6 h postinfection, and the expression of cytokines, chemokines, and influenza A virus *M* gene mRNA were measured by real-time PCR. mRNA expression of each target gene was normalized to  $\beta$ -actin mRNA expression. Results are expressed as mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; comparison was made as indicated.



phosphorylation of IRF3 which dimerizes and translocates into the nucleus leading to IFN- $\beta$  gene transcription. By using flow cytometry analysis with mAb against TBK-1 phosphorylated at Ser<sup>172</sup>, we found that 483/97 virus induced greater activation of TBK-1 (21.5, 29.0, and 17.09% vs mock at 1, 2, and 3 h postinfection, respectively) than 54/98-infected macrophages (11.5, 6.5, and 4.8% vs mock at 1, 2, and 3 h postinfection, respectively) as shown in Table I and Fig. 3.

At 3 h postinfection with 54/98 (H1N1), 483/97 (H5N1), and 3212/04 (H5N1) at a MOI of 2, translocation of IRF3 into the nucleus was detected by an immunofluorescent staining assay, and the number of cells with IRF3 translocation per total cells in three representative low-power fields was counted. The number of cells with IRF3 translocation was greater in 483/97 virus (49 of 100 cells)- and in 3212/04 virus (41 of 100 cells)-infected macrophages than in those infected with 54/98 virus (6 of 100 cells). Moreover,



**FIGURE 2.** Influenza virus induced transcription of IFN- $\beta$ , TNF- $\alpha$ , and IFN- $\lambda$ 1 mRNA is independent of protein synthesis. *A–F*, Differentiated primary human macrophages were infected with 54/98 (H1N1), 483/97 (H5N1), or 3212/04 (H5N1) at a MOI of 2 in the presence of CHX at indicated concentrations. Mock infected cells served as controls. At 3, 6, and 8 h postinfection, cell lysates were collected and analyzed by real-time PCR for mRNA expression of IFN- $\beta$  (*A*), TNF- $\alpha$  (*B*), IFN- $\lambda$ 1 (*C*), MCP-1 (*D*), IP-10 (*E*), and IL-1 $\beta$  (*F*). The mRNA expression of each target gene was normalized by  $\beta$ -actin mRNA expression. *G*, The concentrations of TNF- $\alpha$  in the culture supernatant were measured by ELISA at the indicated time points after 54/98 (H1N1), 483/97 (H5N1), or 3212/04 (H5N1) infection at a MOI of 2 in two different donors.

treatment of the cells with CHX did not prevent the nuclear translocation of IRF3 after 54/98 (11 of 100 cells), 483/97 (51 of 100 cells) or 3212/04 (54 of 100 cells) infection (Fig. 4A). Western blotting results also showed that in the absence of CHX treatment, both H5N1 viruses induced more IRF3 translocation into the nuclei than 54/98 virus. In the presence of CHX treatment, both H5N1 viruses again induced more nuclear translocation of IRF3 than 54/98 (H1N1) virus (Fig. 4B). Taken together, these findings indicate that IRF3 activation and translocation are directly induced by influenza viruses and that H5N1 viruses are more potent than H1N1 viruses in this regard.

#### Dependence of cytokine and chemokine expression on IRF3

To confirm the involvement of IRF3 in the expression of cytokines and chemokines induced by the H1N1 (54/98) and H5N1 (483/97 and 3212/04) viruses, siRNA targeted specifically against the mRNA of IRF3 was transfected into primary human macrophages. The efficiency of transfection was measured by using a FITC-conjugated siRNA and flow cytometric analysis. FITC-positive cells were regarded as transfected cells and the average of transfected cells was ~61% of the whole population in three independent experiments. To define the effects of siRNA transfection on the

Table I. Phosphorylation of TBK-1 (serine 172) after influenza virus infection in human macrophages

Postinfection Period (h)	Increase of MFI (%) <sup>a</sup>	
	54/98 (H1N1)	483/97 (H5N1)
1	11.51 ± 1.15	21.52 ± 4.58 <sup>*b</sup>
2	6.47 ± 3.69	29.02 ± 6.48 <sup>**c</sup>
3	4.8 ± 4.41	17.09 ± 9.34

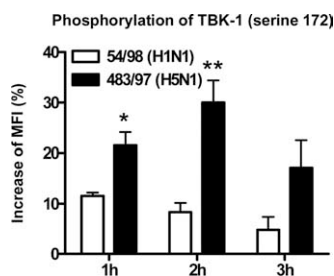
<sup>a</sup> Percentage increase of the median fluorescent intensity (MFI) was calculated by dividing the difference of MFI between infected and uninfected samples by that of the uninfected sample and multiplied by 100 at the corresponding time points.

<sup>b</sup> \*,  $p < 0.05$  compared with 54/98 at corresponding time points.

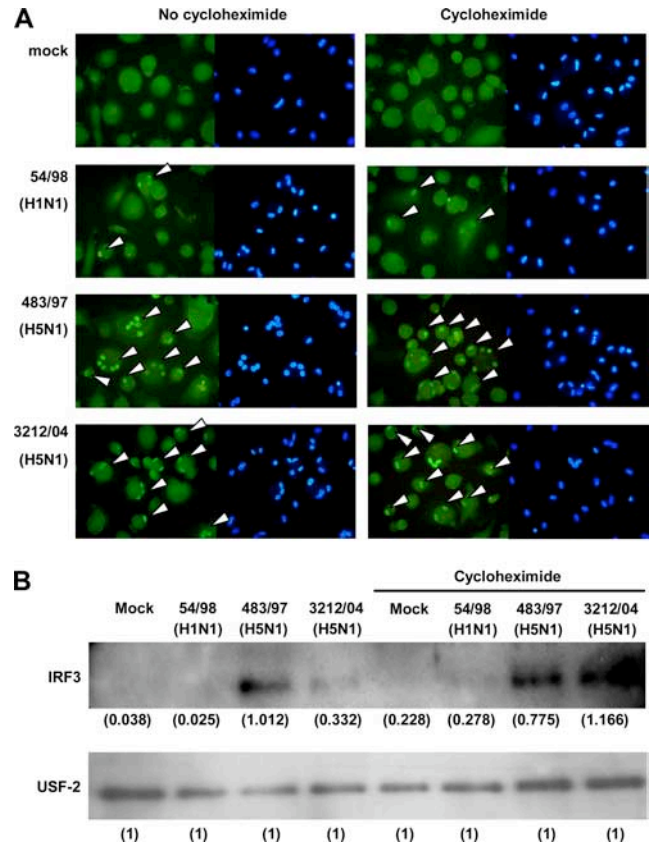
<sup>c</sup> \*\*,  $p < 0.01$  compared with 54/98 at corresponding time points.

cytokine mRNA kinetics on the host cells, we first quantitated the mRNA levels of IFN- $\beta$ , TNF- $\alpha$ , and RANTES as well as the target gene *IRF3* at 2, 4, 8, and 10 days after the transfection by real-time PCR analysis. At 4 days posttransfection, *IRF3* gene expression was still reduced by 57% compared with cells transfected with control siRNA (Fig. 5A), and the protein level of IRF3 in siRNA-transfected cells were determined by Western blotting (Fig. 5B). After 4 days posttransfection, the cells retained good viability and there was no detectable up-regulation of IFN- $\beta$ , TNF- $\alpha$ , and RANTES mRNA response resulting from the siRNA transfection. The cells were challenged with 54/98 (H1N1), 483/97 (H5N1), or 3212/04 (H5N1) viruses to determine the impact of siRNA gene silencing on virus induced innate host responses. Three siRNAs targeting different regions of the *IRF3* gene were used separately in the experiments, and the experiments were repeated in macrophages isolated from three different donors. The cytokine expression profiles after virus infection were similar among the three siRNAs. A representative result using IRF3 siRNA 1 is shown in Fig. 5, C–E.

The IRF3 dependence of 3212/04 (H5N1) virus-induced cytokine responses was comparable with those of 483/97 (H5N1), as shown in Fig. 5. The expression profiles of the cytokines following IRF3 knockdown could be categorized into four groups (Fig. 5C). In the first group, the mRNA expression of IFN- $\beta$ , IFN- $\lambda$ 1, and IP-10 decreased substantially by >80% in the IRF3-silenced cells at both 3 and 6 h postinfection with 483/97 (H5N1). These findings indicated that H5N1 virus-induced IFN- $\beta$ , IFN- $\lambda$ 1, and IP-10 production was largely dependent on IRF3. This is in agreement with previous reports that IRF3 is a transcription factor of IFN- $\beta$ , IFN- $\lambda$ 1, and IP-10 (29, 30). However, the reduction of IP-10 expression



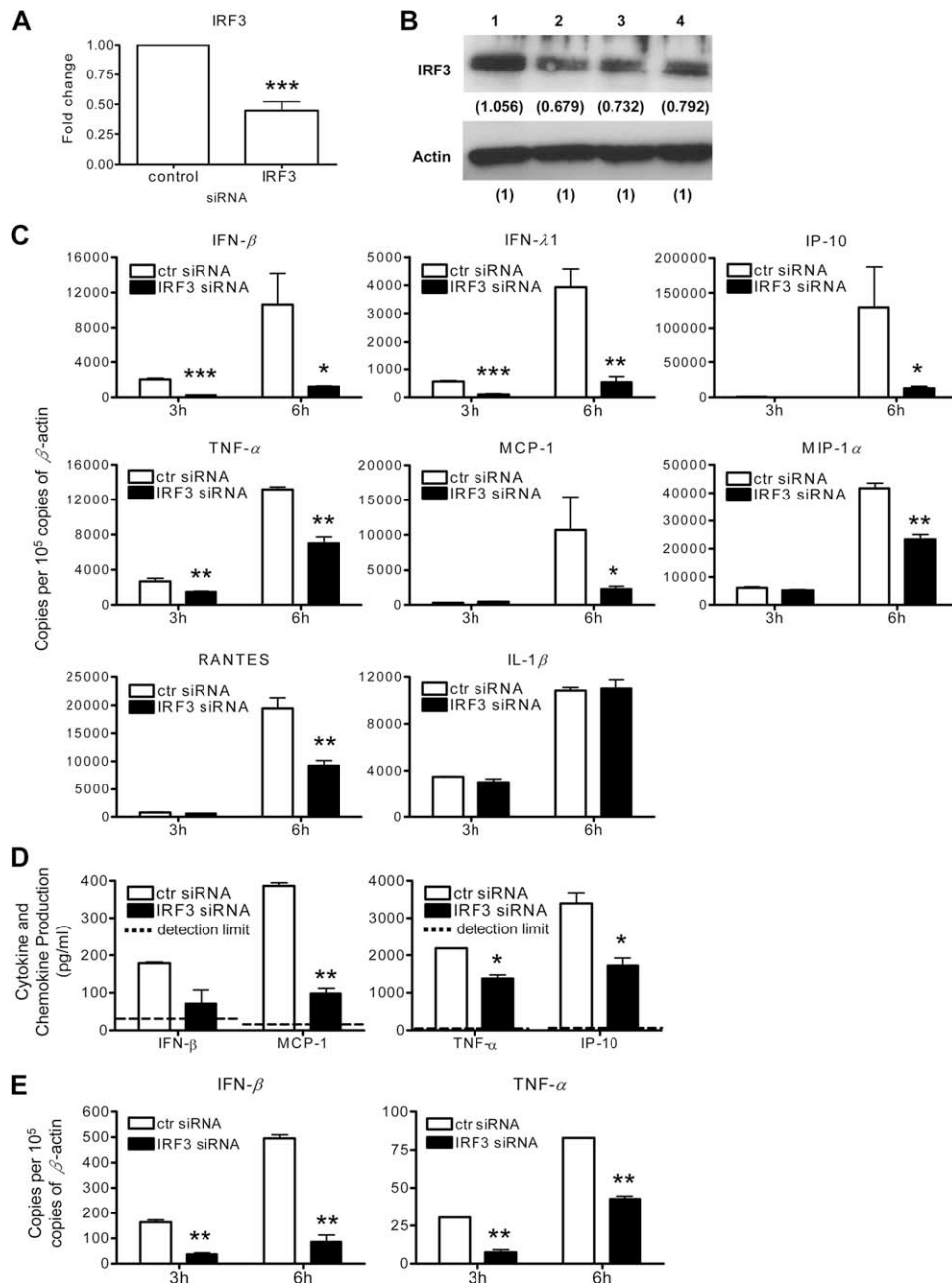
**FIGURE 3.** H5N1 virus differentially activates TBK-1 compared with H1N1 virus. Primary human macrophages were infected with 54/98 (H1N1) or 483/97 (H5N1) at a MOI of 2. Uninfected cells served as controls. Cells were fixed at different periods after infection as indicated. Phosphorylation of TBK-1 was measured using mAb against phosphorylated TBK-1 at Ser<sup>172</sup> and analyzed on a flow cytometer. Results are the percentage increase in MFI ± SD of three separate experiments, compared with that of uninfected samples. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; compared with 54/98 at corresponding time points.



**FIGURE 4.** H5N1 viruses differentially activate IRF3 translocation compared with H1N1 virus. Differentiated primary human macrophages were infected with 54/98 (H1N1), 483/97 (H5N1), or 3212/04 (H5N1) at a MOI of 2 in the absence or presence of 30  $\mu$ M CHX. Mock infected cells served as controls. A, Immunofluorescence staining assay of IRF3 translocation. At 3 h postinfection, the cells were fixed with 4% paraformaldehyde. Permeabilized cells were stained with anti-IRF3 Ab and FITC-conjugated secondary Ab. Cell nuclei were stained with 4',6'-diamidino-2-phenylindole. Arrowheads, Cells with IRF3 translocation. B, Western blotting of IRF3 in nuclear protein fraction. After 3 h of infection, nuclear fractions were collected, and the translocation of IRF3 was analyzed by Western blotting. USF-2 was used as a marker for nuclear content and loading control. Results are of a representative experiment taken from three separate experiments. The density of the protein band was measured by using Alpha Ease FC software (Alpha Innotech). Values in parentheses are relative densities of IRF3 to USF-2.

may also be secondary to the inhibition of IFN- $\beta$ . The second group comprised genes that were partially inhibited by IRF3 silencing and included TNF- $\alpha$  which was inhibited 35–48% in 483/97 (H5N1)-infected cells (Fig. 5C). Thus, IRF3 and/or its downstream gene products contribute to the activation of signaling pathways, leading to TNF- $\alpha$  production by H5N1 viruses, but this is not the only pathway activating this cytokine. The third group of cytokines, MCP-1, MIP-1 $\alpha$ , and RANTES, was reduced by 63, 32, and 53%, respectively, at 6 h postinfection. These were shown to be chemokines that appeared later after virus infection, and the expression of MCP-1 required de novo protein synthesis (i.e., inhibited by CHX shown in Fig. 2). Taken together, the above results suggest that MCP-1 expression may be induced by autocrine/paracrine pathways such as those mediated by IFN- $\beta$ , TNF- $\alpha$ , IFN- $\lambda$ 1, or all of them. The fourth group of cytokines, such as IL-1 $\beta$ , was not inhibited by IRF3 knockdown (Fig. 5C).

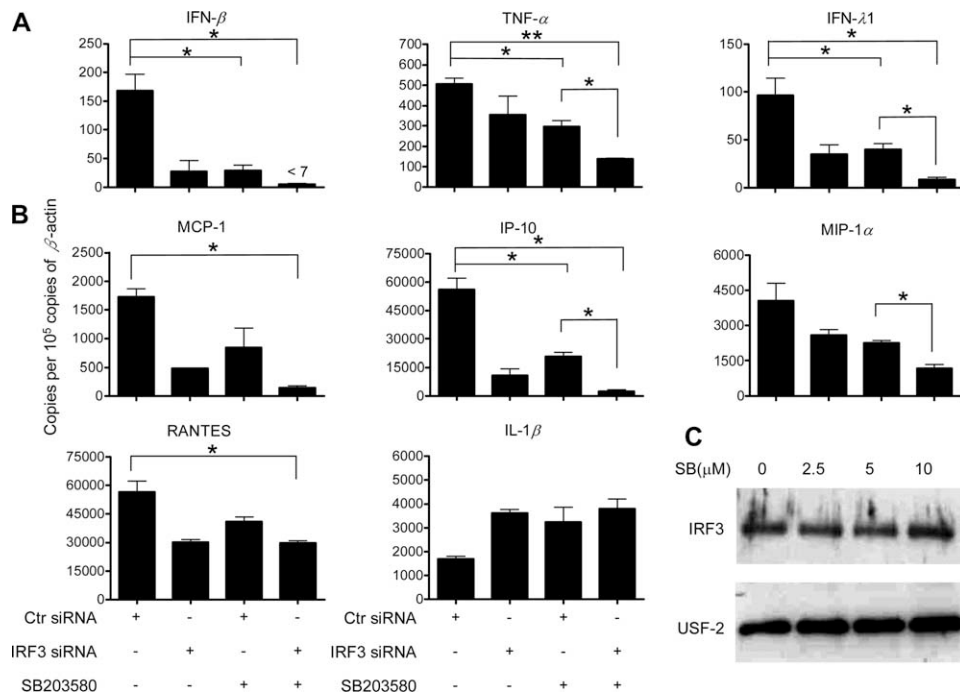
Protein concentrations of IFN- $\beta$ , TNF- $\alpha$ , MCP-1, and IP-10 were measured in culture supernatants of siRNA-transfected and



**FIGURE 5.** H5N1-mediated cytokine and chemokine expression exhibits differences in their dependence on IRF3. *A* and *B*, IRF3 knockdown by RNA interference. Primary human macrophages differentiated for 10 days *in vitro* were transfected with 3  $\mu$ g of siRNA targeting IRF3 (IRF3 siRNA) or control (ctr) nontargeting siRNA. Four days after transfection, IRF3 mRNA was measured by real-time PCR. After normalization to the  $\beta$ -actin mRNA level, the knockdown efficiency was calculated by comparison with cells transfected with control siRNA. Results are presented as mean  $\pm$  SEM from three independent experiments. \*\*\*,  $p < 0.001$ , compared with control siRNA (ctr siRNA)-transfected cells. *B*, Whole-cell lysates were collected 4 days posttransfection, and the protein level of IRF3 was determined by Western blotting. Human primary macrophages were transfected with control nontargeting siRNA (lane 1), IRF3 siRNA 1 (lane 2), IRF3 siRNA 2 (lane 3), and IRF3 siRNA 3 (lane 4). Actin was used as a loading control. Results are of a representative experiment taken from three separate experiments. *C* and *D*, Effect of IRF3 knockdown on cytokine and chemokine expression after H5N1 infection. Four days after transfection with IRF3 or control siRNA, macrophages were infected with 483/97 (H5N1). *C*, Cell lysates were collected at 3 and 6 h postinfection and analyzed by real-time PCR for mRNA expression. mRNA expression of each target gene was normalized to  $\beta$ -actin mRNA expression. Results are expressed as mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; compared with control siRNA-transfected cells at corresponding time points. *D*, The concentrations of cytokine and chemokine in the culture supernatant were measured by ELISA at 18 h of 483/97 infection. Results are of a representative experiment taken from two separate experiments. *E*, Effect of IRF3 knockdown on cytokine expression after H1N1 infection. The transfection conditions and experimental setup were the same as 483/97 infection in *C*. Cell lysates were collected 3 and 6 h postinfection and analyzed by real-time PCR for mRNA expression. mRNA expression of each target gene was normalized to  $\beta$ -actin mRNA expression. Chemokines including MCP-1 and IL-1 $\beta$  were not up-regulated at 6 h postinfection after 54/98 infection, and their data are not shown. Results are expressed as mean  $\pm$  SEM. \*\*,  $p < 0.01$  compared with control siRNA transfected cells at corresponding time points.

483/97 (H5N1)-infected macrophages by ELISA. During 483/97 infection, supernatants from IRF3 siRNA-transfected macrophages had lower concentrations of IFN- $\beta$ , TNF- $\alpha$ , MCP-1, and IP-10

(Fig. 5D) than did control siRNA-transfected macrophages. Infection with 54/98 (H1N1) showed a similar dependence on IRF3 in the transcription of cytokines although the level of induction was



**FIGURE 6.** Combining IRF3 knockdown and p38 kinase inhibitor treatment further reduces H5N1-mediated expression of cytokine and chemokine. *A* and *B*, Primary human macrophages were allowed to differentiate 10 days *in vitro*. The cells were transfected with 3  $\mu$ g of siRNA-targeting IRF3 (IRF3 siRNA) or control nontargeting siRNA (ctr siRNA). Four days after transfection, the macrophages were preincubated with p38 kinase inhibitor SB203580 (SB) at 2.5  $\mu$ M for 45 min or mock treated before H5N1 virus challenge. Cell lysates were collected at 3 and 6 h of infection and analyzed by real-time PCR for mRNA expression. The data for early cytokines IFN- $\beta$ , TNF- $\alpha$ , and IFN- $\lambda$ 1 (*A*) are shown at 3 h, and those for the late cytokines MCP-1, IP-10, MIP-1 $\alpha$ , RANTES, and IL-1 $\beta$  (*B*) are shown at 6 h postinfection. The categorization of cytokine induction as early and late was based on data from Fig. 1. The mRNA expression of each target gene was normalized to  $\beta$ -actin mRNA expression. Results are expressed as mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; comparison was made as indicated. *C*, IRF3 translocation is not inhibited by the p38 kinase inhibitor. Differentiated primary human macrophages were preincubated with p38 kinase inhibitor SB203580 and throughout the infection process at concentrations indicated for 45 min or mock treated before 483/97 (H5N1) virus challenge at a MOI of 2. At 3 h postinfection, nuclear fractions were collected, and the translocation of IRF3 was analyzed by Western blotting. USF-2 was used as a marker for nuclear content and loading control. Results are from a representative experiment and repeated at least three times.

lower than 483/97 (H5N1; Fig. 5*E*). The mRNA expression of IFN- $\beta$  was greatly dependent on IRF3 (77.85 and 82.96% at 3 and 6 h postinfection, respectively), whereas TNF- $\alpha$  gene expression was partially inhibited by 39–53% in IRF3 knockdown macrophages.

#### Combination effect of IRF3 knockdown and SB203580 on cytokine expression

We have previously reported that p38 kinase is involved in 483/97 (H5N1)-mediated TNF- $\alpha$  expression (18); we now report that IRF3 knockdown caused partial inhibition of H5N1-induced TNF- $\alpha$  expression, and we investigated the combined effects of these two interventions on H5N1-induced cytokine expression.

Both IRF3 and p38 kinase play a role in the regulation of the expression of 483/97 (H5N1)-induced cytokines and chemokines. Either IRF3 knockdown or SB203580 treatment individually caused >80% (the average of three independent experiments) decrease in IFN- $\beta$  mRNA expression after 3 h of infection (Fig. 6*A*). A combination of these two interventions further reduced IFN- $\beta$  mRNA expression by ~14%. The effect of IRF3 knockdown or SB203580 treatment on the expression of IFN- $\lambda$ 1 was similar to that of IFN- $\beta$ . H5N1-induced TNF- $\alpha$  transcription was inhibited 39% by IRF3 knockdown and 43% by SB203580 treatment separately, at 3 h postinfection. Combination of both IRF3 knockdown and SB203580 treatment caused an additive effect on the inhibition of TNF- $\alpha$  mRNA expression (59% in 3212/04-infected cells, 67% in 483/97-infected cells). Cytokines and chemokines expressed at 6 h postinfection showed different patterns of depen-

dence on p38 kinase. The inhibition of MCP-1 and IP-10 transcription by SB203580 treatment was greater than that of MIP-1 $\alpha$  and RANTES (Fig. 6*B*). Combination treatment strongly suppressed the expression of both MCP-1 and IP-10. In contrast, IL-1 $\beta$  mRNA level was not inhibited by either intervention separately or both in combination. The SB203580 treatment did not prevent H5N1-induced translocation of IRF3 into the nuclei at concentrations up to 10  $\mu$ M at 3 h postinfection (Fig. 6*C*).

## Discussion

A recently isolated H5N1 strain from Vietnam (3212/04) and one isolated during the outbreak in Hong Kong in 1997 (483/97) share the high cytokine inducing phenotype even though they belong to two different genotypes of H5N1 virus (24). These viruses were found to differentially up-regulate IFN- $\beta$ , TNF- $\alpha$ , IFN- $\lambda$ 1, MIP-1 $\alpha$ , MCP-1, IL-1 $\beta$ , IP-10, and RANTES in human primary macrophages when compared with H1N1 (54/98)-infected cells. Hyperinduction of cytokine may therefore contribute to pathogenesis and be one of a number of different ways in which influenza viruses become highly pathogenic. We showed here and in previously published data (2) that other H1N1 (Fig. 1*A*) and H3N2 viruses share the low-cytokine-inducing phenotype. Viral replication was examined by measuring the mRNA level of influenza A *M* gene (Fig. 1*B*). The higher cytokine and chemokine expression induced by 483/97 and 3212/04 in comparison with 54/98 (H1N1) was not necessarily associated with higher levels of virus replication. Moreover, infection with 54/98 (H1N1) at a MOI of 20 raised the *M* gene expression, but this high viral replication level did not

Table II. Summary of cytokine and chemokine mRNA expression after H5N1 (483/97) infection of macrophages

Cytokine or Chemokine	Expression Kinetics <sup>a</sup>	Direct Up-Regulation by Virus Infection <sup>b</sup>	Effect of IRF3 Knockdown <sup>c</sup>	Effect of p38 Inhibitor <sup>c</sup>	Effect of IRF3 Knockdown and p38 Inhibitor <sup>c</sup>
IFN- $\beta$	Early	Yes	↓↓↓	↓↓↓	↓↓↓
TNF- $\alpha$	Early	Yes	↓↓↓	↓↓↓	↓↓↓
IFN- $\lambda$ 1	Early	Yes	↓↓↓	↓↓↓	↓↓↓
CCL2/MCP-1	Late	No	↓↓↓	↓↓↓	↓↓↓
CXCL10/IP-10	Late	No	↓↓↓	↓↓↓	↓↓↓
CCL3/MIP-1 $\alpha$	Late	I	↓↓↓	↓↓↓	↓↓↓
CCL5/RANTES	Late	I	↓↓↓	↓↓↓	↓↓↓
IL-1 $\beta$	Late	I	— <sup>d</sup>	— <sup>d</sup>	— <sup>d</sup>

<sup>a</sup> "Early" represents the up-regulation of the gene  $\geq 6$ -fold when compared to mock-infected cells at 3 h postinfection. "Late" represents those genes that were up-regulated  $< 6$ -fold compared to mock-infected cells at 3 h but with  $\geq 6$ -fold up-regulation at 6 h postinfection.

<sup>b</sup> "I" represents indeterminate.

<sup>c</sup> The reduction of mRNA expression following IRF3 knockdown and/or p38 inhibitor treatment is expressed as follows: ↓,  $< 25\%$  reduction; ↓↓, 26–50% reduction; ↓↓↓, 51–75% reduction; ↓↓↓↓, 76–90% reduction; ↓↓↓↓↓,  $> 91\%$  reduction.

<sup>d</sup> No inhibition.

lead to greater levels of cytokines than in those infected with 483/97 (H5N1) and 3212/04 (H5N1) at a MOI of 2 (data not shown).

The two H5N1 viruses induced mRNA expression of IFN- $\beta$ , TNF- $\alpha$ , and IFN- $\lambda$ 1 at an earlier time point (3 h postinfection), whereas MCP-1, IL-1 $\beta$ , IP-10, MIP-1 $\alpha$ , and RANTES (Fig. 1B) were expressed at 6 h postinfection. The time course of their appearance suggested that IFN- $\beta$ , TNF- $\alpha$ , and IFN- $\lambda$ 1 were probably the cytokines directly induced by the virus (Table II).

The protein synthesis inhibitor, CHX did not suppress the mRNA expression of IFN- $\beta$  (Fig. 2A), TNF- $\alpha$  (Fig. 2B), and IFN- $\lambda$ 1 (Fig. 2C) in response to viral infection, indicating that these cytokines were induced directly by the virus even in the absence of de novo protein synthesis. The observation that mRNA levels of some cytokines were enhanced after CHX treatment in virus-infected cells may be related to the removal of negative feedback mechanism of cytokine induction as a result of blocking protein synthesis. In any event, IFN- $\beta$ , TNF- $\alpha$ , and IFN- $\lambda$ 1 are not autocrine/paracrine responses induced by other cytokines nor do they require expression of viral proteins for their induction (Table II). On the other hand, the up-regulation of MCP-1 (Fig. 2D) and IP-10 (Fig. 2E) mRNA was blocked by CHX treatment, suggesting that this activation of genes following virus infection requires protein synthesis. The human influenza virus 54/98 (H1N1) induced similar cytokine kinetics and susceptibility to inhibition by CHX compared with H5N1 although the responses were lower in magnitude. Because IFN- $\beta$  and IFN- $\lambda$ 1 are the early cytokines differentially up-regulated by H5N1 viruses, we then focused on one of their transcription factors, IRF3. Although there are previous studies showing that influenza virus induced proinflammatory cytokines via the activation of NF- $\kappa$ B (31, 32) and p38 MAPK (18), there is not much information on the role of IRF3 in the induction of TNF- $\alpha$ .

Immunofluorescence staining and Western blotting of IRF3 in influenza virus-infected human macrophages revealed that both H5N1 and H1N1 viruses activated IRF3 and caused its translocation into the nucleus but the magnitude of this effect was greater with the H5N1 viruses. The difference correlated well with the IFN- $\beta$  mRNA expression levels induced by these viruses, suggesting that the IRF-3 translocation was associated with functional activity. IRF3 translocation was not inhibited by the presence of CHX, indicating that de novo protein synthesis was not required for its activation by the virus (Fig. 4). Taken together, these findings demonstrated that H5N1 differentially activated IRF3, that this IRF3 was functional, and that this activation was directly mediated by the virus.

To evaluate the dependence of H5N1- and H1N1-induced cytokine production on IRF3, siRNA specific to mRNA of IRF3 was transfected into human primary macrophages. Because the transfection efficiency (61%) in the human primary macrophage is comparable with the overall reduction of IRF3 mRNA in the whole-cell population (57%), it is likely that there is close-to-complete knockdown of IRF3 mRNA in cells that were successfully transfected. Therefore, the incomplete knockdown of IRF3 in the whole-cell population is more likely to be due to the incomplete transfection efficiency of these primary cells rather than the silencing capability of the IRF3 siRNA. Even though the knockdown of IRF3 was not complete, IFN- $\beta$  and IFN- $\lambda$ 1 (Fig. 5C) transcription was almost completely blocked by siRNA treatment. IRF3 is the key transcription factor in their induction (30, 33), and our results show the regulatory role of IRF3 in H5N1-induced IFN- $\beta$  and IFN- $\lambda$ 1 expression. Furthermore, we provide evidence that influenza virus-induced activation of IRF3 regulates TNF- $\alpha$  induction. In contrast to IFN- $\beta$ , the expression of TNF- $\alpha$  was only modestly inhibited in IRF3-knocked down macrophages. This may be due to the incomplete knockdown of IRF3, but the comparison with the effect on IFN- $\beta$  suggests that TNF- $\alpha$  expression is only partly mediated via IRF3. A similar pattern of IRF3 dependence was observed in 54/98 (H1N1)-induced mRNA expression of IFN- $\beta$  and TNF- $\alpha$ , although the level of induction by 54/98 was much lower than that of 483/97 (Fig. 5E).

The IRF3 knockdown also decreased the transcription of many chemokines including IP-10, RANTES, MCP-1, and MIP-1 $\alpha$  (Fig. 5C). However, based on the kinetics of cytokine and chemokine induction after influenza virus infection and the experiments with CHX treatment, these chemokines appear to be a result of an autocrine or paracrine secondary cascade. IRF3 knockdown suppressed IP-10 expression to an extent similar to that of IFN- $\beta$ ; but because of its dependence on de novo protein synthesis, it is probably induced by secreted mediators (possibly IFN- $\beta$  and/or IFN- $\lambda$ 1) rather than directly by H5N1 infection. This hypothesis is supported by the results of others showing that neutralizing Abs against IFN- $\alpha/\beta$  substantially abolished IP-10 expression induced by influenza A virus in macrophages (34). Both IRF3 and NF- $\kappa$ B regulate RANTES expression (35–37). We found that IRF3 knockdown caused only partial inhibition of RANTES expression, suggesting that IRF3 only partly accounts for H5N1-induced RANTES expression. Evidence that IFN- $\alpha/\beta$  induces MCP-1 (38) may explain the substantial suppression of MCP-1 expression after 6 h of H5N1 infection in IRF3 knockdown macrophages (Table II). The effect of IRF3 knockdown on the production of cytokine and chemokine mRNA after 483/97 infection

was confirmed by assaying IFN- $\beta$ , TNF- $\alpha$ , IP-10, and MCP-1 protein concentrations in culture supernatants by ELISA. IRF3 siRNA transfection led to a reduction in the production of secreted cytokines (IFN- $\beta$  and TNF- $\alpha$ ) and chemokines (IP-10 and MCP-1) in the supernatants following 483/97 infection when compared with supernatants from cells transfected with control siRNA (Fig. 5D). Although 54/98 (H1N1) induced less cytokines and chemokines when compared with 483/97 (H5N1) and 3212/04 (H5N1) viruses (Fig. 1), the H1N1 virus exhibited a similar dependence on IRF3 for the induction of IFN- $\beta$  and TNF- $\alpha$  mRNA expression (Fig. 5E).

Proinflammatory cytokine induction was independent of de novo protein synthesis. Because UV-irradiated virus elicited little or no response (Fig. 1C), we conclude that viral replication is required to trigger the innate immune sensing, leading to the induction of cytokines or chemokines in 483/97 (H5N1) and 3212/04 (H5N1) infection. Retinoic acid-inducible gene I (*RIG-I*) and MDA-5, which are innate immune sensing receptors of ssRNA situated in the cytoplasm, are likely candidates for initiating IRF3 activation. The role of these receptors in H5N1 virus-associated hyperinduction of cytokines and chemokines needs further investigation.

Previous reports have shown that p38 kinase regulates influenza virus-induced RANTES expression in bronchial epithelial cells (39) and is involved in TNF- $\alpha$  production during H5N1 infection (18). Therefore, we explored the effect of IRF3 knockdown and p38 kinase inhibitor treatment both singly and in combination on H5N1-induced cytokine and chemokine expression. Western blot analysis on H5N1-infected macrophages revealed that treatment of these cells with p38 kinase inhibitor did not affect the translocation of IRF3 (Fig. 6C). Whereas IRF3 knockdown or p38 kinase inhibitor treatment alone caused partial reduction of TNF- $\alpha$  expression after 483/97 or 3212/04 infection at 3 h, combination treatment led to a further reduction of TNF- $\alpha$  expression (Fig. 6A). The partial reduction of TNF- $\alpha$  expression by these interventions suggests that 483/97-induced TNF- $\alpha$  may be also regulated by other transcription factors and/or kinases. In contrast to TNF- $\alpha$ , IFN- $\beta$  expression was approximately 80% abolished by either p38 kinase inhibitor (SB203580) or IRF3 knockdown but was further suppressed by a combination of the two interventions. IFN- $\lambda$ 1 showed a similar pattern of expression to IFN- $\beta$ . These results indicate that both IRF3 and p38 pathways separately play a role in H5N1-induced TNF- $\alpha$ , IFN- $\beta$ , and IFN- $\lambda$ 1 expression. Although IRF3 and p38 kinase contribute to the expression of TNF- $\alpha$  and IFN- $\beta$ , there are significant differences in their regulation (Table II). Data shown in Fig. 6B indicate that those later expressed (6 h postinfection) cytokines and chemokines were regulated by IRF3 and p38 kinase to a different degree.

In this study, we have shown that the up-regulation of MCP-1 and IP-10 expression in response to 483/97 (H5N1) and 3212/04 (H5N1) was reduced in the absence of protein synthesis and is likely to be induced as part of the autocrine/paracrine cascade. Partial inhibition of MCP-1 and IP-10 by IRF3 knockdown and p38 kinase inhibition implies that mediators under the regulation of these pathways are playing a role on the transcription of MCP-1 and IP-10. MIP-1 $\alpha$  and RANTES transcription were partially dependent on IRF3 and/or p38 kinase, suggesting that they regulate the mRNA expression of the induction of these cytokines by 483/97. The mRNA expression of IL-1 $\beta$  after 483/97 infection is not reduced by IRF3 knockdown or p38 kinase inhibition, indicating that IL-1 $\beta$  is regulated by different mechanisms. Neither IRF3 nor p38 kinase appear to affect IL-1 $\beta$  and activation of the inflammasome.

H1N1 induced a smaller extent of IRF3 activation than with the two H5N1 virus (483/97 and 3212/04)-infected macrophages. Because IRF3 can be activated by interaction of dsRNA with TLR-3 or *RIG-I* during virus replication (40–42), one may also hypothesize that more competent viral replication may provide stronger signals for triggering innate sensing receptors, which may lead to a stronger induction of cytokines and chemokines. However, as discussed previously, the high-cytokine-induction phenotype of 483/97 (H5N1) and 3212/04 (H5N1) viruses seems not to be solely related to more competent viral replication.

It has been shown that NS1 protein of human influenza viruses allows the virus to evade the antiviral defenses of the host by inhibiting IRF3 activation and the consequent transcription of IFN- $\beta$  (23, 43). This may be the reason for the lower capacity of H1N1 to induce cytokine and chemokine responses. A chimeric H1N1 virus containing the *NS1* gene of H5N1 was associated with a partial but not complete reconstitution of the high-cytokine phenotype in human macrophages (2). Furthermore, mice infected with H1N1 (WSN) virus possessing the 1997 H5N1 *NS* gene had increased amounts of proinflammatory cytokine production (44). Whether the *NS1* gene of the avian H5N1 virus directly promotes the activation of IRF3 (and the subsequent IFN- $\beta$  production) or alternatively is less able to perform its role as an IFN antagonist in mammalian cells as efficiently as human H1N1 virus which has evolved to coexist with the mammalian host, remains to be investigated. In addition to the *NS* gene, other viral internal genes or the combination of those genes may also contribute to the hyperinduction of cytokine by H5N1 viruses.

We recently reported that mediators from H5N1 virus-infected macrophages amplify and broaden the cytokine cascade through their interaction with alveolar epithelial cells. We also showed that cyclooxygenase 2 (COX2) plays a regulatory role in this cytokine cascade and that COX2 inhibitors modulate this response (45). In mice infected with H5N1 virus, others have shown that COX2 inhibitors enhanced the beneficial effect of antiviral drugs (46). Therefore, the inhibition of the early secreted cytokines will markedly attenuate the downstream cytokine cascade in H5N1-infected macrophages.

In conclusion, our results suggest that TNF- $\alpha$ , IFN- $\beta$ , and IFN- $\lambda$ 1 are directly induced by H5N1 virus and in turn play an important role in the subsequent autocrine/paracrine cytokine cascade. We demonstrate that IRF3 and p38 kinase play important roles in H5N1-mediated up-regulation of IFN- $\beta$ , TNF- $\alpha$ , and IFN- $\lambda$ 1 transcription. These results also indicate that there are differences in the pathways leading to the induction of TNF- $\alpha$  and IFN- $\beta$  by H5N1. These pathways may potentially be exploited for selective therapeutic interventions that may attenuate TNF- $\alpha$  induction without completely abrogating the antiviral IFN- $\beta$  induction. If confronted with an H5N1 influenza pandemic, therapies that minimize immunopathology associated with innate immunity without impairing effective host defense may be invaluable adjuncts to antiviral therapy (47).

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## Disclosures

The authors have no financial conflict of interest.

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