Hyperinduction of Cyclooxygenase-2–Mediated Proinflammatory Cascade: A Mechanism for the Pathogenesis of Avian Influenza H5N1 Infection

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The mechanism for the pathogenesis of H5N1 infection in humans remains unclear. This study reveals that cyclooxygenase-2 (COX-2) was strongly induced in H5N1-infected macrophages in vitro and in epithelial cells of lung tissue samples obtained during autopsy of patients who died of H5N1 disease. Novel findings demonstrated that COX-2, along with tumor necrosis factor α and other proinflammatory cytokines were hyperinduced in epithelial cells by secretory factors from H5N1-infected macrophages in vitro. This amplification of the proinflammatory response is rapid, and the effects elicited by the H5N1-triggered proinflammatory cascade are broader than those arising from direct viral infection. Furthermore, selective COX-2 inhibitors suppress the hyperinduction of cytokines in the proinflammatory cascade, indicating a regulatory role for COX-2 in the H5N1-hyperinduced host proinflammatory cascade. These data provide a basis for the possible development of novel therapeutic interventions for the treatment of H5N1 disease, as adjuncts to antiviral drugs.

The emergence and spread of the highly pathogenic avian influenza viruses (H5N1) in poultry and wild birds with repeated zoonotic transmission to humans has raised widespread concern. Outbreaks of H5N1 infection among poultry have now been reported on 3 continents, and at the time this article was written, over 370 cases of infection among humans had been reported, with an overall case fatality rate of over 60% [1]. The H5N1 virus appears to have the capacity to cause severe disease in previously healthy young children and adults

[2, 3], and it is reminiscent of the strain that caused the 1918 influenza pandemic in this respect. The explanation for the severity of H5N1-induced lung pathology may include increased viral replication competence or enhanced inflammatory responses, and the relative importance of these remains to be elucidated [4–6]. Although there is a paucity of autopsy investigation of patients with H5N1 disease, the available data has consistently found very few virus-infected lung epithelial cells, despite widespread histopathological damage [7–10], which supports the argument that host inflammatory responses are important, at least in maintaining ongoing lung pathology in these patients.

We previously found that the H5N1 viruses hyperinduced proinflammatory cytokines and chemokines (by >10 fold) in macrophages and alveolar epithelial cells in vitro, compared with H1N1-infected cells [11, 12], suggesting that this differential host response may contribute to the pathogenesis of infection with H5N1 viruses in humans. Recently, others have reported that H5N1 virus induces lower levels of type-1 interferon (IFN) from differentiated bronchial epithelium [13]. These differences

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in results may relate to the experimental cell phenotype, specifically to differentiated bronchial epithelium as compared with macrophage or alveolar epithelium, or they may relate to the way that the viruses were propagated.

The hyperinduction of proinflammatory cytokines in vitro is paralleled by data showing that some of these cytokines are markedly elevated in the serum and lung tissue of patients with H5N1 disease [4, 7]. High levels of secretory proinflammatory cytokines in the lung is believed to set off a potent inflammatory cascade leading to rapidly progressive pneumonia and acute respiratory distress syndrome (ARDS), the terminal pathology seen in many patients with H5N1 disease.

Recently, we have used microarray analysis to compare gene expression in macrophages infected with either H5N1 or H1N1 influenza virus. In addition to proinflammatory cytokines, H5N1 viruses differentially induced cyclooxygenase-2 (COX-2), but not COX-1 (S.M.Y.L., unpublished data). COX-2 is a component of the arachidonic-acid cascade thought to be involved in the inflammatory process and immune response [14–16] and is the target of anti-inflammatory drugs [17–19]. Recent studies have suggested that COX-2 may be relevant to the pathogenesis of H3N2 virus infection; compared with wild type or COX-1 deficient mice, COX-2 deficient mice had better survival rates and lower levels of proinflammatory cytokines despite having higher levels of virus [20]. Taking this information together, we therefore hypothesized that the hyperinduction of COX-2 may play an important role in the pathogenesis of infection with H5N1 viruses.

To our knowledge, the present study provides the first data obtained by use of an appropriate human cell model to mimic the microenvironment of the lung, together with studies on autopsy tissue samples from H5N1-infected patients. We combined these methods to address the interaction between macrophages and epithelial cells after influenza virus infection, so as to determine the importance of host response in the pathogenesis of H5N1 lung disease.

METHODS

Viruses. The viruses used were A/Hong Kong/483/97 (483/97) (H5N1), an influenza virus isolated from a patient with fatal influenza A H5N1 disease in Hong Kong in 1997; A/Vietnam/3212/04 (3212/04) (H5N1), a virus from a patient with H5N1 disease in Vietnam during 2004; and A/Hong Kong/54/98 (H1N1), a human H1N1 virus. The viruses were grown and titrated in Madin-Darby canine kidney cells, as described elsewhere [11]. Virus infectivity was expressed as $TCID_{50}$.

Influenza virus infection of macrophages. Peripheral-blood leukocytes were separated from buffy coat (obtained from healthy blood donors; Hong Kong Red Cross Blood Transfusion Service) by centrifugation on a Ficoll-Paque density gradient (Pharmacia Biotech) and purified by adherence, as reported

elsewhere [11]. The research protocol was approved by the ethics committee of the University of Hong Kong. The cells were allowed to differentiate for 14 days in vitro. Differentiated macrophages were infected at an MOI of 2. After 30 min of virus adsorption, the virus inoculum was removed, and the cells were washed with warm culture medium and incubated in macrophage serum-free medium (Invitrogen) supplemented with 0.6 mg/L penicillin and 60 mg/L streptomycin.

Influenza virus infection of A549. A549 cells were obtained from ATCC and maintained in culture using Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS), as well as 0.6 mg/L penicillin and 60 mg/L streptomycin, as recommended by the supplier. Cells were incubated in a humidified atmosphere (5% CO_2; 37°C). Cells were infected with influenza A viruses by use of the same methods used for macrophages (see above), except that DMEM supplemented with 10% FCS and 0.6 mg/L penicillin and 60 mg/L streptomycin was used for incubation after infection.

Interaction between macrophages and epithelial cells. To investigate the paracrine effects of virus-infected macrophages, supernatants of influenza H5N1-infected, H1N1-infected, and mock-infected macrophages were collected 6 h after infection. Supernatants were filtered using a 100-kDa pore filter (Millipore) to remove any virus and added to fresh (uninfected) A549 cells. The A549 cells were harvested, and RNA was extracted with RNeasy Mini kits (Qiagen) to study COX-2 and cytokine gene expression. The expression of influenza matrix (M) gene in the A549 cells was measured to ensure that no virus was carried over onto the A549 cells.

Treatment of cells with selective COX-2 inhibitors. To investigate the effects of selective COX-2 inhibitors on cytokine induction in macrophages or epithelial cells, the cells were pretreated for 1 h with nimesulide or NS-398 (Cayman) dissolved in a vehicle consisting of 0.1% DMSO prior to virus infection or application of filtered culture supernatant from infected macrophages. Cells were incubated in the presence of selective COX-2 inhibitor or drug vehicle (as controls) throughout the experiment.

COX-2 and cytokine gene expression by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated and the cDNA was synthesized from mRNA with poly(dT) primers and Superscript III reverse transcriptase (Invitrogen). Transcript expression was monitored using a Power SYBR Green PCR master mix kit (Applied Biosystems) with specific primers. The fluorescence signals were measured in real time during the extension step with MX3000P QPCR System (Stratagene). The specificity of the SYBR Green PCR signal was confirmed by melting curve analysis. The threshold cycle (C_T) was defined as the fractional cycle number at which the fluorescence reached 10 times the standard deviation of the baseline (from cycle 2 to 10). The ratio change in target gene relative

to the β-actin control gene was determined by the $2^{-\Delta\Delta C_T}$ method, as described elsewhere [21].

Immunohistochemical study of COX-2 in autopsy samples from patients infected with H5N1. Single and double staining of COX-2 in human lung tissue was performed as follows. Tissue sections were microwaved in 10 mmol/L citrate buffer (pH 6.0) for 20 min and blocked with 3% H_2O_2 in methanol for 30 min, followed by treatment with an avidin/biotin blocking kit (Vector Lab). After blocking with 10% normal rabbit serum for 10 min at room temperature, the sections were incubated with a 1/100 dilution of COX-2 antibody (Cayman) overnight at 4°C, followed by biotinylated rabbit anti–mouse (Dako Cytomation) diluted 1/100 for 30 min at room temperature. After incubation with Strep-ABC Complex (Dako Cytomation) diluted 1/100 for 30 min at room temperature, the sections were developed with 0.5 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.02% H_2O_2 for 20 min. When double labeling was used, the sections were incubated with CD68 (Dako Cytomation) at a 1/10 dilution for 1 h at room temperature and then with fluorescein isothiocyanate (FITC)–conjugated donkey anti–mouse (Jackson Lab) at a 1/100 dilution for 1 h at room temperature. The nuclei were counterstained with 1.5 μmol/L propidium iodide (Sigma) for 4 min and the sections were then mounted in fluorescent mounting medium (Dako Cytomation). For colocalization of COX-2 with epithelial cell markers, the FITC-conjugated CD68 was replaced with AE1/AE3 (Dako) at 1/30 for 1 h at room temperature followed by incubation with 1/50 FITC-conjugated donkey anti–mouse (Jackson Lab). Images were captured sequentially with a Nikon epifluorescence microscope with brightfield for the DAB, and then with fluorescence using a FITC filter. The sequential images were overlaid by using Photoshop (version 7.0; Adobe Systems) to produce a merged image that showed DAB (brown) for COX-2 and FITC (green) for the second antigen.

Quantification of cytokines by enzyme immunoassay. The levels of secreted cytokines in cell culture supernatant were measured by specific enzyme immunoassay (R & D Systems). For safety reasons, samples were irradiated with ultraviolet light (CL-100 UltraViolet Crosslinker; UVP) for 15 min to inactivate any infectious virus before performing the enzyme immunoassay in the Biosafety Level 3 facility. Previous research had confirmed that the dose of ultraviolet light used did not affect cytokine concentrations as measured by enzyme immunoassay [11].

RESULTS

Hyperinduction of COX-2 and proinflammatory cytokines in H5N1-infected macrophages, compared with that in H1N1-infected macrophages. Gene expression of COX-2 in primary human macrophages after influenza infection was quantified using real-time PCR. We found that COX-2 was differentially upregulated by A/HongKong/483/97 (483/97) (H5N1), compared
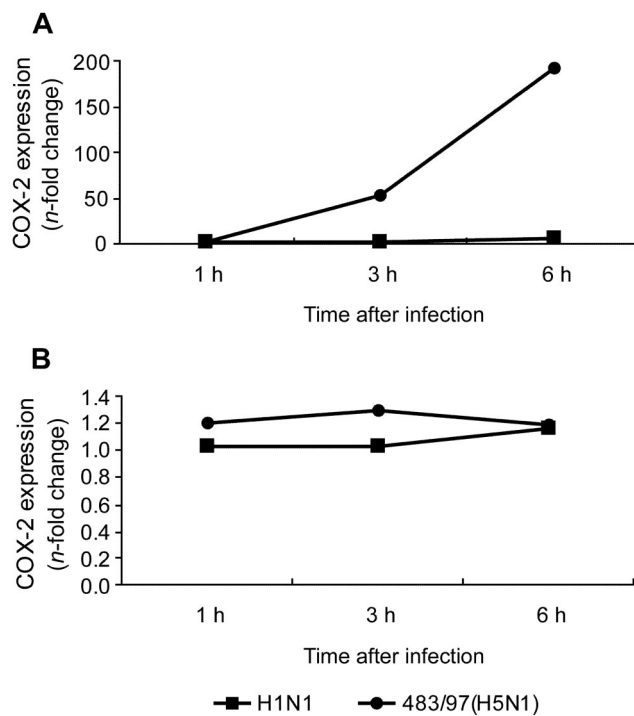

Figure 1. Differential induction of cyclooxygenase-2 (COX-2) in H5N1-infected macrophages, compared with H1N1-infected macrophages, and lack of COX-2 induction in influenza virus–infected epithelial cells. Human monocyte-derived macrophages and type II alveolar epithelial A549 cells were infected with 483/97 (H5N1) or H1N1 virus (MOI = 2). RNA was extracted from the cells at 1, 3, and 6 h after infection. After cDNA synthesis, COX-2 expression was assayed by real-time polymerase chain reaction. *A,* In macrophages, COX-2 was markedly upregulated by 483/97 (H5N1) within 3 h, with a further increase by 6 h. H1N1 virus induced a much weaker response. *B,* In A549 epithelial cells, there was no induction of COX-2 by either virus. Data shown are the *n*-fold change of COX-2 expression relative to mock-infected controls, after normalizing to β-actin in each sample. Representative data for duplicate experiments (for macrophages, 2 different donors were employed) and means of triplicate assays are shown.

with A/Hong Kong/54/98 (H1N1), as early as 3 h after infection (figure 1*A*). Human H1N1 virus infection also led to induction of COX-2 with similar kinetics but at much lower magnitude. As the induction of COX-2 in virus-infected cells was abolished by pretreatment of cells with cycloheximide (data not shown), its induction is likely to be due to autocrine or paracrine effects of cellular secretory mediators rather than to direct stimulation by the virus. In contrast with virus-infected macrophages, COX-2 was not upregulated in human alveolar epithelial (A549) cells infected with either H5N1 or H1N1 (figure 1*B*).

Because the currently prevalent Z-genotype H5N1 viruses differ from 483/97 (H5N1) in their gene constellation [22], we also tested a recent Z genotype clade 1 H5N1 virus, A/Vietnam/3212/04 (3212/04) (H5N1). As with 483/97 (H5N1), infection with the 3212/04 (H5N1) virus hyperinduced COX-2 and other cytokine genes tested (tumor necrosis factor [TNF]–α, CCL-2/

MCP-1, CCL-5/RANTES, CXCL-10/IP-10, interleukin [IL]–1β, IL-6, IFN-α1, IFN-β, and TRAIL) in macrophages, compared with macrophages infected with H1N1 (figure 2*A*–2*J*). Thus, despite the difference in viral genotype, these 2 H5N1 viruses share a common biological phenotype with regard to the induction of proinflammatory host responses.

Extensive expression of COX-2 in epithelial cells from autopsy samples of lung tissue from patients infected with H5N1. To determine whether COX-2 was induced in patients infected with H5N1, we performed immunohistochemical analysis of COX-2 protein expression for autopsy samples of lung tissue specimens from 3 patients with confirmed H5N1 disease and from 3 control patients who died of nonrespiratory causes. There was strong cytoplasmic immunostaining for COX-2 in the respiratory bronchial epithelial cells and pneumocytes of all 3 H5N1-infected patients (figure 3*A* and 3*B*). COX-2 expression colocalized with cytokeratin antigen and was found in basal cells and ciliated cells of the respiratory epithelium (figure 3*C*). COX-2 was identified in cytokeratin-positive alveolar epithelial cells (figure 3*D*) but was not observed in macrophages, as identified by the CD68 macrophage marker (figure 3*E*). The reason for this absence or low level of COX-2 expression in alveolar macrophages may be that autopsies were carried out late in the course of the illness, at which time there were few H5N1-infected cells [7–9]. Because macrophages found in the lung at this late stage of the illness may have been recently recruited to the lung via the cytokine cascade rather than being the original resident alveolar marcrophages, they may not have been exposed to the stimuli that trigger COX-2 expression to the same extent as structural resident epithelial cells.

COX-2 expression was also found to be induced in the lung epithelial cells of patients with fatal bacterial pneumonia (figure 3*F*) and again was not widely present in macrophages (figure 3*G*). This suggests that the mechanism of pathogenesis for H5N1 and fatal bacterial lung inflammation may share a common pathway that involves COX-2 induction. Interestingly, there was no COX-2 expression in macrophages or epithelial cells from patients who died of a noninflammatory condition, namely pulmonary edema secondary to ischemic heart disease. The lungs of patients who died of nonrespiratory causes showed very weak or no cytoplasmic staining for COX-2 in the alveoli (figure 3*H*).

Soluble factors secreted by H5N1-infected macrophages are potent inducers of COX-2 and cytokines in alveolar epithelial cells. Because we determined that H5N1 infection of lung epithelial cells does not directly lead to induction of COX-2, the extensive expression of COX-2 in lung epithelial cells in autopsy samples from patients infected with H5N1 is unlikely to be caused directly by viral infection. Indeed, viral antigen was scarcely detectable in the autopsy specimens from these patients [7–9]. Because the expression of COX-2 can be induced by inflammatory mediators such as cytokines, we developed an in vitro cell model to investigate the proinflammatory cascade,

mimicking the macrophage–epithelial cell interaction. Culture supernatants of human macrophages infected with influenza A virus were collected 6 h after infection, filtered to remove any infectious virus, and added to uninfected human epithelial (A549) cells. COX-2 expression was markedly upregulated in epithelial cells challenged with supernatant from H5N1-infected macrophages, more so than in those challenged with supernatants from H1N1-infected cells (figure 4*A*). There was no evidence of virus infection in these epithelial cells, as determined by RT-PCR for virus M gene. Together with the results suggesting that COX-2 induction in macrophages is dependent on de novo protein synthesis, these findings demonstrate that the hyperinduction of COX-2 is triggered in an autocrine and/or paracrine manner.

We had previously reported increased expression of the proinflammatory cytokine TNF-α in epithelial cells from autopsy samples of lung tissue from patients infected with H5N1 [7]. In contrast, H5N1 virus infection of lung epithelial cells did not lead to induction of TNF-α in vitro [12]. We can now explain these apparently contradictory observations with the novel finding that TNF-α was strongly upregulated in epithelial cells treated with supernatants collected from H5N1-infected macrophages (figure 4*B*). In addition to COX-2 and TNF-α, CCL-2/MCP-1, CXCL-10/IP-10, IL-1β, IL-6, and TRAIL were hyperinduced by secretory factors from H5N1, compared with H1N1-infected cells (figure 4*C*–4*G*). The hyperinduction of COX-2 and the cytokines peaked at 3 h after treatment, with the exception of TRAIL, which peaked later.

Soluble mediators secreted by lipopolysaccharide (LPS)-treated macrophages are potent inducers of COX-2 and cytokines in alveolar epithelial cells. Because COX-2 was also upregulated in lung epithelial cells from patients who had fatal acute bacterial pneumonia, we investigated whether COX-2 expression in epithelial (A549) cells in vitro is directly stimulated by LPS or is the result of paracrine effects of soluble factors from LPS-stimulated macrophages. Although LPS alone had little effect, the supernatant of LPS-treated macrophages significantly upregulated the expression of COX-2 and other cytokines in A549 cells (figure 5). This suggests that the proinflammatory cascade may also contribute to the prominent COX-2 expression observed in epithelial cells in fatal acute bacterial pneumonia.

Attenuation of cytokine expression by selective COX-2 inhibitors. The selective COX-2 inhibitor nimesulide was used to further investigate the role of COX-2 in the proinflammatory response hyperinduced by H5N1. Nimesulide attenuated expression of cytokines such as CCL-2/MCP-1, at both mRNA and protein levels, in a dose-dependent manner (figure 6*A* and 6*B*). Expression of all cytokines tested was significantly attenuated by 600 μmol/L nimesulide in macrophages infected with either H5N1 (483/97 [H5N1] or 3212/04 [H5N1]) or H1N1 (figure 6*C*). Another selective COX-2 inhibitor, NS-398, showed a similar effect at 400 μmol/L (data not shown).
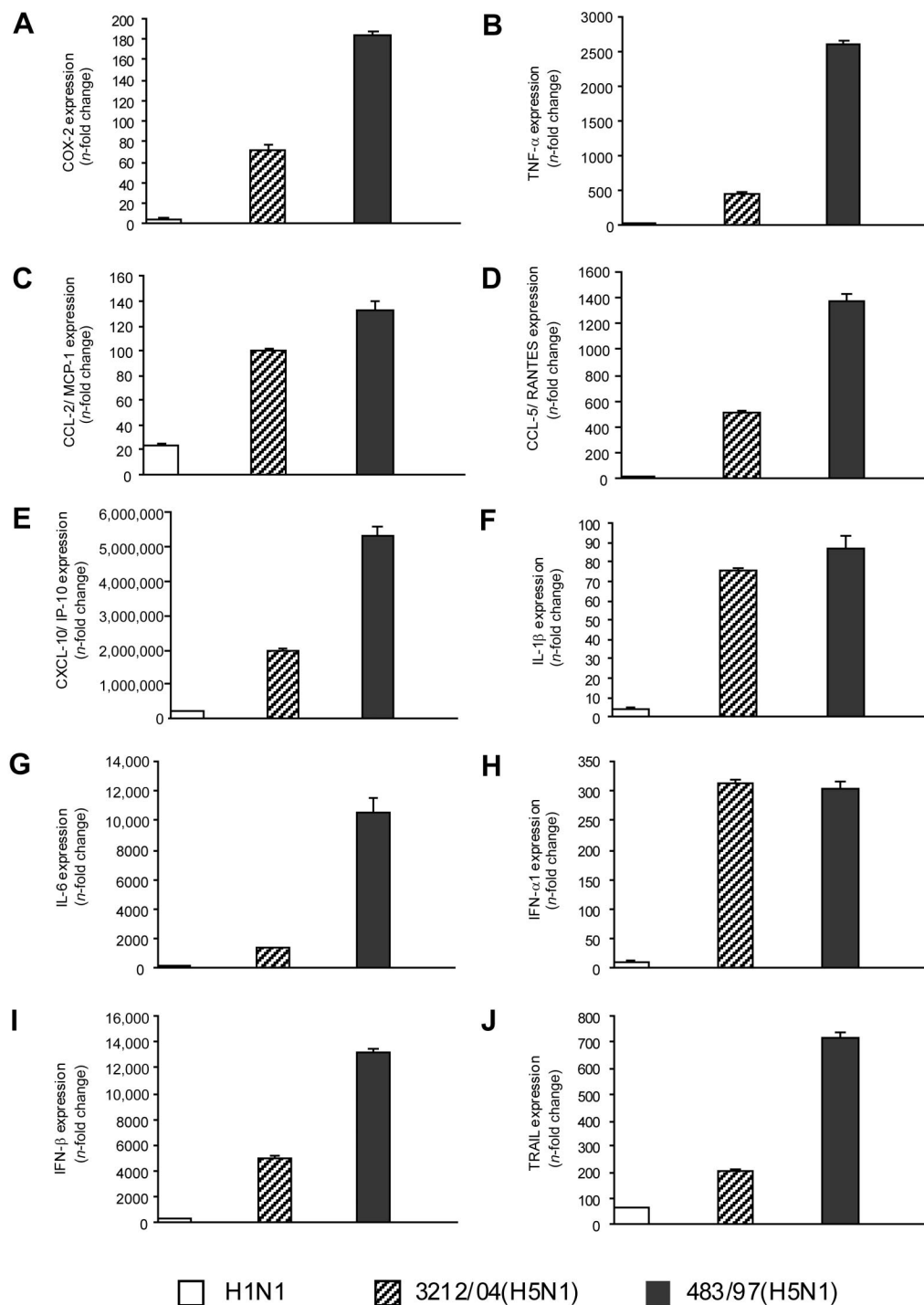
Figure 2. Induction of cyclooxygenase-2 (COX-2) and cytokines in macrophages infected with H1N1 virus or H5N1 virus (3213/04 [H5N1] or 483/97 [H5N1]). Human monocyte–derived macrophages were infected with 3212/04 (H5N1), 483/97 (H5N1) or H1N1 (MOI = 2). RNA was extracted from the cells 6 h after infection. After cDNA synthesis, mRNA expression of (A) COX-2, (B) tumor necrosis factor (TNF)–α, (C) CCL-2/MCP-1, (D) CCL-5/RANTES, (E) CXCL-10/IP-10, (F) interleukin (IL)–1β, (G) IL-6, (H) interferon (IFN)–α1, (I) IFN-β, and (J) TRAIL was assayed by real-time polymerase chain reaction. Both 483/97 (H5N1) and 3212/04 (H5N1) induced markedly higher levels of COX-2 and cytokines than did H1N1. Data shown are the n-fold changes of gene expression relative to mock-infected controls, after normalizing to β-actin in each sample. Representative data for duplicate experiments in which 2 different macrophage donors were used and means of triplicate assays are shown.
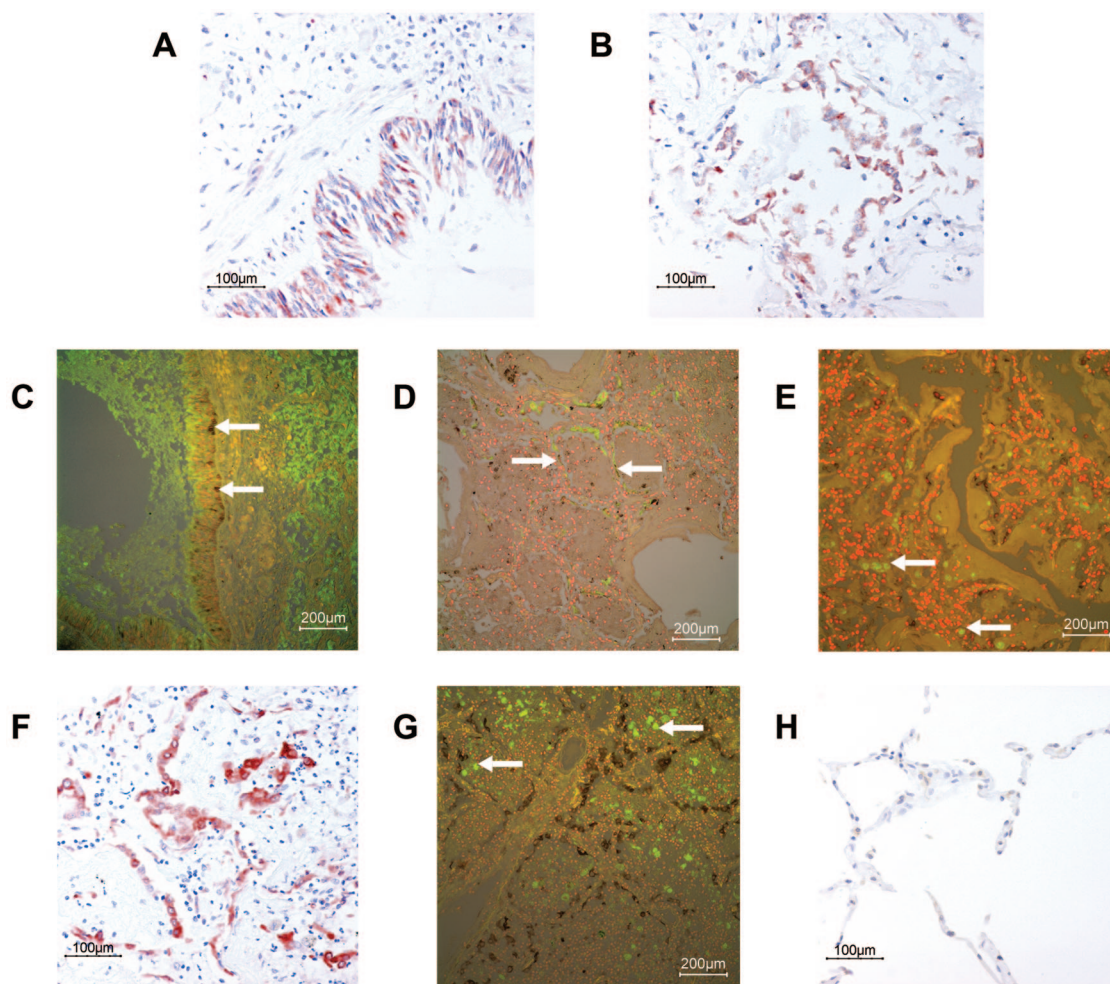
Figure 3. Cyclooxygenase-2 (COX-2) expression in the lung epithelial cells of persons who died of H5N1 infection. Immunohistochemistry for COX-2 protein in H5N1-infected lungs showed strong cytoplasmic staining in (A) bronchial epithelial cells and (B) pneumocytes. C, Double staining for COX-2 (3,3'-diaminobenzidine tetrahydrochloride [DAB], brown) and cytokeratin antigen (fluorescein isothiocyanate [FITC], green) in cells from a patient with fatal H5N1 pneumonia demonstrated cytoplasmic staining of basal cells and ciliated cells (white arrows). COX-2 was expressed in (D) cytokeratin-positive alveolar epithelial cells (white arrows) but not in (E) macrophages, as identified by the CD68 macrophage marker (FITC, green) (white arrows). F, COX-2 was strongly expressed in pneumocytes in fatal acute bacterial pneumonia. G, Double staining for COX-2 (DAB, brown) and CD68 macrophage marker (FITC, green) in cells from a patient with fatal acute bacterial pneumonia showed COX-2 expression in epithelial cells but little in macrophages (white arrows). H, Representative data from 2 lung tissue samples obtained from persons who died of nonrespiratory causes showed little or no staining for COX-2.

Because the expression of viral M gene was also suppressed by nimesulide in a dose-dependent manner (figure 6D), it is uncertain whether or not the observed reduction in the induction of cytokines was partly or wholly due to suppression of viral replication. To further investigate the role of COX-2 in the proinflammatory cascade distinct from the confounding effect of viral replication, virus-filtered supernatants from human macrophages infected with influenza A virus with or without nimesulide treatment were added to uninfected epithelial cells and the resulting effect on cytokine expression was determined. Supernatant from influenza A virus–infected macrophages that had been pretreated with nimesulide induced lower levels of cytokine expression in epithelial cells than did supernatant from macrophages not treated with nimesulide (figure 7A). Further-more, treatment of uninfected epithelial cells with nimesulide (figure 7B) led to suppression of the cytokines induced by treating these cells with supernatants from influenza A virus–infected macrophages.

DISCUSSION

In this study, we have provided evidence that the effects of the proinflammatory cascade are rapid and that the proinflammatory mediators induced by the cascade are more diverse than those induced by direct influenza virus infection. Moreover, we found that lethal H5N1 viruses induce the proinflammatory cascade to levels that are much higher than those induced by a seasonal H1N1 virus, which may explain, at least in part, why H5N1 virus is so pathogenic in humans. This increased patho-
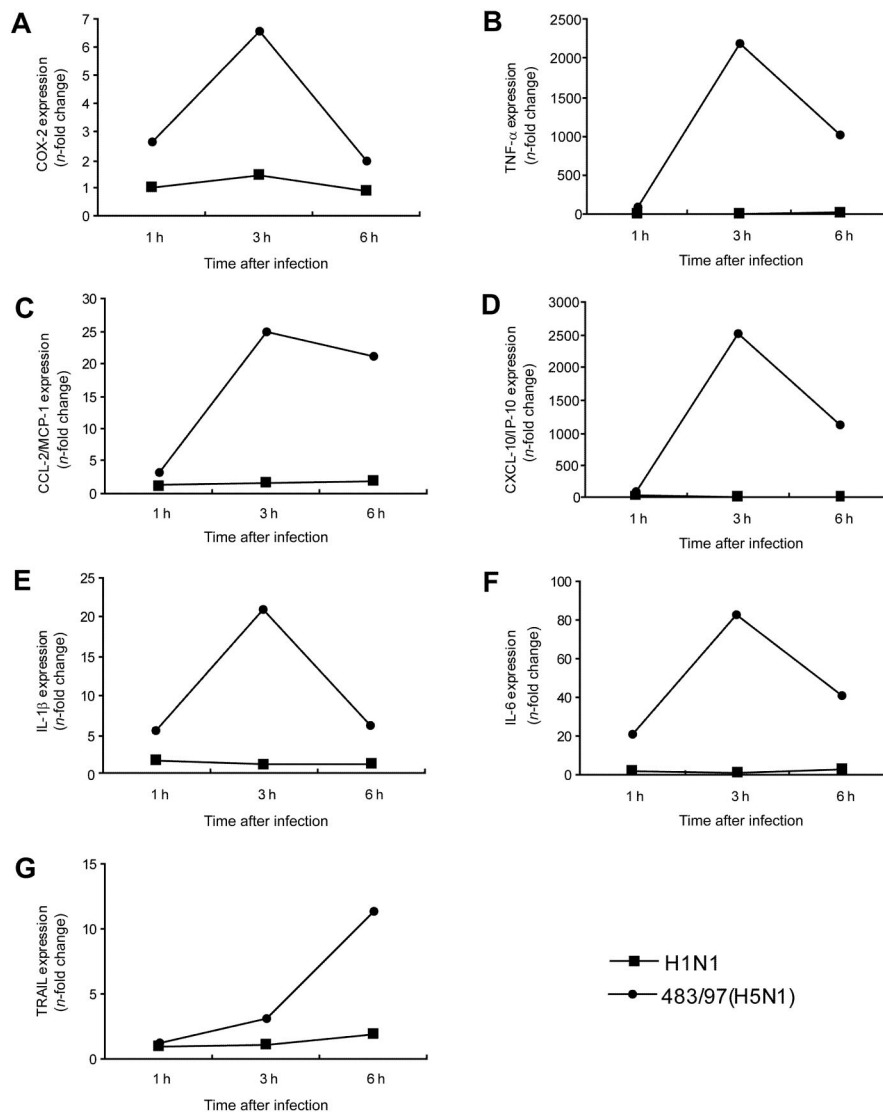
Figure 4. Induction of cyclooxygenase-2 (COX-2) and cytokines in alveolar epithelial cells by soluble mediators secreted by H5N1-infected macrophages. Human monocyte–derived macrophages were infected with 483/97 (H5N1) or H1N1 (MOI = 2) or mock infected. At 6 h after infection, supernatant was harvested and filtered to remove viruses. The filtrate was applied to alveolar type-II epithelial A549 cells. RNA was extracted from the A549 cells at 1, 3, and 6 h after treatment, reverse transcribed to cDNA, and the expression of COX-2 and cytokine mRNA was assayed by real-time polymerase chain reaction. *A,* COX-2 expression was markedly upregulated by the supernatant collected from 483/97 (H5N1)–infected cells, peaking at 3 h after challenge. Supernatant collected from H1N1-infected macrophages caused only a small increase in COX-2 expression. Comparable findings were observed for other cytokines such as *(B)* tumor necrosis factor (TNF)–α, *(C)* CCL-2/MCP-1, *(D)* CXCL-10/IP-10, *(E)* interleukin (IL)–1β, *(F)* IL-6, and *(G)* TRAIL. Data shown are *n*-fold changes of gene expression relative to mock-infected controls, after normalizing to β-actin in each sample. Representative data for duplicate experiments (for macrophages, 2 different donors were employed) and means of triplicate assays are shown.

genicity is unlikely to be due to differences in virus replication competence, because we were able to show that the levels of viral M gene for H1N1 and H5N1 infection are comparable, indicating that there are no major differences in viral gene transcription between these viruses.

Moreover, our novel findings demonstrate that soluble factors from H5N1-infected macrophages trigger a cascade of cytokine production in uninfected bystander epithelial cells, as well as other potent proinflammatory mediators, such as COX-2 and TNF-α, that are not normally induced by direct influenza infec-

tion in epithelial cells in vitro, as shown in our previous study [12]. Therefore, not only is it the case that the proinflammatory cascade triggered is rapid, but the range of proinflammatory mediators is also broader than that induced by direct viral infection. In addition, these findings in vitro are paralleled by those in vivo, as we have shown that autopsy tissue from patients with H5N1 disease shows extensive induction of COX-2 and TNF-α [7] in epithelial cells. Because virus antigen was scarce in the lung tissues that show COX-2 and TNF-α expression, these findings support the hypothesis that the cytokine cascade sustains itself
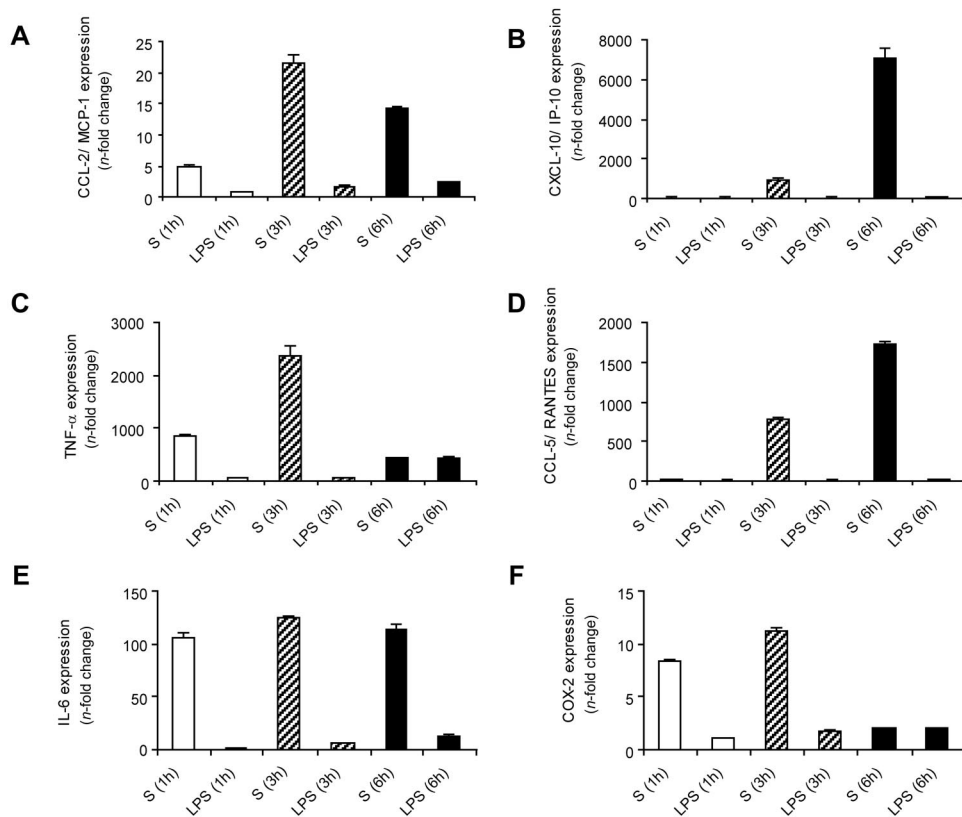
Figure 5. Induction of cyclooxygenase-2 (COX-2) and cytokines in alveolar epithelial cells by soluble mediators secreted by lipopolysaccharide (LPS)–treated macrophages and by direct LPS stimulation. Human monocyte–derived macrophages were treated with LPS at 10 ng/mL for 1 h; the supernatant was then harvested and used to treat A549 alveolar type-II epithelial cells. A549 cells were also treated with 10 ng/mL LPS for comparison. RNA was extracted from the cells at 1, 3, and 6 h after infection; after cDNA synthesis, COX-2 and cytokine expression was assayed by real-time polymerase chain reaction. Expression of *(A)* CCL-2/MCP-1, *(B)* CXCL-10/IP-10, *(C)* tumor necrosis factor (TNF)–α, *(D)* CCL-5/RANTES, *(E)* interleukin (IL)–6, and *(F)* COX-2 was markedly more upregulated after challenge with supernatant from the LPS-stimulated macrophages than after direct challenge with LPS. Data shown are *n*-fold changes of gene expression relative to mock-infected controls, after normalizing to β-actin in each sample. Representative data for duplicate experiments (for macrophages, 2 different donors were employed) and means of triplicate assays are shown. S, supernatant from LPS-stimulated macrophages.

even when viral replication has largely been controlled [7–9]. Because we have shown that influenza virus infection of epithelial cells does not directly trigger COX-2 or TNF-α induction, it must be concluded that the expression of these mediators in the lung in the absence of significant viral replication is more likely due to a self-sustaining cytokine cascade than ongoing viral replication, at least in the later stages of the illness. Taken together, these findings provide strong evidence supporting the hypothesis that excessive induced host inflammatory responses indeed play a major role in contributing to the severity of human H5N1 disease.

The activation of the proinflammatory cascade will result in increased chemotaxis, vascular permeability, and other inflammatory responses. Such responses may be protective at the level of activation observed with H1N1 infection, but the much greater proinflammatory activity seen during H5N1 infection could well have pathological consequences, such as ARDS, and it may explain the high mortality rate that is frequently associated

with human H5N1 infection [3, 23]. It is accepted that the hyperinduction of cytokines contributes to the pathophysiology of ARDS in sepsis [24, 25]. In this study, we found extensive COX-2 expression in lung epithelial cells from autopsy specimens from patients with fatal acute bacterial pneumonia, which provides parallels between the role of COX-2 in causing ARDS in patients with bacterial sepsis as well as H5N1 disease.

The ability of selective COX-2 inhibitors to attenuate the induction of a number of proinflammatory cytokines in influenza virus–infected macrophages suggests that COX-2 has a role in the regulation of the proinflammatory cascade. Nimesulide was also able to decrease the transcription of viral M gene, suggesting that nimesulide may suppress viral replication. It is possible that the decrease in cytokine induction could, at least in part, be attributed to decrease in viral replication. To further demonstrate that COX-2 is indeed involved in mediating the induction of proinflammatory cytokines, we designed experiments to show that nimesulide reduces the induction of proinflammatory cyto-
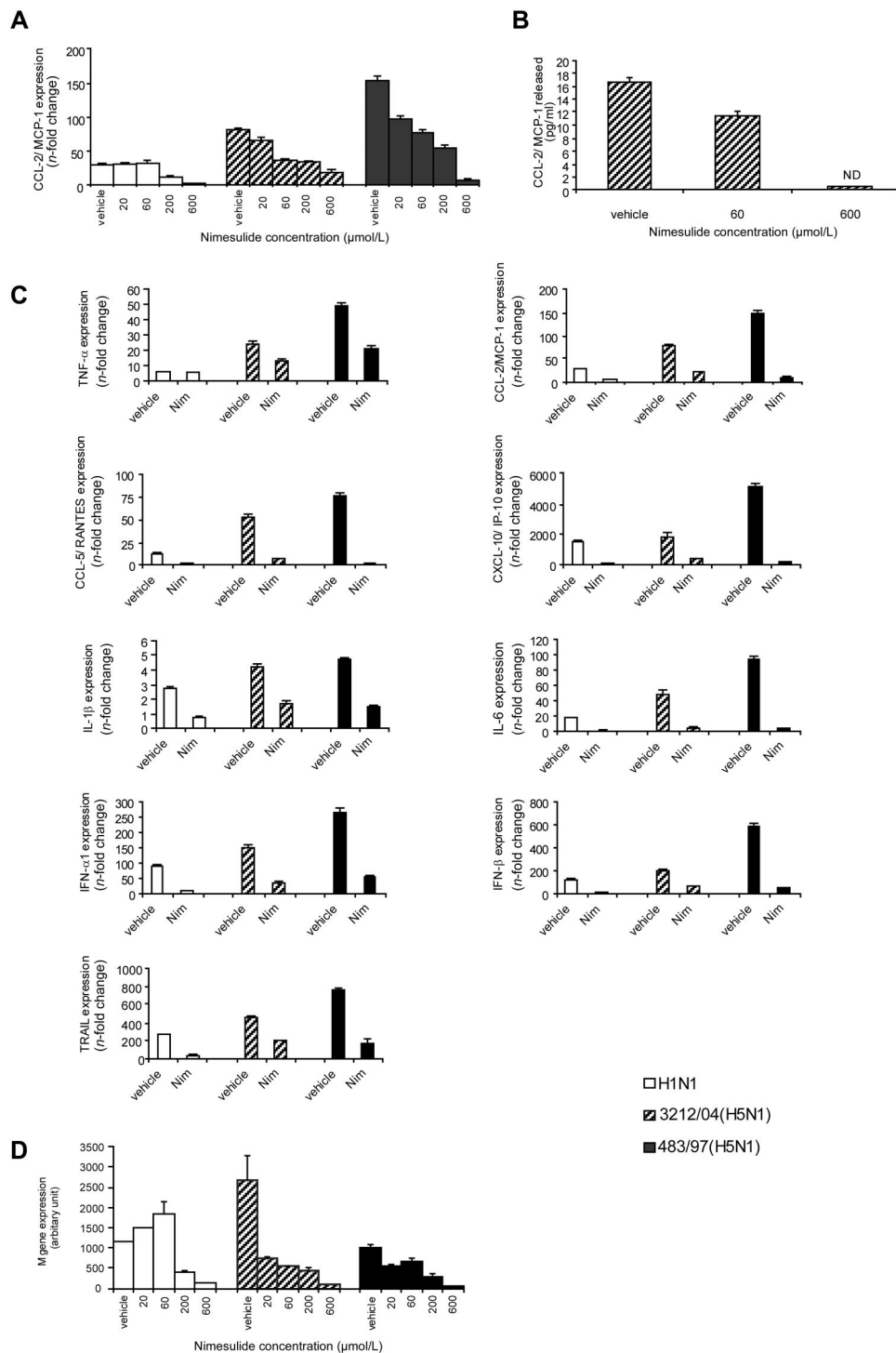
Figure 6. Nimesulide's effect on cytokine expression and secretion. *A*, mRNA level. Human monocyte–derived macrophages were pretreated with different concentrations of nimesulide or drug-vehicle control for 1 h before infection with H5N1 or H1N1 virus (MOI = 2). After 6 h of infection in the presence of nimesulide, RNA was extracted from the cells, and after cDNA synthesis, cytokine expression was assayed by real-time polymerase chain reaction. Cytokines (CCL-2/MCP-1) induced by 3212/04 (H5N1), 483/97 (H5N1), and H1N1 were attenuated by nimesulide in a dose-dependent manner. Data shown are *n*-fold changes of gene expression relative to mock-infected controls, after normalizing to β-actin in each sample. Representative data for duplicate experiments in which 2 different macrophage donors were used and the means of triplicate assays are shown. *B*, Secretory protein level. Human monocyte-derived macrophages were pretreated with nimesulide or drug-vehicle control for 1 h before infection with 3212/04 (H5N1) viruses (MOI = 2). After 24 h of infection in the presence of nimesulide, the culture supernatant was harvested and the secreted cytokines were assayed in duplicate by enzyme immunoassay. CCL-2/MCP-1 release was attenuated by nimesulide in a dose-dependent manner. Data shown are the mean of duplicate macrophage culture (±SD). *C*, A panel of cytokines tested in this study showed attenuation in expression level at 600 μmol/L nimesulide after influenza A infection in macrophages. *D*, Viral M gene transcription in influenza A–infected macrophages was attenuated by nimesulide in a dose-dependent manner. ND, not detectable.
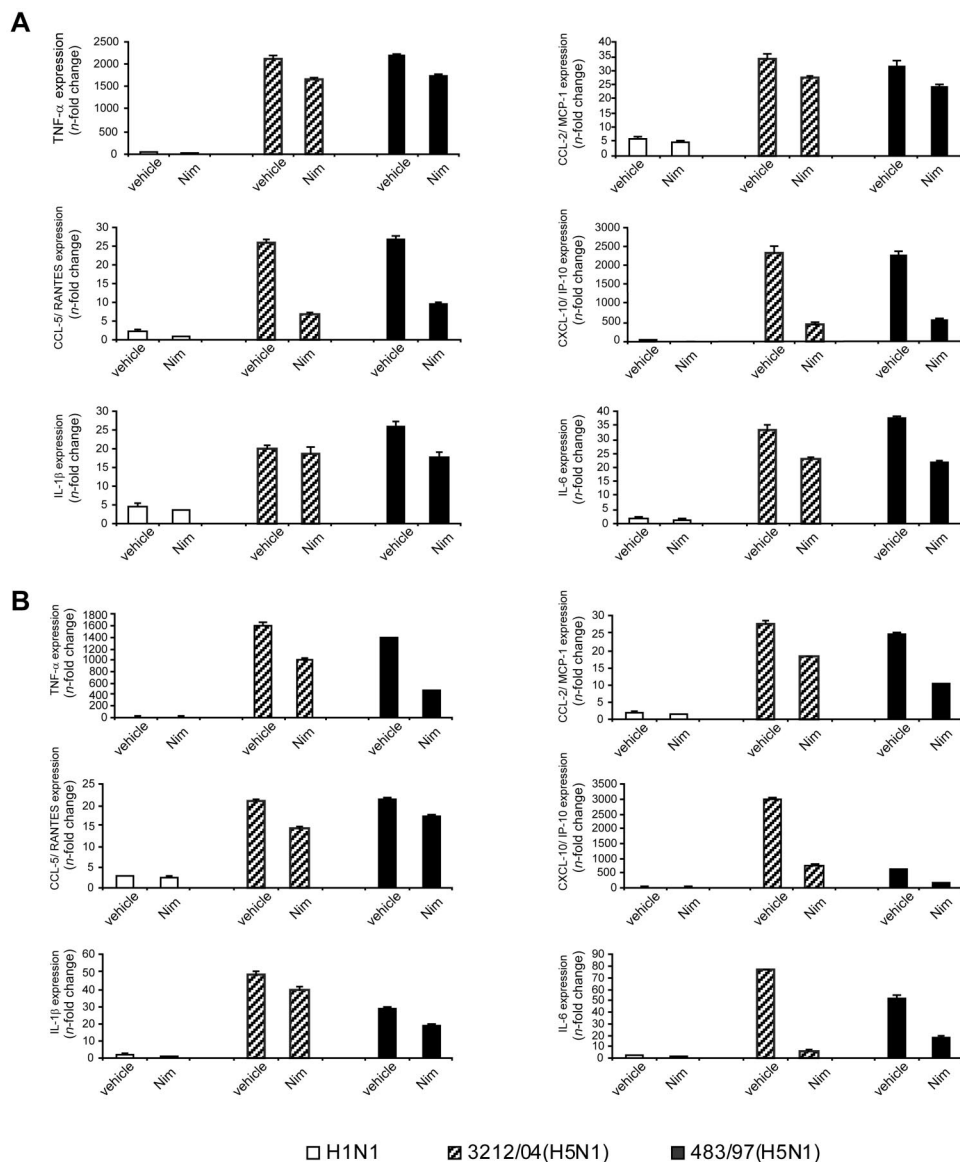
Figure 7. Influence of nimesulide (Nim) on cytokine expression in the proinflammatory cascade. *A,* Cytokine expression was attenuated in epithelial cells challenged with supernatants from influenza A–infected macrophages that had been treated with 200 μmol/L nimesulide. *B,* Epithelial cells treated with 200 μmol/L nimesulide showed attenuation in cytokine expression similar to that induced by supernatants from influenza A–infected macrophages. Data shown are *n*-fold changes of gene expression relative to corresponding mock-infected controls, after normalizing to β-actin in each sample. Representative data for duplicate experiments that used 2 different macrophage donors and the means of triplicate assays are shown.

kines elicited by soluble factors derived from H5N1-infected macrophages. These experiments avoid the confounding effect of viral infection because the macrophage supernatants have been filtered to remove infectious virus. The current paradigm is that COX-2 is induced by cytokines [26, 27], whereas here we demonstrate that COX-2 drives and maintains the proinflammatory cascade via a complex positive feedback loop during H5N1 infection.

At present, early antiviral therapy with oseltamivir is the mainstay for managing patients with H5N1 disease. However, the clinical response to antiviral therapy has been variable, and this has been attributed to a number of causes, including delayed

commencement of therapy, development of antiviral resistance [28], and poor bioavailability of the oral drug in severely ill patients. Our finding that the cytokine cascade is maintained even in the absence of significant virus infection in the lung suggests that in addition to antiviral therapy, interventions to selectively modulate this cascade may be needed. Our data points to one such potential intervention: the inhibition of the COX-2 pathway, which would attenuate the proinflammatory cascade and possibly the pathology associated with it. Such an approach may be more beneficial than attenuating the action of a single cytokine such as TNF-α by use of direct antagonists. COX-2 inhibitors are either already registered for clinical use or undergoing

late phase clinical trials, and COX-2 inhibitors may also have the added benefit of inhibiting viral replication.

References

1. World Health Organization. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO. Available at: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_03_18/en/index.html. Accessed 31 March 2008.
2. Yuen KY, Chan PK, Peiris M, et al. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet 1998; 351:467–71.
3. Beigel JH, Farrar J, Han AM, et al. Avian influenza A (H5N1) infection in humans. N Engl J Med 2005; 353:1374–85.
4. de Jong MD, Simmons CP, Thanh TT, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 2006; 12:1203–7.
5. Peiris JS, de Jong MD, Guan Y. Avian influenza virus (H5N1): a threat to human health. Clin Microbiol Rev 2007; 20:243–67.
6. Salomon R, Hoffmann E, Webster RG. Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. Proc Natl Acad Sci U S A 2007; 104:12479–81.
7. Peiris JS, Yu WC, Leung CW, et al. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 2004; 363:617–9.
8. To KF, Chan PK, Chan KF, et al. Pathology of fatal human infection associated with avian influenza A H5N1 virus. J Med Virol 2001; 63:242–6.
9. Uiprasertkul M, Puthavathana P, Sangsiriwut K, et al. Influenza A H5N1 replication sites in humans. Emerg Infect Dis 2005; 11:1036–41.
10. Gu J, Xie Z, Gao Z, et al. H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet 2007; 370:1137–45.
11. Cheung CY, Poon LL, Lau AS, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 2002; 360:1831–7.
12. Chan MC, Cheung CY, Chui WH, et al. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir Res 2005; 6:135.
13. Zeng H, Goldsmith C, Thawatsupha P, et al. Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type I interferon response in polarized human bronchial epithelial cells. J Virol 2007; 81:12439–49.
14. Claria J. Cyclooxygenase-2 biology. Curr Pharm Des 2003; 9:2177–90.
15. Futagami S, Hiratsuka T, Tatsuguchi A, et al. Monocyte chemoattractant protein 1 (MCP-1) released from *Helicobacter pylori* stimulated gastric epithelial cells induces cyclooxygenase 2 expression and activation in T cells. Gut 2003; 52:1257–64.
16. Khanapure SP, Garvey DS, Janero DR, Letts LG. Eicosanoids in inflammation: biosynthesis, pharmacology, and therapeutic frontiers. Curr Top Med Chem 2007; 7:311–40.
17. Seibert K, Masferrer J, Zhang Y, et al. Mediation of inflammation by cyclooxygenase-2. Agents Actions Suppl 1995; 46:41–50.
18. van Ryn J, Trummlitz G, Pairet M. COX-2 selectivity and inflammatory processes. Curr Med Chem 2000; 7:1145–61.
19. Vasoo S, Ng SC. New cyclooxygenase inhibitors. Ann Acad Med Singapore 2001; 30:164–9.
20. Carey MA, Bradbury JA, Seubert JM, Langenbach R, Zeldin DC, Germolec DR. Contrasting effects of cyclooxygenase-1 (COX-1) and COX-2 deficiency on the host response to influenza A viral infection. J Immunol 2005; 175:6878–84.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C_T)$ method. Methods 2001; 25:402–8.
22. Li KS, Guan Y, Wang J, et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 2004; 430:209–13.
23. Bauer TT, Ewig S, Rodloff AC, Muller EE. Acute respiratory distress syndrome and pneumonia: a comprehensive review of clinical data. Clin Infect Dis 2006; 43:748–56.
24. Cohen J. The immunopathogenesis of sepsis. Nature 2002; 420:885–91.
25. Bhatia M, Moochhala S. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. J Pathol 2004; 202:145–56.
26. Bartlett SR, Sawdy R, Mann GE. Induction of cyclooxygenase-2 expression in human myometrial smooth muscle cells by interleukin-1β: involvement of p38 mitogen-activated protein kinase. J Physiol 1999; 520:399–406.
27. Fong CY, Pang L, Holland E, Knox AJ. TGF-β1 stimulates IL-8 release, COX-2 expression, and PGE(2) release in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2000; 279:L201–7.
28. de Jong MD, Tran TT, Truong HK, et al. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med 2005; 353:2667–72.