MAJOR ARTICLE

H5N1 Influenza Virus–Induced Mediators Upregulate RIG-I in Uninfected Cells by Paracrine Effects Contributing to Amplified Cytokine Cascades

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Highly pathogenic avian influenza H5N1 viruses cause severe disease in humans, and dysregulation of cytokine responses is believed to contribute to the pathogenesis of human H5N1 disease. However, mechanisms leading to the increased induction of proinflammatory cytokines by H5N1 viruses are poorly understood. We show that the innate sensing receptor RIG-I is involved in interferon regulatory factor 3 (IRF3), NF-κB nuclear translocation, p38 activation, and the subsequent interferon (IFN) β, IFN-λ1, and tumor necrosis factor α induction during H5N1 infection. Soluble mediators from H5N1-infected human macrophages upregulate RIG-I, MDA5, and TLR3 to much higher levels than those from seasonal H1N1 in uninfected human macrophages and alveolar epithelial cells via paracrine IFNAR1/JAK but not IFN-λ receptor signaling. Compared with H1N1 virus–induced mediators, H5N1 mediators markedly enhance the cytokine response to PolyIC and to both seasonal and H5N1 virus infection in a RIG-I–dependent manner. Thus, sensitizing neighboring cells by upregulation of RIG-I contributes to the amplified cytokine cascades during H5N1 infection.

Influenza H5N1 viruses remain endemic in poultry in a number of countries and continue to pose a pandemic threat. These viruses occasionally infect humans, leading to severe viral pneumonia associated with high case fatality. The mechanisms underlying the pathogenesis of human H5N1 disease remain uncertain. Patients with H5N1 disease [1, 2], as well as experimentally infected ferrets and macaques in vivo, show evidence of cytokine dysregulation [3, 4]. Primary human macrophages (Mφ) and respiratory epithelial cells infected with H5N1 viruses elicit markedly stronger responses of proinflammatory cytokines than does seasonal influenza [5–7]. Cytokine dysregulation is thus believed to contribute to the pathogenesis of human H5N1 disease [2, 5, 8, 9]. However, there are little data on the innate sensing and downstream signaling pathways involved in H5N1-mediated cytokine dysregulation. In previous studies, we identified that interferon regulatory factor 3 (IRF3) and p38 mitogen-activated protein kinase (MAPK) were separate pathways involved in the induction of the first-wave cytokines (interferon [IFN] β, IFN-λ1, and tumor necrosis factor [TNF] α) and were differentially activated by H5N1 viruses compared with seasonal H1N1 virus [10]. However, the role of innate sensing receptors in H5N1-mediated cytokine induction has not been defined.

Toll-like receptors (TLRs) and retinoic acid inducible gene I (RIG-I)–like helicases (RLHs) are pattern-recognition receptors that sense invading pathogens and trigger the innate immune response. TLR3 recognizes

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double-stranded RNA (dsRNA), and TLR8 detects single-stranded RNA (ssRNA). RIG-I and melanoma differentiation-associated gene 5 (MDA5) belong to RLHs and are cytoplasmic sensors of viral nucleic acids. RIG-I is believed to recognize 5′-triphosphate RNA with base-paired structures [11], while MDA5 senses long dsRNA [12]. The engagement of the receptor by their cognate ligands leads to the recruitment of the adaptor molecules IFN-β promoter stimulator 1 (IPS-1) to RLHs, TRIF to TLR3, and MyD88 to TLR8, respectively, and activates the signaling cascades, resulting in the expression of interferons. TLR3 and RIG-I are shown to recognize infection of influenza H3N2 virus, leading to the expression of IFN-β and proinflammatory cytokines in a bronchial epithelial cell line [13]. RIG-I and TLR7 contribute to recognition and response to influenza infection in a subset of murine dendritic cells (FL-DCs) [14]. However, there is a lack of data on the role of these innate sensing receptors in highly pathogenic avian influenza (HPAI) H5N1 infection. Furthermore, the paracrine effects on innate sensing receptor induction and activation in adjacent uninfected cells have not been investigated.

EXPERIMENTAL PROCEDURES

Cell Culture and Virus Infection

Human peripheral blood monocyte–derived Mφ, primary type I–like alveolar epithelial cells, and human alveolar epithelial A549 cells were isolated and cultured as described elsewhere [10, 15, 16]. This study was approved by the local ethics committee, and written informed consent was acquired for the use of primary type I–like alveolar epithelial cells. For supernatant challenge assays, primary type I–like alveolar epithelial cells and A549 cells were seeded at 1×10^5 cells/well in 24-well plates 1 day before the experiment. Influenza A viruses A/HK/483/97 (H5N1) and A/HK/54/98 (H1N1) were isolated from patients with H5N1 disease and seasonal flu, respectively. These viruses were cultured in Madin-Darby canine kidney cells. The stock virus was filtered on a 100-kDa filter to remove cytokine in the culture supernatants (Amicon) [15]. Mφ were infected at a multiplicity of infection (MOI) of 2 as described previously [10].

Drug Treatment

NF-κB inhibitor (Bay 11-7085), JAK inhibitor I, or p38 inhibitor (SB203580, Calbiochem) was applied 45 minutes before infection, and the same concentrations were maintained throughout the infection process.

Small Interfering RNA–Mediated Gene Silencing

Small interfering RNAs (siRNAs) against human RIG-I, MDA5, TLR3, TLR8, IPS-1, TRIF, MyD88 (Qiagen), and IFNAR1 and IL-28RA (Dharmacon) and corresponding control siRNA were transiently transfected to macrophages using Human Macrophage Nucleofector Kit (Lonza) and A549 cells Nucleofector Kit T VCA-1002, as previously described [10]. Three days after transfection, cells were infected with influenza virus at an MOI of 2, and the cytokine expression was measured by real-time polymerase chain reaction (PCR). One day after transfection, A549 cells were challenged with virus-free supernatants, as mentioned below.

Real-Time PCR

Total RNA was extracted, reverse transcribed with poly dT or uni-12 primers (5′-AGCAAAAGCAGG-3′) [17], and used for real-time PCR with SYBR green (Roche), as described previously [10]. Primers used can be provided upon request.

Immunofluorescence

Macrophages were fixed and stained as described elsewhere [10]. IRF3 and NF-κB p65 subunit were stained with rabbit polyclonal antibodies (Santa Cruz Biotechnology) and fluorescein-conjugated anti-rabbit immunoglobulin (Ig) G antibody (Molecular Probes). Nuclei of cells were stained with 1μg/mL of 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich).

Western Blotting

Whole cell extracts and Western blotting were performed as previously described [10]. Cellular lysates (30 μg protein) were subjected to immunoblot analysis with anti–RIG-I, MDA5 (Alexis), phosphorylated-p38 (P-p38), p38 (Cell Signaling), or actin (Chemicon) and detected with horseradish peroxidase–coupled anti-rabbit or anti-mouse IgG antibodies (GE Healthcare) and ECL Plus solution (GE Healthcare).

Cytokine Assays

Cytokine proteins in culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). The detection limits for the ELISA development kits are as follows: TNF-α, 16–1000 pg/mL, and IP-10, 31–2000 pg/mL.

Virus-Free Supernatant Challenge Assay

Culture supernatants from infected Mφ were collected at 6 hours after infection and filtered through a 100-kDa pore filter (Millipore) to remove virus particles. The virus-free supernatants were added to uninfected cells. The expression of the influenza A M gene was monitored to ensure no virus carryover from the supernatants. Supernatant-treated cells were subsequently transfected (Qiagen) with PolyIC (Merck) or infected with H1N1 or H5N1 virus.

Statistical Analysis

The 2-tailed Student *t* test was used. Results were considered statistically significant with *P* values $<.05$.

RESULTS

H5N1 and H1N1 Induce Cytokine Mainly via RIG-I

Human Mφ constitutively expressed relatively high levels of MDA5, TLR1, TLR2, TLR8, TLR4, and RIG-I, whereas the

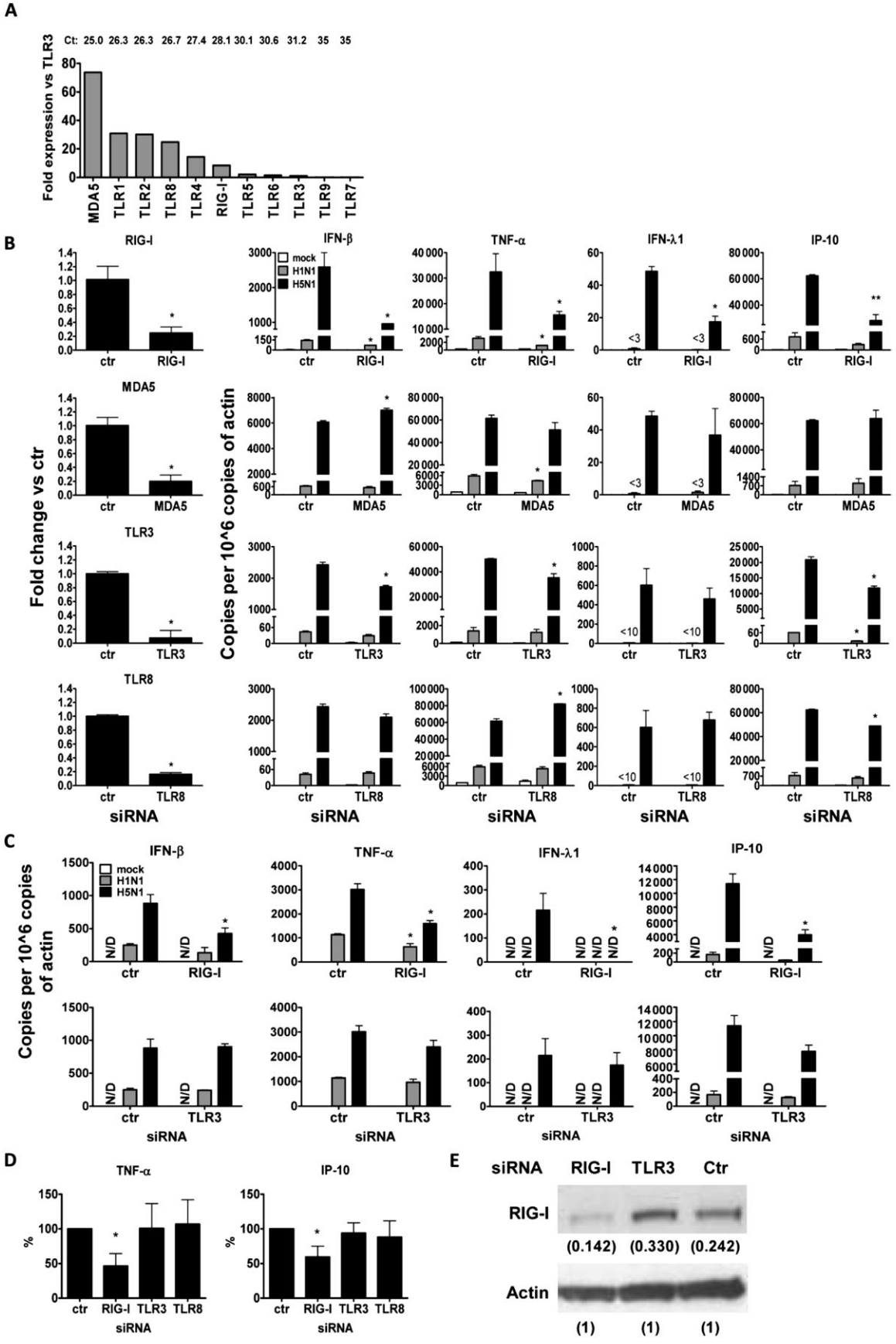

Figure 1.

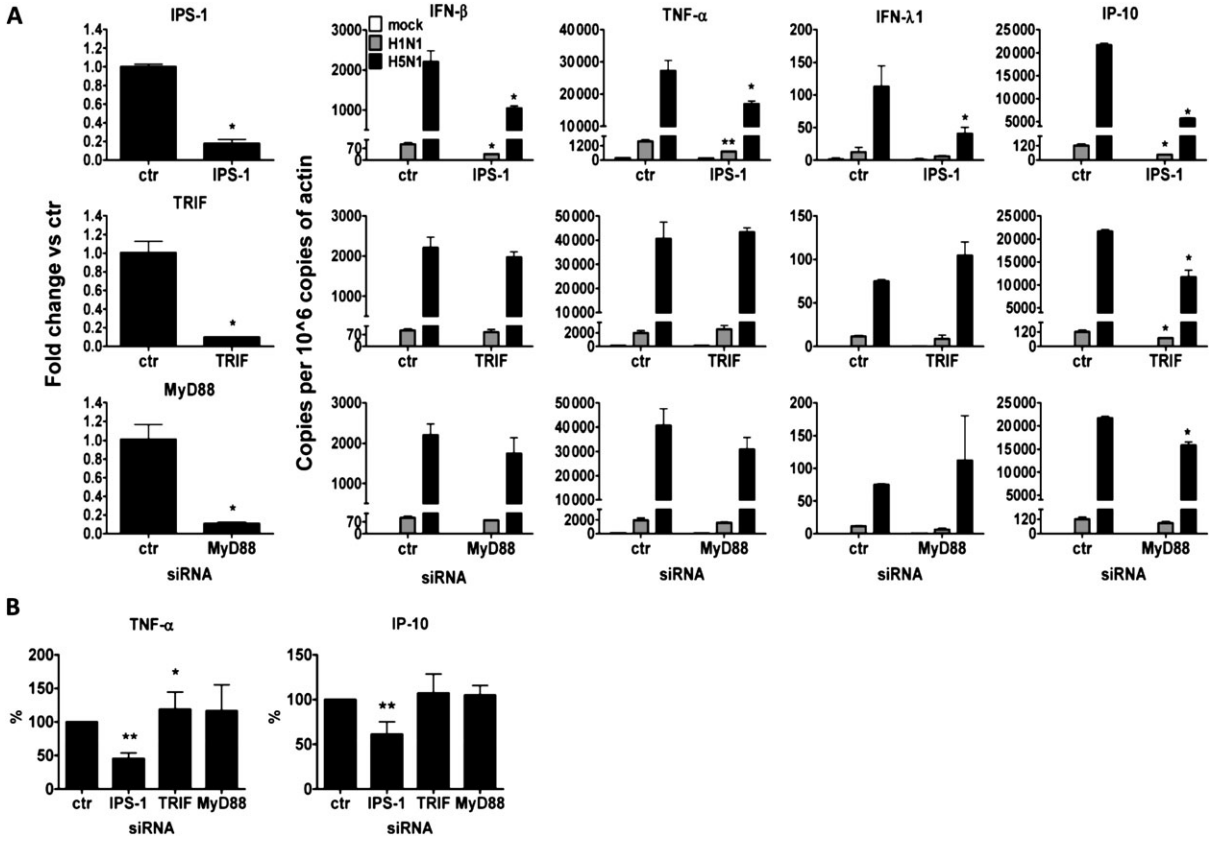

Figure 2. Both H5N1 and H1N1 influenza viruses induce cytokines via the IPS-1 pathway. *A* and *B*, Human Mφ were transfected with control (ctr), IPS-1, TRIF, or MyD88 siRNAs. Three days after transfection, the cells were infected with mock, H1N1, or H5N1 virus at a multiplicity of infection (MOI) of 2. (*A*) The knockdown efficiencies and cytokine expression at 6 h after infection were assessed by real-time PCR. Data are means ± SD of a representative experiment taken from 3 separate experiments (*$P < .05$; **$P < .01$) and compared with control siRNA–transfected and corresponding virus-infected cells. *B*, Culture supernatants were collected for ELISA of TNF-α and IP-10 at 18 hours after infection. Data are mean percentages ± SD of at least 3 independent experiments (*$P < .05$; **$P < .001$) and were compared with control siRNA–transfected and H5N1 virus–infected cells.

expression of TLR5, TLR6, and TLR3 was relatively low and that of TLR7 and TLR9 was undetectable (Figure 1*A*).

In the present study, we focused on expression of the first-wave cytokines (IFN-β, IFN-λ1, and TNF-α), together with the chemokine IP-10 and the innate sensing receptors involved in cytokine induction by H5N1 virus. Seasonal H1N1 54/98 (H1N1) virus was included for comparison. As previously reported, H5N1 induces these cytokines more strongly than does seasonal H1N1 (Figure 1*B*). Compared to nontargeting controls, siRNA knockdown of RIG-I in Mφ significantly reduced by 52%–64% the IFN-β, IFN-λ1, TNF-α, and IP-10 induction elicited by 483/97 (H5N1) and 54/98 (H1N1) virus infection at 6 hours after infection. TLR3 silencing marginally reduced IFN-β, TNF-α, and IFN-λ1 (<32%), except for IP-10, which was reduced by >43% at 6 hours after infection. Silencing of MDA5 or TLR8 had no significant effect (<25% change) on these cytokines and chemokines by either virus with the exception of a marginal increase in TNF-α following H5N1 infection. At 18 hours after infection, RIG-I silencing continued to significantly suppress cytokine induction, but the effect of TLR3 silencing was marginal and without statistical significance (Figure 1*C*). The role of RIG-I, but not TLR3, in cytokine induction was also observed with other

Figure 1 continued. Both H5N1 and H1N1 influenza viruses induce cytokines predominantly via RIG-I pathway. *A*, Basal mRNA expression of RIG-I, MDA5, and TLR1-TLR9 in human Mφ was assessed by real-time PCR. Results are representative of 2 separate experiments. *B–D*, Human Mφ were transfected with control (ctr), RIG-I, MDA5, TLR3, or TLR8 siRNAs. Three days after transfection, the cells were infected with mock, H1N1, or H5N1 virus at a multiplicity of infection (MOI) of 2. The knockdown efficiencies and cytokine expression at 6 hours (*B*) and 18 hours (*C*) after infection were assessed by real-time PCR. Data are means ± SD of 1 representative experiment of 3 (*$P < .05$; **$P < .01$) and were compared with control siRNA–transfected and corresponding virus-infected cells. N/D, not detected. *D*, Culture supernatants were collected for ELISA of TNF-α and IP-10 at 18 h after infection. Data are means ± SD of at least 3 independent experiments (*$P < .001$) and were compared with control siRNA–transfected and H5N1 virus–infected cells. *E*, RIG-I protein was detected by Western blotting.

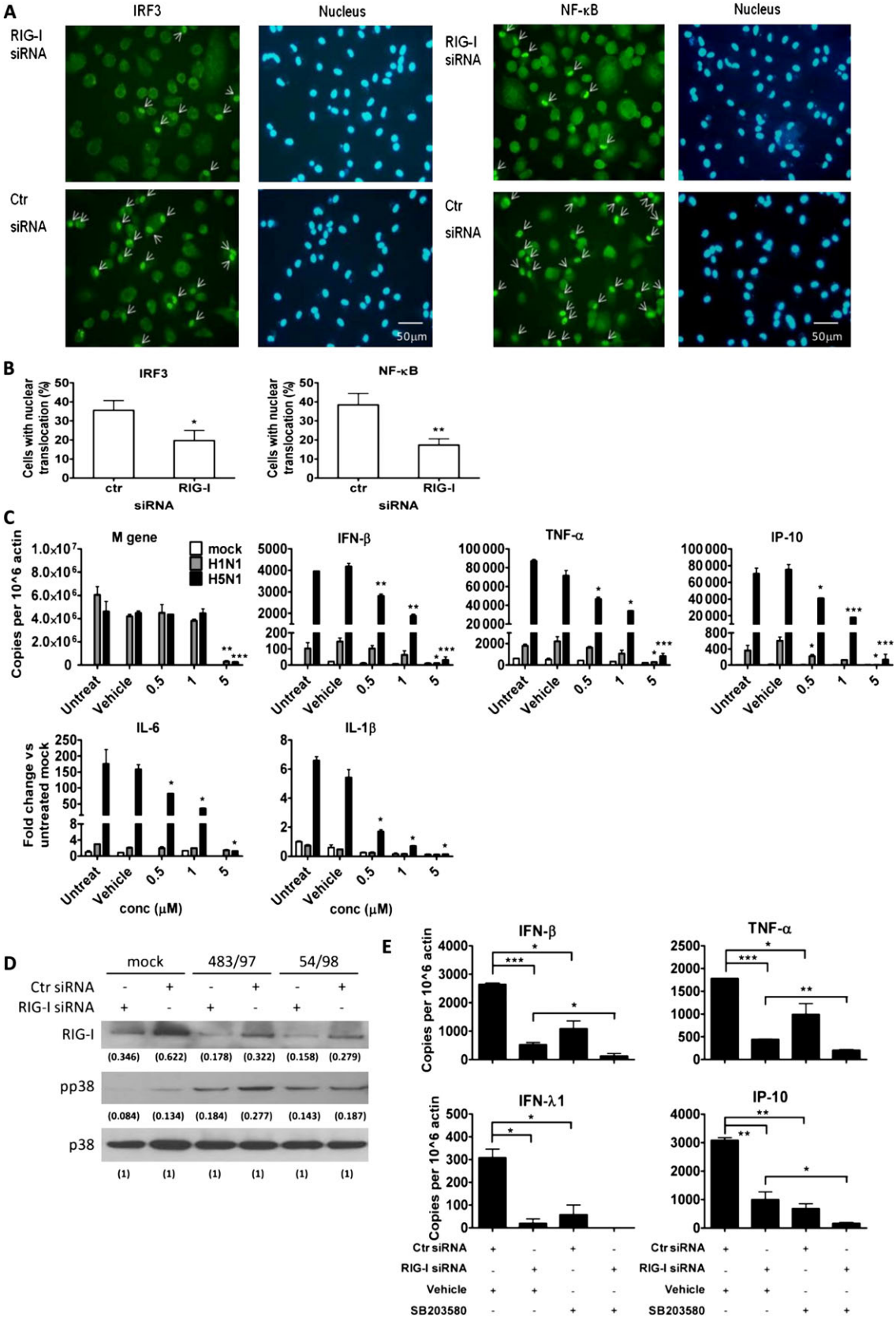

Figure 3. RIG-I leads to activation of IRF3, NF-κB, p38 MAPK, and the subsequent cytokine induction. *A*, Human Mφ were transfected with control (ctr) or RIG-I siRNAs. Three days after transfection, the cells were infected with H5N1 virus at a multiplicity of infection (MOI) of 2 for 3 hours, fixed, and

viruses of H5N1 subtype (A/Vietnam/1203/04) and H1N1 subtype (A/Hong Kong/415742/09, pandemic H1N1) (data not shown). These effects on messenger RNA (mRNA) transcription were confirmed by assays of protein expression; siRNA-mediated silencing of RIG-I, but not of other receptors, was associated with a reduction of both TNF-α and IP-10 protein expressed in culture supernatants by 53% and 41%, respectively (Figure 1*D*). The effect of each siRNA in silencing RIG-I, MDA5, TLR3, and TLR8 mRNA is shown in Figure 1*B*. The extent of reduction of RIG-I protein is shown in Figure 1*E*. Because baseline levels of TLR3 in macrophages were too low to be demonstrated by Western blotting, we demonstrated the extent of TLR3 knockdown by showing lack of IFN response to PolyIC treatment (data not shown).

Next, we investigated the key adapters of these innate sensing receptors. IPS-1 knockdown significantly reduced both H5N1- and H1N1-induced IFN-β by 53%–61%, IFN-λ1 by 52%–64%, TNF-α by 38%–54%, and IP-10 by 61%–74% (Figure 2*A*). TRIF silencing partially reduced H5N1- and H1N1-induced IP-10 expression by 43%–46%, while MyD88 knockdown caused marginal reduction of 27% H5N1-induced IP-10 expression with no significant effect on the other cytokines investigated. ELISA quantitation of cytokine proteins confirmed that IPS-1 silencing led to reduction of TNF-α and IP-10 protein expression in H5N1 virus–infected culture supernatants by 54% and 39%, respectively (Figure 2*B*).

RIG-I Is Required for Activation of IRF3, NF-κB, p38, and Cytokine Induction by H5N1 Virus

IRF3 and NF-κB are 2 major transcription factors activated by RLH pathways. We therefore hypothesized that knockdown of RIG-I would lead to an impact on H5N1-induced activation of IRF3 and NF-κB. RIG-I knockdown significantly reduced the nuclear translocation of IRF3 and NF-κB compared with control siRNA–transfected cells (Figure 3*A* and *B*). We have previously shown the IRF3-dependent induction of IFN-β, TNF-α, and IP-10 after H5N1 infection [10]. Here, we demonstrated that the NF-κB inhibitor significantly suppressed induction of a number of cytokines in a dose-dependent manner (Figure 3*C*). The suppressive effects of the drug at 0.5–1.0 μM concentrations on IFN-β, TNF-α, and IP-10 expression, a dose at which there is no discernible effect on viral M gene expression, suggests that NF-κB is a key transcription factor in H5N1 virus–induced cytokine expression. The NF-κB inhibitor at 1 μM reduced H1N1-induced IFN-β (68%) and TNF-α (61%), but these changes were not statistically significant. Moreover, knocking down RIG-I led to the reduction of p38 activation and the subsequent induction of cytokines (Figure 3*D* and *E*). The combination of RIG-I silencing and p38 inhibitors additively reduced the induction of the cytokine tested (Figure 3*E*).

H5N1 Induces Cytokine via Autocrine and Paracrine JAK Signaling

Our previous study showed that IP-10 and MCP-1 expression is protein-synthesis dependent [10]. We now demonstrate that the induction of IP-10 and MCP-1 by H5N1 is dependent on JAK activity. Treatment of human Mφ with a JAK inhibitor marginally suppressed H5N1 virus–induced TNF-α and dramatically suppressed induction of IP-10 and MCP-1 in H5N1 and H1N1 virus infection (Figure 4*A*), but the expression of virus M gene was not affected.

Virus-free cell supernatants from H5N1-infected human Mφ (H5S) induced higher mRNA levels of IP-10 and MCP-1 from uninfected human Mφ when compared with H1N1 supernatants (H1S) and mock-infected cell supernatants (MS) (Figure 4*B*). The paracrine induction of IP-10 and MCP-1 by H5S or H1S was reduced by 99% or >75%, respectively, by pretreating the cells with JAK inhibitor.

RIG-I, TLR3, and MDA5 Are Differentially Upregulated by H5N1 Supernatants via Jak and IFNAR1 but not IL-28RA Signaling

We next investigated the effect of H5N1 and H1N1 infection and their culture supernatants on the expression of RLHs and TLRs. The mRNA expression of RIG-I, MDA5, and TLR3 was markedly upregulated directly by H5N1 infection (Figure 4*A*) and also by treatment of uninfected cells with virus-free H5S (Figure 4*B*). H1N1 infection or H1S caused markedly less upregulation of these receptors than did H5N1. Western blotting for RIG-I and

Figure 3 continued. immunostained with anti-IRF3 (green) or anti–NF-κB p65 antibodies (green) and AlexFluor488-conjugated secondary antibody. Cell nuclei were visualized with DAPI stain (blue). Arrowheads indicate nuclei with either IRF3 or NF-κB p65 staining. Data shown are representative of 4 independent experiments. Scale bar = 50 μm. *B*, Human Mφ were treated as described in Figure 3*A*. Percentages of cells with nuclear translocation were calculated in 3 low-power fields of each treatment, and >700 cells were counted per treatment. Data are means ± SD of a representative experiment taken from 4 separate experiments (*$P < .01$; **$P < .001$) and compared with cells transfected with ctr siRNA and infected with H5N1 virus. *C*, Human Mφ were left untreated or treated with vehicle (DMSO) or NF-κB inhibitor (Bay 11-7085) 45 min before and throughout the infection process. The cells were infected with mock, H1N1, or H5N1 virus at an MOI of 2 for 6 hours. Cell lysates were collected and analyzed by real-time PCR. Data are means ± SD of a representative experiment taken from 3 separate experiments (*$P < .05$; **$P < .01$; ***$P < .001$) and compared with cells treated with vehicle and infected with corresponding virus. *D*, Human Mφ transfected with ctr or RIG-I siRNA were infected with H5N1 virus 3 days later. Protein lysates were collected at 3 hours after infection and analyzed by Western blotting for the expression of RIG-I, P-p38, and p38. Data are representative of 2 independent experiments. *E*, Human Mφ transfected with ctr or RIG-I siRNA were treated with vehicle (DMSO) or p38 inhibitor (SB203580) at 2.5 μM. The cells were infected with mock or H5N1 virus at an MOI of 2 for 6 hours. Cell lysates were collected and analyzed by real-time PCR. Data are means ± SD of a representative experiment taken from 2 separate experiments (*$P < .05$; **$P < .01$; ***$P < .001$) as indicated.

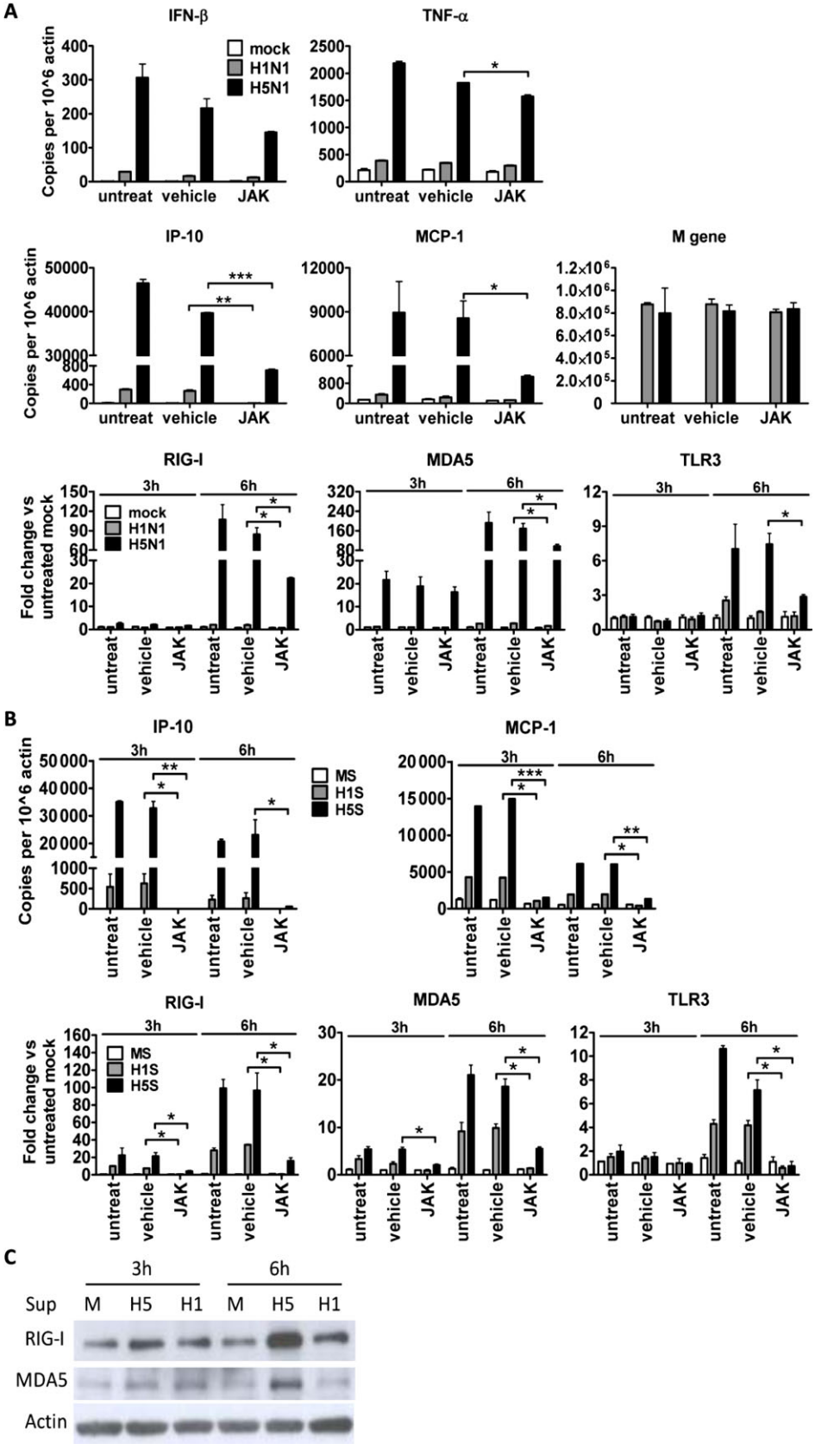

Figure 4. Cytokines and innate sensing receptors were hyperinduced by H5N1 infection via JAK signaling in human Mφ. *A*, Human Mφ were left untreated or treated with vehicle (DMSO) or JAK inhibitor (1 μM) 45 min before and throughout infection. The cells were infected with mock, H1N1, or

MDA5 protein confirmed these mRNA changes (Figure 4*C*). Of the TLR1–TLR9 investigated, only TLR3 was significantly induced by the virus-infected cell culture supernatants (data not shown). These upregulations were dramatically suppressed by JAK inhibitor.

Type I alveolar epithelial cells are a major cell type found in the human lung and cover >80% of the alveolar surface. We therefore investigated the effect of H5S or H1S from infected human Mφ on primary human type I–like alveolar epithelial cells. These cells constitutively expressed MDA5, TLR2–5, and RIG-I mRNA, but TLR1, 6, 8, and 9 were undetectable (Figure 5*A*). Virus-free culture supernatants from influenza virus–infected Mφ triggered marked upregulation of RIG-I, MDA5, and TLR3 in type I–like alveolar epithelial cells (Figure 5*B*). The effect of H5S was markedly more potent than that of H1S. A similar effect was observed in A549 cells. As with human Mφ, the upregulation of RIG-I, MDA5, and TLR3 by H5S or H1S in type I–like alveolar epithelial cells and A549 cells was dependent on JAK signaling (Figure 5*C*).

Although the regulation of IFN-λ1 gene is similar to that of the IFN-β gene [18], type III IFNs activate the JAK/STAT pathway via a distinct receptor that consists of IL-10 receptor β (IL-10Rβ) and IL-28 receptor alpha (IL-28RA) [19]. IFNAR1 silencing by siRNA significantly suppressed the upregulation of RIG-I, MDA5 and TLR3 by H1S or H5S, while IL-28RA silencing only slightly reduced (25%) the induction of MDA5 by H1S treatment (Figure 6*A*). Other H5N1 (A/Vietnam/1203/04) and H1N1 (A/Hong Kong/415742/09 pandemic) viruses were tested in IFNAR1-silenced macrophages with comparable results (data not shown). Similar findings were observed in A549 cells treated with H5S or H1S (Figure 6*B*).

Upregulation of RIG-I Enhances the Cytokine Expression to Poly IC and Virus Infection

We investigated the effects of treating uninfected human Mφ with H1S or H5S, followed by challenge with PolyIC. Transfection of PolyIC (t-PIC) into the H5S (and to a lesser degree, H1S) supernatant-treated cells led to more potent induction of IFN-β and TNF-α (Figure 7*A*). Untreated human Mφ and those pretreated with MS, H1S, or H5S were subsequently infected with either H1N1 or H5N1 virus. Viral M gene mRNA and viral RNA (vRNA) in the untreated, MS- and H1S-treated cells challenged with live H1N1 virus were comparable (Figure 7*B*), while H5S-treated cells challenged with live H5N1 virus had >69% suppression of viral M mRNA and vRNA at 7 hours after infection or later. Although the H5S had a suppressive effect on viral gene expression, it significantly increased expression of IFN-β and TNF-α in response to live-virus challenge compared with MS-treated or untreated cells. H1S also enhanced the expression of IFN-β and TNF-α induced by H1N1 infection, but to a lesser extent. Furthermore, H5S-treated cells had significant enhancement of IFN-β and TNF-α expression in response to live H1N1 challenge compared with H1S-treated cells (Figure 7*C*). The enhancement of cytokine responses of H5N1 live-virus challenge in H5S-treated cells was suppressed when RIG-I (but not the other 2 receptors) was knocked down with siRNA (Figure 7*D*).

DISCUSSION

We demonstrated the predominant role of RIG-I/IPS-1, compared with that of TLR3/TRIF, in the induction of the primary cytokines (IFN-β, IFN-λ1, and TNF-α) and the subsequent chemokine induction via the cytokine cascade after H5N1 infection. RIG-I also plays a role in H1N1-mediated IFN-β and TNF-α induction. On the other hand, MDA5 and TLR8/MyD88 are not involved in cytokine induction by H5N1 virus, and silencing TLR8 appears to have a paradoxical effect of increasing TNF-α responses following H5N1 infection. TLR7 has been reported to play a role in IFN induction in murine DCs [14], but it was not detectable in human Mφ. Although H5N1 infection is associated with a more potent cytokine response compared with seasonal H1N1 virus, both H5N1 and H1N1 viruses require the same innate sensor, RIG-I, to induce cytokines.

RIG-I is the major innate sensing receptor for cytokine induction in influenza-infected human Mφ. Although TLR3 silencing marginally reduced cytokine mRNA at 6 hours after infection, it has no detectable effect on cytokine proteins at 18 hours after infection. These results are consistent with the lack of effect following TRIF silencing. RIG-I contributes to nuclear translocation of IRF3 and NF-κB and activation of p38 MAPK in influenza-infected cells. We previously reported that IRF3 is involved in H5N1-induced IFN-β, TNF-α, and IFN-λ1 but not

Figure 4 continued. H5N1 virus at a multiplicity of infection (MOI) of 2. Cell lysates were collected and analyzed by real-time PCR for cytokines and M gene at 6 h after infection and innate sensing receptors at 3 and 6 hours after infection. Data are means ± SD of a representative experiment taken from 3 separate experiments (*$P < .05$; **$P < .01$; ***$P < .001$). *B*, Culture supernatants were collected from human Mφ infected with mock, H1N1, or H5N1 virus at an MOI of 2 for 6 hours. Virus-free supernatants (mock [MS], H1N1 [H1S], and H5N1 [H5S]) were prepared by filtering through a 100-kDa pore filter for supernatant challenge assay. Human Mφ were left untreated or treated with vehicle (DMSO) or JAK inhibitor (1 μM) 45 min before and throughout the incubation period with virus-free supernatants. Cells were treated with virus-free supernatants, and cell lysates were collected at 3 and 6 hours after incubation for analysis by real-time PCR for chemokines and innate sensing receptors. Data are means ± SD of a representative experiment taken from 2 separate experiments. (*$P < .05$; **$P < .01$; ***$P < .001$). *C*, Human Mφ were treated for 3 and 6 hours with virus-free supernatants prepared as described in Figure 4*B*. Protein lysates were collected and analyzed by Western blotting for the expression of RIG-I and MDA5. Data shown are representative of 2 independent experiments.

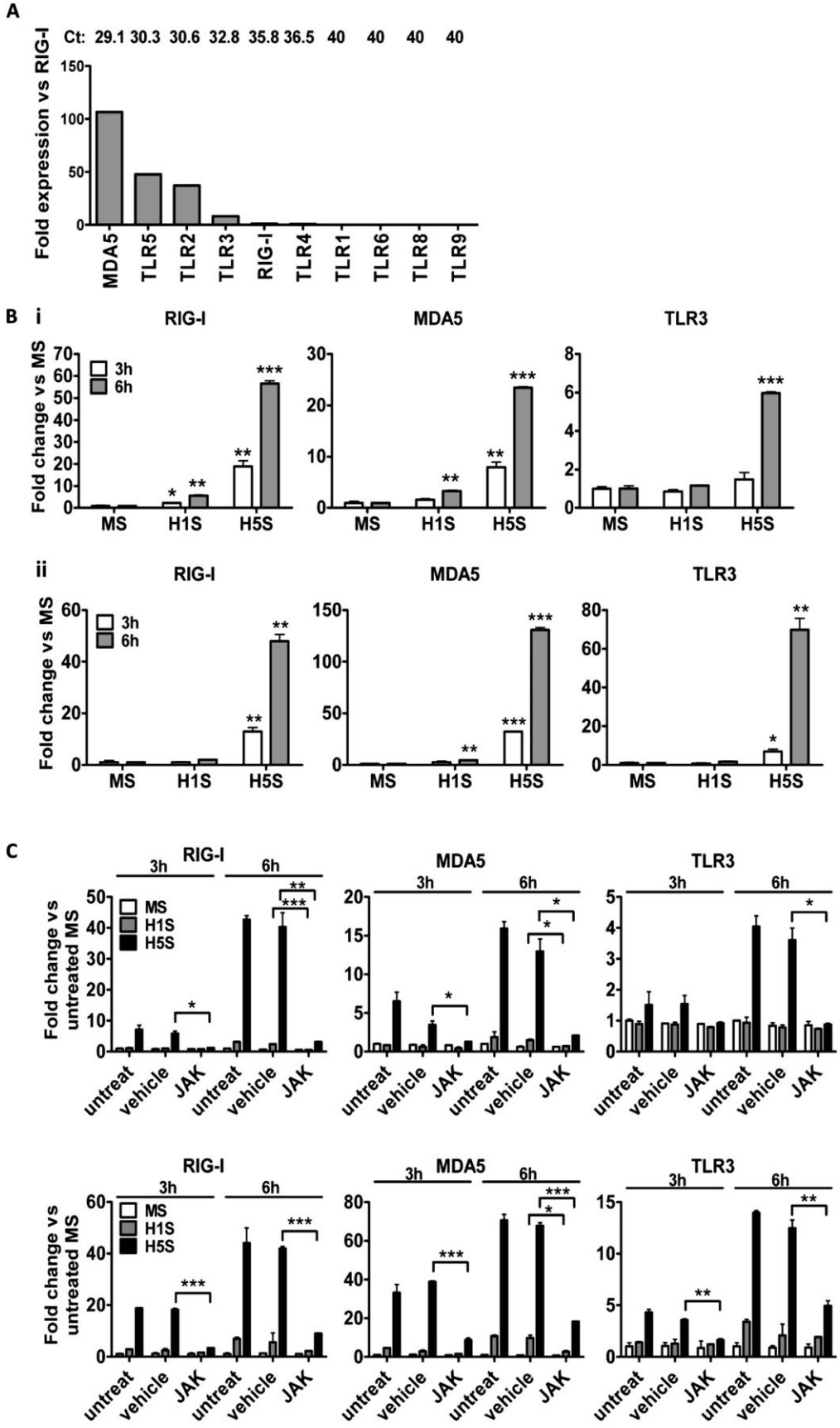

Figure 5. Innate sensing receptors are hyperinduced by H5N1 infection via JAK signaling in human alveolar type I–like pneumocytes and A549 cells.

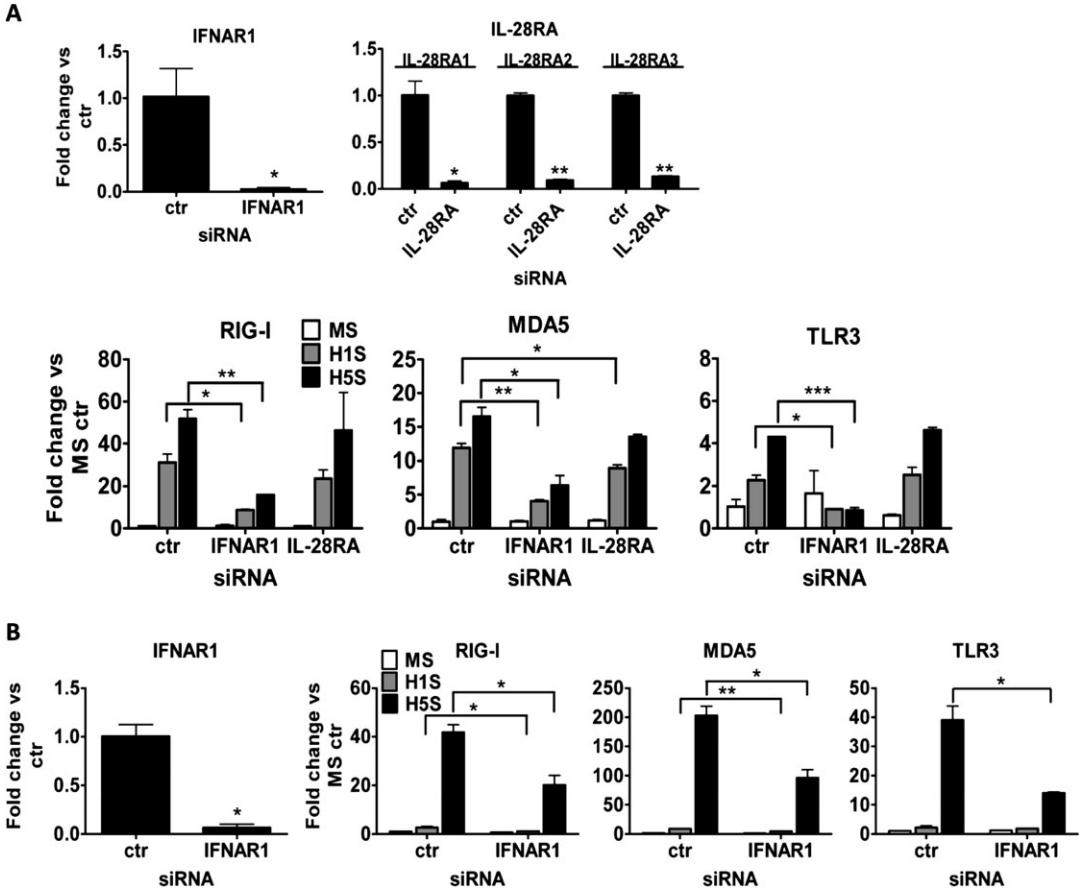

Figure 6. RIG-I, MDA5, and TLR3 are upregulated through IFNAR1 but not IL-28RA. *A*, Human Mφ were transfected with control (ctr), IFNAR1, or IL-28RA siRNAs. The knockdown efficiencies of the siRNAs were analyzed at 3 days after transfection by real-time PCR. Data are means ± SD of a representative experiment taken from 2 separate experiments (*$P < .05$; **$P < .001$) and compared with cells transfected with ctr siRNA. Transfected cells were treated with virus-free supernatants prepared as described in Figure 4*B*. Six hours after incubation, cell lysates were collected and analyzed by real-time PCR. Data are means ± SD of a representative experiment taken from 2 separate experiments (*$P < .05$; **$P < .01$; ***$P < .001$). *B*, A549 cells were transfected with ctr or IFNAR1 siRNAs. The knockdown efficiency of the siRNA was analyzed at 1 day after transfection by real-time PCR. Data are means ± SD of a representative experiment taken from 2 separate experiments (*$P < .01$) and compared with cells transfected with ctr siRNA. Transfected A549 cells were treated with virus-free supernatants prepared as described in Figure 4*B*. Six hours after incubation, cell lysates were collected and analyzed by real-time PCR. Data are means ± SD of a representative experiment taken from 2 separate experiments (*$P < .05$; **$P < .01$).

IL-1β mRNA induction [10]. NF-κB activity has been reported to be critical for influenza virus replication in lung epithelial A549 cells [20, 21]. We demonstrated that H5N1-induced cytokine expression is blocked at levels of NF-κB inhibitor, which does not affect virus M gene mRNA expression. NF-κB activation contributes to both antiviral (IFN-β and IFN-λ1) and proinflammatory cytokine (such as TNF-α and IL-1β) expression induced by H5N1 virus. Furthermore, we demonstrated that RIG-I regulates, at least in part, the activation of p38 MAPK in influenza A infection, which leads to the subsequent induction of both antiviral (IFN-β and IFN-λ1) and proinflammatory cytokines (TNF-α and IP-10).

We confirmed previous transcriptomic data in which there appears to be no qualitative difference between H5N1 and H1N1 viruses in the signaling pathways triggered in infected cells [7]. Thus, the enhanced capacity for cytokine induction by H5N1 is

Figure 5 continued. A, Basal mRNA expression of RIG-I, MDA5, and TLR1–TLR9 in human alveolar type I–like pneumocytes was assessed by real-time PCR. Data shown are from a representative experiment taken from 2 separate experiments. *B*, Human alveolar type I–like pneumocytes (*i*) and A549 cells (*ii*) were treated for 3 and 6 h with virus-free supernatants from infected human Mφ prepared as described in Figure 4*B*. Cell lysates were collected and analyzed by real-time PCR. Data are means ± SD of a representative experiment taken from 3 separate experiments (*$P < .05$; **$P < .01$; ***$P < .001$) and compared with MS-treated cells at 3 hours. *C*, Human alveolar type I–like pneumocytes (*i*) and A549 cells (*ii*) were left untreated or treated with vehicle (DMSO) or JAK inhibitor (1 μM) 45 min before and throughout the incubation period with virus-free supernatants, which were prepared as described in Figure 4*B*. Cell lysates were collected after 3 and 6 hours incubation and analyzed by real-time PCR. Data are means ± SD of a representative experiment taken from 2 separate experiments (*$P < .05$; **$P < .01$; ***$P < .001$).

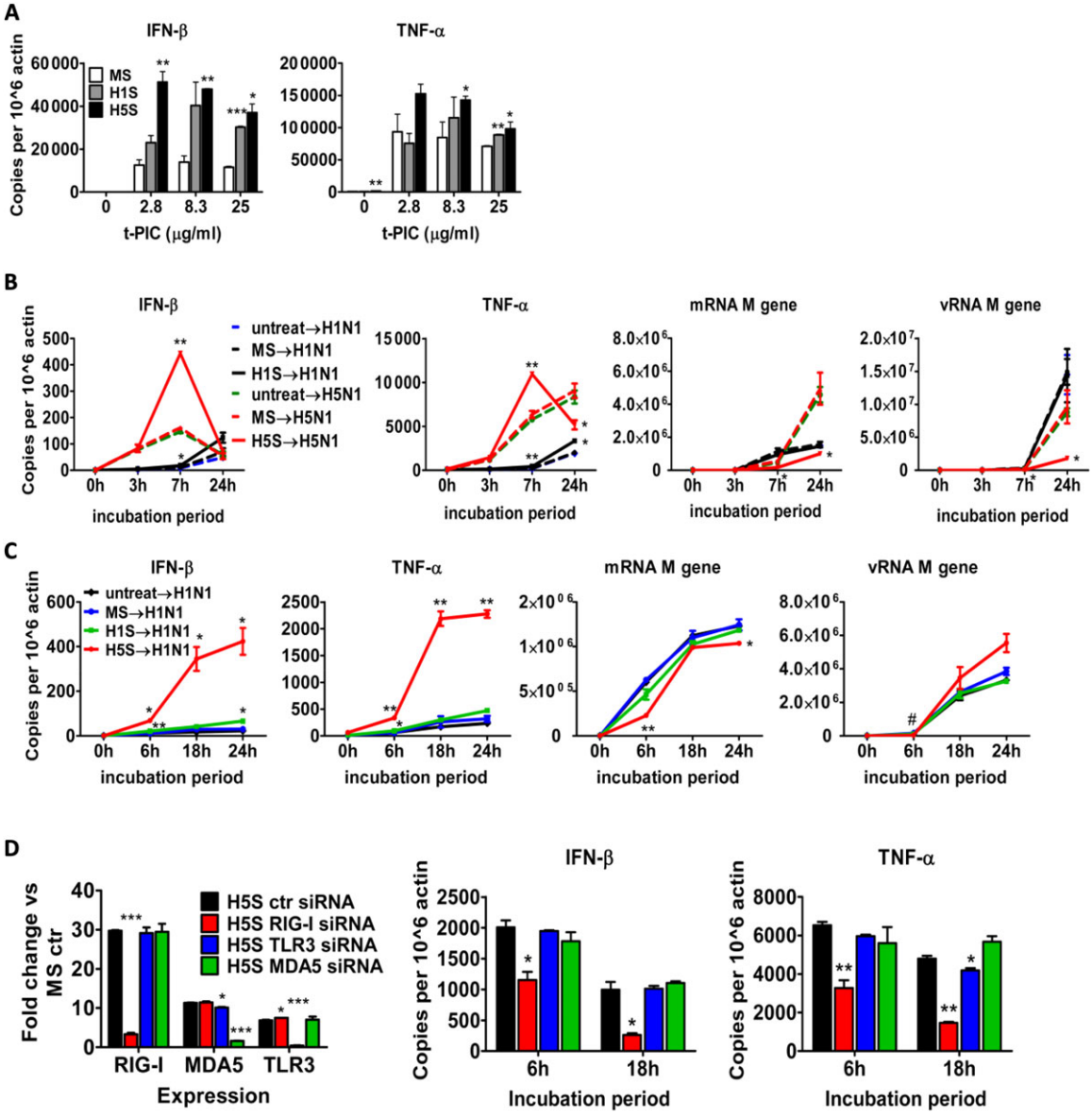

Figure 7. H5N1 supernatant enhances cytokine expression to PolyIC (PIC) and virus infection via paracrine upregulation of RIG-I. *A*, Human Mφ were pretreated for 6 hours with virus-free supernatants prepared as described in Figure 4*B*, and subsequently transfected with PIC (t-PIC) at indicated concentrations for 3 hours. Cell lysates were collected and analyzed by real-time PCR. Data are means ± SD of 1 representative experiment of 3 (*$P < .05$; **$P < .01$; ***$P < .001$) and were compared with cells treated with MS at 0 μg/mL of PIC. *B*, Human Mφ left untreated or pretreated for 6 hours with MS, H1S, or H5S were infected with corresponding viruses. H5S-treated cells were infected with H5N1 virus, and untreated cells were separately infected with H1N1 or H5N1 virus at a multiplicity of infection (MOI) of 2 for indicated periods. Cell lysates were collected and analyzed by real-time PCR. Data represent means ± SD of a representative experiment taken from 2 separate experiments (*$P < .05$; **$P < .01$) and compared with cells treated with MS that were infected with corresponding virus at the same time point. *C*, Human Mφ left untreated or pretreated for 6 h with MS, H1S, or H5S were infected with H1N1 virus at an MOI of 2 for indicated periods. Cell lysates were analyzed in a similar fashion as described in Figure 7*B*. Data represent means ± SD of 1 representative experiment of 2 (*$P < .05$; **$P < .01$) and were compared with cells treated with MS and infected with H1N1 virus at the same time point (#$P < .05$ in H1S → H1N1 and H5S → H1N1). *D*, Human Mφ were transfected with control (ctr), RIG-I, TLR3, or MDA5 siRNAs. Three days after transfection, the cells pretreated for 6 hours with MS or H5S were infected with H5N1 virus at an MOI of 2 for 6 and 18 hours. Cell lysates were collected and analyzed by real-time PCR. Data are means ± SD of a representative experiment taken from 3 separate experiments (*$P < .05$; **$P < .01$) and compared with H5S ctr cells infected with H5N1 virus at the same time point.

likely due to its potency in activating the innate immune sensors rather than differences in the type of innate immune sensors or signaling pathways activated. The manner in which H5N1 is more potent at activation of RIG-I induction, leading to hyperinduction of cytokines, has not been clarified. Recent studies have shown that NS1 protein containing an E at position 196 blocks the phosphorylation of IRF3 at serine 396 [22]. Although the 483/97 (H5N1) strain we used in this study contains

196E in the NS1 (data not shown), it increased activation and nuclear translocation of IRF3 and enhanced IFN-β expression compared with seasonal H1N1 virus in human Mφ [10]. One explanation may be that the phosphorylation at serine 386, instead of serine 396, on IRF3 is critical for its dimerization and binding with CBP/p300 [23]. Another possibility is that the ability of the virus to activate the pathway overweighs the inhibitory effects of NS1 protein.

The mutation of K to E at position 627 on PB2 of H5N1 virus dramatically reduced cytokine induction compared with wild-type H5N1 virus [24]. At the molecular level, PB2 protein of human seasonal influenza virus localizes to mitochondria, while avian PB2 protein has a nonmitochondrial localization [25]. Mutation from N to D at amino acid position 9 on PB2 abolishes the mitochondrial targeting property, and the mutant virus induces higher level of IFN-β compared with wild-type virus. PB2 has been reported to interact with IPS-1 and hence inhibits IFN-β promoter activity [26]. Further investigation is needed to elucidate the effects of PB2 of HPAI H5N1 and seasonal influenza strains on the RIG-I pathways, particularly the interaction with IPS-1.

We investigated the interactions between different cell populations during HPAI H5N1 infection by treating alveolar epithelial cells with virus-free supernatants from virus-infected Mφ. Detailed mechanistic studies have been done in gene-knockout mice or transformed cell lines treated with recombinant cytokines such as IFN-β and IFN-α. We used primary human Mφ and primary human alveolar epithelial cells together with supernatants collected from virus-infected cells, an approach that can capture a complexity not reflected by pretreatment with a single cytokine. Compared with seasonal H1N1 virus, the H5N1 virus-free cell culture supernatants elicited a more dramatic effect on inducing RIG-I, MDA5, and TLR3 via JAK signaling pathways activated by autocrine and paracrine mediators in uninfected human Mφ and human lung alveolar epithelial cells. Type I IFN signaling, but not type III IFN signaling, was responsible for the upregulation of these innate sensing receptors in both human Mφ and epithelial cells. These results were confirmed with other strains of the same subtypes (1203/04 [H5N1] and 415742/09 [pandemic H1N1]), demonstrating the general relevance of this phenomenon.

We provided evidence that the more effective sensitization of uninfected human Mφ and lung epithelial cells by H5N1-infected cell supernatants results in an enhanced response to dsRNA and to virus infection. Thus both dsRNA from virus-infected and dying cells and virus infection may be relevant in the physiological setting during H5N1 (more so than H1N1 infection) in amplifying proinflammatory cytokine cascades. H5N1-supernatant treated cells, by upregulating RIG-I in adjacent uninfected cells, become more sensitive to even very low levels of RIG-I and TLR3 agonists. More interestingly, priming with H5N1 supernatant also enhanced the cytokine induction by seasonal influenza H1N1 virus challenge, thus demonstrating that the interferon-antagonist effects of seasonal influenza were overridden by the paracrine effects of H5N1-induced mediators.

In summary, RIG-I/IPS-1 signaling, rather than TLR3, plays the more dominant role in the activation of IRF3, NF-κB, p38 MAPK, and cytokine expression by H5N1 infection in human Mφ and primary human alveolar epithelial cells. Although H5N1 and seasonal H1N1 viruses differ in the extent to which they hyperinduce cytokines, they both utilize the common mechanisms for such cytokine induction by following direct virus infection as well as through paracrine effects on uninfected bystander cells. Soluble mediators from H5N1-infected human Mφ upregulated RIG-I, MDA5, and TLR3 more dramatically than those from H1N1-infected cells in both uninfected human Mφ and human alveolar epithelial cells via IFNAR1/JAK but not IFN-λ receptor signaling. Cytokine induction in response to Poly IC or virus infection was enhanced by treatment with H5N1-supernatant compared with H1N1-supernatant treatment and was dependent on upregulation of RIG-I expression. Taken together, these results provide part of the explanation for the stronger proinflammatory cytokine cascades induced in H5N1 infection. A more precise identification of the signaling pathways triggered by H5N1 virus leading to cytokine induction may provide novel options for designing therapeutic strategies for severe human H5N1 influenza and treating other causes of acute respiratory disease syndrome.

Notes

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References

1. Peiris JS, Yu WC, Leung CW, et al. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet **2004**; 363:617–9.
2. 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.
3. Cameron CM, Cameron MJ, Bermejo-Martin JF, et al. Gene expression analysis of host innate immune responses during lethal H5N1 infection in ferrets. J Virol **2008**; 82:11308–17.
4. Baskin CR, Bielefeldt-Ohmann H, Tumpey TM, et al. Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. Proc Natl Acad Sci U S A **2009**; 106:3455–60.
5. 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.

6. 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.
7. Lee SM, Gardy JL, Cheung CY, et al. Systems-level comparison of host-responses elicited by avian H5N1 and seasonal H1N1 influenza viruses in primary human macrophages. PLoS One **2009**; 4:e8072.
8. Peiris JS, Cheung CY, Leung CY, Nicholls JM. Innate immune responses to influenza A H5N1: friend or foe? Trends Immunol **2009**; 30:574–84.
9. Peiris JS, Hui KP, Yen HL. Host response to influenza virus: protection versus immunopathology. Curr Opin Immunol **2010**; 22:475–81.
10. Hui KP, Lee SM, Cheung CY, et al. Induction of proinflammatory cytokines in primary human macrophages by influenza A virus (H5N1) is selectively regulated by IFN regulatory factor 3 and p38 MAPK. J Immunol **2009**; 182:1088–98.
11. Schlee M, Roth A, Hornung V, et al. Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. Immunity **2009**; 31:25–34.
12. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature **2006**; 441:101–5.
13. Le Goffic R, Pothlichet J, Vitour D, et al. Cutting edge: influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. J Immunol **2007**; 178:3368–72.
14. Koyama S, Ishii KJ, Kumar H, et al. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol **2007**; 179:4711–20.
15. Lee SM, Cheung CY, Nicholls JM, et al. Hyperinduction of cyclooxygenase-2-mediated proinflammatory cascade: a mechanism for the pathogenesis of avian influenza H5N1 infection. J Infect Dis **2008**; 198:525–35.
16. Chan MC, Chan RW, Yu WC, et al. Tropism and innate host responses of the 2009 pandemic H1N1 influenza virus in ex vivo and in vitro cultures of human conjunctiva and respiratory tract. Am J Pathol **2010**; 176:1828–40.
17. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol **2001**; 146:2275–89.
18. Osterlund PI, Pietila TE, Veckman V, Kotenko SV, Julkunen I. IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. J Immunol **2007**; 179: 3434–42.
19. Sheppard P, Kindsvogel W, Xu W, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol **2003**; 4:63–8.
20. Wurzer WJ, Ehrhardt C, Pleschka S, et al. NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation. J Biol Chem **2004**; 279:30931–7.
21. Nimmerjahn F, Dudziak D, Dirmeier U, et al. Active NF-kappaB signalling is a prerequisite for influenza virus infection. J Gen Virol **2004**; 85:2347–56.
22. Kuo RL, Zhao C, Malur M, Krug RM. Influenza A virus strains that circulate in humans differ in the ability of their NS1 proteins to block the activation of IRF3 and interferon-beta transcription. Virology **2010**; 408:146–58.
23. Takahasi K, Horiuchi M, Fujii K, et al. Ser386 phosphorylation of transcription factor IRF-3 induces dimerization and association with CBP/p300 without overall conformational change. Genes Cells **2010**; 15:901–10.
24. Mok KP, Wong CH, Cheung CY, et al. Viral genetic determinants of H5N1 influenza viruses that contribute to cytokine dysregulation. J Infect Dis **2009**; 200:1104–12.
25. Graef KM, Vreede FT, Lau YF, et al. The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with the mitochondrial antiviral signaling protein and inhibiting expression of beta interferon. J Virol **2010**; 84:8433–45.
26. Iwai A, Shiozaki T, Kawai T, et al. Influenza A virus polymerase inhibits type I interferon induction by binding to interferon beta promoter stimulator 1. J Biol Chem **2010**; 285:32064–74.