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Gene Expression and Antiviral Activity of Alpha/Beta Interferons and Interleukin-29 in Virus-Infected Human Myeloid Dendritic Cells

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Dendritic cells (DCs) respond to microbial infections by undergoing phenotypic maturation and by producing multiple cytokines. In the present study, we analyzed the ability of influenza A and Sendai viruses to induce DC maturation and activate tumor necrosis factor alpha (TNF-α), alpha/beta interferon (IFN-α/β), and IFN-like interleukin-28A/B (IFN-λ2/3) and IL-29 (IFN-λ1) gene expression in human monocyte-derived myeloid DCs (mDC). The ability of influenza A virus to induce mDC maturation or enhance the expression of TNF-α, IFN-α/β, interleukin-28 (IL-28), and IL-29 genes was limited, whereas Sendai virus efficiently induced mDC maturation and enhanced cytokine gene expression. Influenza A virus-induced expression of TNF-α, IFN-α, IFN-β, IL-28, and IL-29 genes was, however, dramatically enhanced when cells were pretreated with IFN-α. IFN-α priming led to increased expression of Toll-like receptor 3 (TLR3), TLR7, TLR8, MyD88, TRIF, and IFN regulatory factor 7 (IRF7) genes and enhanced influenza-induced phosphorylation and DNA binding of IRF3. Influenza A virus also enhanced the binding of NF-κB to the respective NF-κB elements of the promoters of IFN-β and IL-29 genes. In mDC IL-29 induced MxA protein expression and possessed antiviral activity against influenza A virus, although this activity was lower than that of IFN-α or IFN-β. Our results show that in human mDCs viruses can readily induce the expression of IL-28 and IL-29 genes whose gene products are likely to contribute to the host antiviral response.

Dendritic cells (DCs) are the pivotal antigen-presenting cells, and thus they play an important role in initiating and regulating antigen-specific adaptive immune responses (17, 36). Immature DCs are the cellular sensors that are on alert for microbial pathogens in peripheral tissues. After virus infection or microbial recognition and uptake, DCs undergo a maturation process that is associated with enhanced expression of costimulatory adhesion molecules, production of cytokines, reduced capacity to endocytose, and enhanced antigen presentation by major histocompatibility complex class I and II molecules. During maturation DCs also produce CCL19/MIP-3β and upregulate the expression of its receptor CCR7, which stimulates the cells to leave the peripheral tissue and migrate into the local lymph node where they present captured antigens to T cells (3). In humans there are two DC types, plasmacytoid (pDC) and myeloid (mDC) DCs, which express a different repertoire of pattern recognition receptors and show a differential response to various microbial stimuli (17).

Influenza A virus is a common respiratory pathogen, whose primary cellular targets are the epithelial cells of the upper respiratory tract. However, the virus is also able to infect other cell types such as T cells, monocytes/macrophages, and DCs. Each cell type has a unique response to virus infection (8, 19, 38, 42, 45): influenza A virus-infected epithelial cells produce limited amounts of alpha/beta interferons (IFN-α/β), proinflammatory cytokines (interleukin-1 [IL-1], IL-6, and tumor necrosis factor alpha [TNF-α]), and chemokines RANTES, MCP-1, and IL-8 (19); macrophages, in contrast, produce significant levels of IFN-α/β, IL-1β, TNF-α, chemokines, and IL-18, whereas no IL-12 production is detected (31, 37, 38, 45). Influenza A virus-infected DCs, especially pDCs, have been shown to produce high levels of IFN-α/β (7, 8, 11). In addition to its function as a critical antiviral cytokine, IFN-α/β is involved in the development of Th1 immunity by stimulating natural killer (NK) and T-cell Th1 cytokine receptor gene expression and IFN-γ production (30, 44, 45).

One of the major cellular responses to virus infection is the synthesis of IFN-α/β. They induce the expression of several antiviral proteins, such as double-stranded RNA (dsRNA)-activated protein kinase R, oligoadenylate synthetases, and Mx proteins, which ultimately mediate the antiviral actions of IFNs (51). Thus, the main role of IFNs is to restrict virus replication in virus-infected cells and protect uninfected cells from being infected by the virus. Recently, a new group of IFN-like cytokines, IL-28A, IL-28B (IFN-λ2 and -λ3) and IL-29 (IFN-λ1), was identified (23, 49). Since IL-28 and IL-29 activate STATs (STAT1, STAT2, and STAT3), they contribute to the antiviral response by partly similar mechanisms as those used by IFN-α/β. IFN-α/β gene expression is strictly regulated by several different transcription factor systems. The crucial transcription factors regulating IFN-α/β production are nuclear factor of κB (NF-κB) and IFN regulatory factors 3 (IRF3) and IRF7 (29, 55). In virus infection the replication of the virus activates IκB kinase (IKK) complex, which leads to the activation of NF-κB and IRFs (1, 10, 13, 48). The virus-specific components of IKK

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have been identified to be IKKε and Traf family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), which mediate the induction of antiviral response by activating both IRF3 and IRF7 (13, 32, 48). IKKε and TBK1 are activated by virus-specific dsRNA and Toll-like receptor 3 (TLR3) stimulation (13). Recently, it was shown that the recognition of viral single-stranded RNA (ssRNA), including that of influenza A virus, is mediated through TLR7, TLR8, MyD88, and IRF7 (12, 15, 20, 28). Moreover, signaling through TLR3 and subsequent activation of NF-κB and induction of IFN-α/β is essential for the maturation of DCs during virus infection (26).

In the present study we characterized human monocyte-derived DC responses to influenza A and Sendai virus infections, the latter of which has functioned as a commonly used model virus in cytokine research. We show that influenza A virus induces mDC maturation relatively poorly compared to Sendai virus. In spite of detectable basal expression of signaling molecules and IRFs, which regulate IFN-α/β production, influenza A virus induced TNF-α, IFN-α/β, IL-28 and IL-29 gene expression in a limited fashion. However, pretreatment of the cells with IFN-α lead to a dramatically enhanced expression of IFN-α/β and IL-29 genes, which correlated to enhanced expression of TLR3, TLR7/8, MyD88, TRIF and IRF7 and enhanced the binding of IRF and NF-κB transcription factors to the respective promoter element of the genes. In mDCs, IL-29 was found to have antiviral activity against influenza A virus, and it probably contributes to host resistance against viral infections.

MATERIALS AND METHODS

Differentiation of mDCs. Monocyte-derived DCs were obtained as previously described (53). Briefly, monocytes were isolated from leukocyte-rich buffy coats of healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) by consecutive centrifugations in Ficoll-Paque and Percoll gradients (Amersham Biosciences, Uppsala, Sweden), followed by depletion of the remaining T or B cells with anti-CD3 and anti-CD19 magnetic beads (Dynal, Oslo, Norway). Monocytes were suspended in RPMI 1640 medium in the absence of fetal calf serum (FCS) and 2.5×10^6 cells/well were allowed to adhere to plastic six-well plates (Falcon; Becton Dickinson, Franklin Lakes, NJ). After 1 h of incubation at 37°C nonadherent cells were removed, and the cells were washed with phosphate-buffered saline (PBS). Monocytes were differentiated into immature DCs for 6 days in 2 ml of RPMI 1640 medium supplemented with 0.6 mg of penicillin/ml, 60 mg of streptomycin/ml, 2 mM L-glutamine, 20 mM HEPES, 10% FCS (Integro BV, Dieren, The Netherlands), 10 ng of recombinant human GM-CSF (BioSource, Camarillo, CA)/ml, and 20 ng of recombinant human IL-4 (R&D Systems, Abingdon, United Kingdom)/ml. Fresh medium (1 ml/well) was added every 2 days. Cultured cells were $CD1a^+$, $CD14^-$, $CD80^-$, $CD83^-$, $CD86^+$ and HLA class II^+, and they showed a typical DC morphology (data not shown).

Virus infections. Human pathogenic influenza A virus (strain A/Beijing/353/89 H3N2) originates from the National Institute of Medical Research (London, United Kingdom), and the murine Sendai virus (strain Cantell) is from the National Public Health Institute (Helsinki, Finland). Both viruses were cultured in embryonated hen eggs and stored at −70°C (42). The hemagglutination titers of influenza A and Sendai viruses were 128 and 4096 and the infectivity of the virus stocks in mDCs was 3×10^9 PFU/ml and 6×10^9 PFU/ml, respectively. mDCs were infected with different doses of virus stocks for 18 h, and the cells were collected and processed for flow cytometric and Western blot analyses. For the RNA induction experiments, mDCs were infected with influenza A or Sendai viruses at a multiplicity of infection (MOI) of 5 for 3, 6, and 12 h, and the total cellular RNA was isolated. The experiments were performed with cells obtained from three to four blood donors, and the samples were either pooled or analyzed separately.

Cytokines. Highly purified, endotoxin-free human leukocyte IFN-α and IFN-γ were provided by the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). Recombinant human IFN-α2b and IFN-β were purchased from Schering-Plough (Kenilworth, NJ), TNF-α was purchased from R&D Systems (Minneapolis, MN), and IL-29 was kindly provided by ZymoGenetics (Seattle, WA). mDCs were pretreated with different cytokines or their combinations as indicated in each experiment.

Flow cytometry (FACS). For fluorescence-activated cell sorting (FACS) analysis, the cells were washed with PBS after stimulation experiments and fixed with 1% paraformaldehyde for 15 min at room temperature. Cells were washed twice and nonspecific binding of antibodies was prevented by treating the cells with 2% FCS in PBS. The expression of costimulatory molecules and HLA class II was analyzed by staining mDCs with fluorescein isothiocyanate (FITC)-conjugated anti-CD80, anti-CD83, anti-CD86, and anti-HLA-DR antibodies (Caltag Laboratories, Burlingame, CA). Respective FITC-conjugated mouse isotype controls were used. The expression of viral proteins was analyzed by staining the cells with anti-influenza A H3N2 glycoprotein, anti-influenza A whole virus, or anti-Sendai whole virus-specific antibodies (1:500 dilutions) (18, 21), followed by staining with secondary antibodies (goat anti-rabbit-FITC, 1:100 dilution; Caltag Laboratories, Burlingame, CA). To analyze the expression of MxA protein and intracellular virus proteins, virus-infected cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Rabbit anti-MxA antibodies (41) were used in a 1:500 dilution, and the secondary anti-rabbit-FITC was used in a 1:100 dilution (Caltag Laboratories, Burlingame, CA). In every step, the cells were stained at 4°C for 40 min and washed twice with PBS plus 2% FCS. Cells were analyzed with FACScan using CellQuest software (Becton Dickinson, San Diego, CA).

Gel electrophoresis and Western blot analysis. For Western blot analyses, virus-infected mDCs were collected and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 25% ethylene glycol, 1 mM Na_3VO_4, and protease inhibitor mixture Complete (Roche Diagnostics, Mannheim, Germany). Protein samples were separated (10 or 20 μg of protein/lane) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8 or 12% gel by using the Laemmli buffer system (24). Proteins separated on gels were transferred onto Immobilon-P membranes (Millipore, Bedford, MA) with an Isophor electrotransfer apparatus (Hoeffer Scientific Instruments, San Francisco, CA) at 300mA for 3 h. The membranes were blocked with PBS containing 5% nonfat milk. The blots were stained with rabbit anti-influenza A virus (1:500), rabbit anti-parainfluenza 1 (1:500), rabbit anti-human MxA (1:2000), rabbit anti-human IKKε (1:500), mouse anti-human TBK1 (1:1,000) (Imgenex, San Diego, CA), rabbit anti-human IRF3 (1:2000), rabbit anti-human phospho-IRF3 S396 (1:500, 47), guinea pig anti-human IRF7 (1:2,000), rabbit anti-human p50 (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA), and goat anti-human p65 (1:2,000; Santa Cruz) antibodies. Anti-IKKε, anti-IRF3, and anti-IRF7 antibodies were prepared by immunizing rabbits or guinea pigs with baculovirus-expressed, preparative SDS-PAGE (Bio-Rad) purified proteins (four immunizations, 20 μg/immunization). Peroxidase-conjugated goat anti-rabbit (1:2,000), rabbit anti-guinea pig (1:1,000; Dako, Glostrup, Denmark), or rabbit anti-mouse (1:5,000; Jackson Immunoresearch Laboratories, Inc., Jackson, PA) antibodies were used in secondary staining. The primary and secondary antibody binding was carried out in PBS containing 5% nonfat milk for 1 h at room temperature. After primary and secondary staining, blots were washed three times for 10 min. The protein bands were visualized on Amersham Hyper-Max films by an enhanced chemiluminescence system as recommended by the manufacturer (Amersham, Buckinghamshire, United Kingdom).

RNA isolation and Northern blot analysis. For isolation of total cellular RNA, cells were collected, washed once with PBS, and lysed in guanidinium isothiocyanate (9), followed by centrifugation through a CsCl cushion (14). RNA was quantified photometrically, and samples containing equal amounts (10 μg) of total cellular RNA were size fractionated on 1% formaldehyde-agarose gels and transferred onto Hybond-N nylon membranes (Amersham Biosciences). To control equal RNA sample loading, ethidium bromide staining was used. The membranes were hybridized with human CCL19 and CCL20 (kindly provided by A. Zlotnik); TNF-α (American Type Culture Collection [ATCC], Manassas, VA); IFN-α1 and IFN-β (C. Weissman); influenza A nucleoprotein and NS1 (R. Krug); IRF7 (J. Pagano); TLR3, TLR7, and TLR8 (34); and MyD88, TRIF, IKKε, TBK1, IL-28A/B, and IL-29 (50) cDNA probes. The probes for Northern blot analysis were labeled with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham Biosciences) by using a random primed DNA labeling kit (Boehringer, Mannheim, Germany). Hybridizations were performed in Ultrahyb buffer (Ambion, Austin, TX). After hybridization, membranes were washed three times with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.01% SDS at 42°C for 30 min and once at 65°C for 30 min. Membranes were exposed to Kodak X-Omat AR films (Eastman Kodak, Rochester, NY) at −70°C with intensifying screens.

TNF-α ELISA and biological IFN assay. TNF-α levels from cell culture supernatants were analyzed by sandwich-enzyme-linked immunosorbent assay

(ELISA) as previously described (33). TNF-α levels were determined with antibody pairs and standard obtained from BD Pharmingen (San Diego, CA). For the IFN-α/β assay, medium for cell cultures was harvested and dialyzed against acidic glycine buffer (pH 2), followed by two dialyses against PBS. IFN-α/β titers in treated samples were assayed by vesicular stomatitis virus plaque reduction in Hep2 cells (6). The sensitivity of the assay was <1 IU/ml when an international control IFN-α preparation was used as an internal laboratory standard.

DNA affinity binding. Virus-induced activation of transcription factors was studied by DNA affinity binding method with interferon response element (ISRE) and NF-κB oligonucleotide sequences from the IFN-α, IFN-β, and IL-29 promoters. Cells were collected and nuclear extracts were prepared by lysing the cells first in a buffer containing 10 mM HEPES-KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM Na_3Vo_4, and protease inhibitors (Complete; Roche Diagnostics) and thereafter by lysing the remaining nuclei in a buffer containing 10 mM HEPES, 400 mM KCl, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 0.01% Triton X-100, 0.5 mM dithiothreitol, 1 mM Na_3VO_4, and protease inhibitors (Complete; Roche Diagnostics). The forward oligonucleotide was 5′-biotinylated, and a BamHI site was added as a spacer between the biotin and transcription factor binding sequence. The oligonucleotides used were IFN-α14 ISRE (PRD-like; 5′-GGATCCGGAAAGCCAAAAGAGAAGTAGAAAAAAA), IFN-β ISRE (PRDI-III; 5′-GGATCCGAAAACTGAAAGGGAGAAGTGAAAGTG), IFN-β NF-κB (PRDII; 5′-GGATCCGGAATTTCCCGGAATTTCCC), putative IL-29 ISRE [at position −223-(−213); 5′-GGATCCTCAAGAAGGAAAGAGAAACTGAAATC], and putative IL-29 NF-κB [at −438-(−429); 5′-GGATCCGGCCTCAGGGAGTCCCTGACGGA]. All oligonucleotides were obtained from DNA Technology A/S (Aarhus, Denmark). Oligonucleotides were annealed in 0.5 M NaCl for 30 min at room temperature and incubated with streptavidin-agarose beads (Pierce, Rockford, IL) at 4°C for 2 h. The unbound oligonucleotide was washed, after which equal amounts of protein samples were incubated with the agarose-bound oligonucleotides for 2 h at 4°C. After washings, the bound proteins were boiled in SDS sample buffer and separated by SDS-PAGE, followed by Western blotting. Proteins were visualized by using specific antibodies.

RESULTS

Infectivity and maturation of mDCs by influenza A virus and Sendai virus infection. To analyze the susceptibility of mDCs to virus infection, human monocyte-derived mDCs were infected with influenza A/Beijing/353/89 (H3N2) or Sendai viruses. After overnight infection (18 h), the expression of viral proteins was determined by FACS analysis and Western blotting. Based on FACS analyses, both influenza A and Sendai viruses infected mDCs very efficiently and nearly 100% infectivity was seen in cells infected with high MOI values (Fig. 1A). Also in immunoblot analysis a clear virus dose-dependent expression of viral proteins was seen (Fig. 1B). To assess the ability of influenza A or Sendai virus to induce antiviral MxA protein expression as an indirect measure of IFN-α/β production, immunoblotting with anti-MxA protein antibodies, demonstrated that both viruses readily induced MxA protein expression in mDCs. However, the ability of Sendai virus to induce MxA protein expression was much better than that of influenza A virus (Fig. 1B).

Since the maturation of DCs is an essential step in their cytokine production and antigen presentation capacity, the ability of influenza A and Sendai viruses to induce DC maturation was analyzed. Virus-induced maturation of mDCs was analyzed by FACS with antibodies to CD80, CD83, CD86, and HLA class II. Sendai virus-infected mDCs showed a clear increase in the expression of costimulatory molecules and HLA class II, whereas influenza A virus evoked only a weak increase in the expression of CD86 and HLA class II (Fig. 2A and B). Furthermore, the expression of CD86 and HLA class II was elevated even at low Sendai virus doses (MOI = 0.2). The expression of CCL20 and CCL19 chemokine genes is also

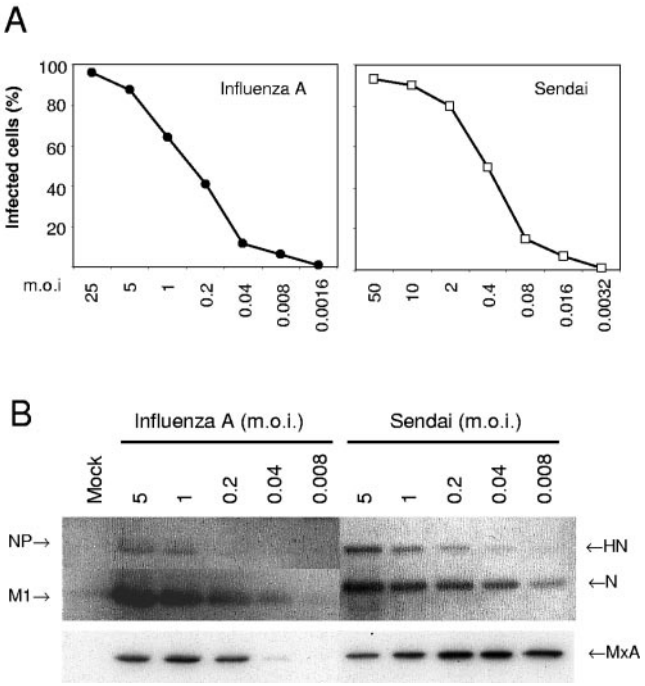

FIG. 1. Infectivity of influenza A and Sendai viruses in human monocyte-derived mDCs. (A) Monocyte-derived mDCs were infected with influenza A/Beijing/353/89 H3N2 or Sendai (Cantell strain) viruses with different virus doses as indicated in the figure. Virus-infected cells were fixed with 1% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 5 min, and stained with whole virus-specific antibodies and FITC-labeled secondary antibodies, followed by FACS analysis. The results are the means of two donor mDCs. (B) mDCs from three separate donors were infected with different viral doses. Cells were collected and pooled, and protein samples (10 μg/lane) were separated by SDS-PAGE, followed by Western blot analysis with virus and MxA protein-specific antibodies. Virus doses, viral proteins, and MxA protein as indicated in the figure. m.o.i., MOI.

associated with the maturation of DCs, with CCL20 expressed at early stages and with CCL19 at late stages of maturation (3, 43). Interestingly, at 3, 6, and 12 h after infection Sendai virus, but not influenza A virus, was able to induce the expression of CCL20 and CCL19 mRNAs (Fig. 2C). Elevated CCL20 mRNA levels were observed 3 h after Sendai virus infection peaking at 12 h. The expression of CCL19 mRNA was found to steadily increase during the 12 h infection (Fig. 2C).

Induction of TNF-α, IFN-α/β, IL-28, and IL-29 in virus-infected mDC. One of the cellular responses to virus infection is the synthesis of cytokines, including TNF-α and IFN-α/β. In addition to its proinflammatory functions, TNF-α also regulates the maturation of DCs (3). IFN-α/β, as well as the recently identified IL-28 and IL-29 (23, 49), is likely to account for the majority of antiviral response in virus-infected cells. Therefore, we concentrated on analyzing TNF-α, IFN-α/β, IL-28, and IL-29 gene expression in virus-infected mDCs. Influenza A virus infection induced a relatively weak TNF-α mRNA expression at early times (3 h) of infection. Similarly, in influenza A virus-infected mDCs only a weak signal for IFN-β mRNA was observed, and no detectable expression of IFN-α or IL-28 mRNAs was seen. Interestingly, IL-29 mRNA expres-

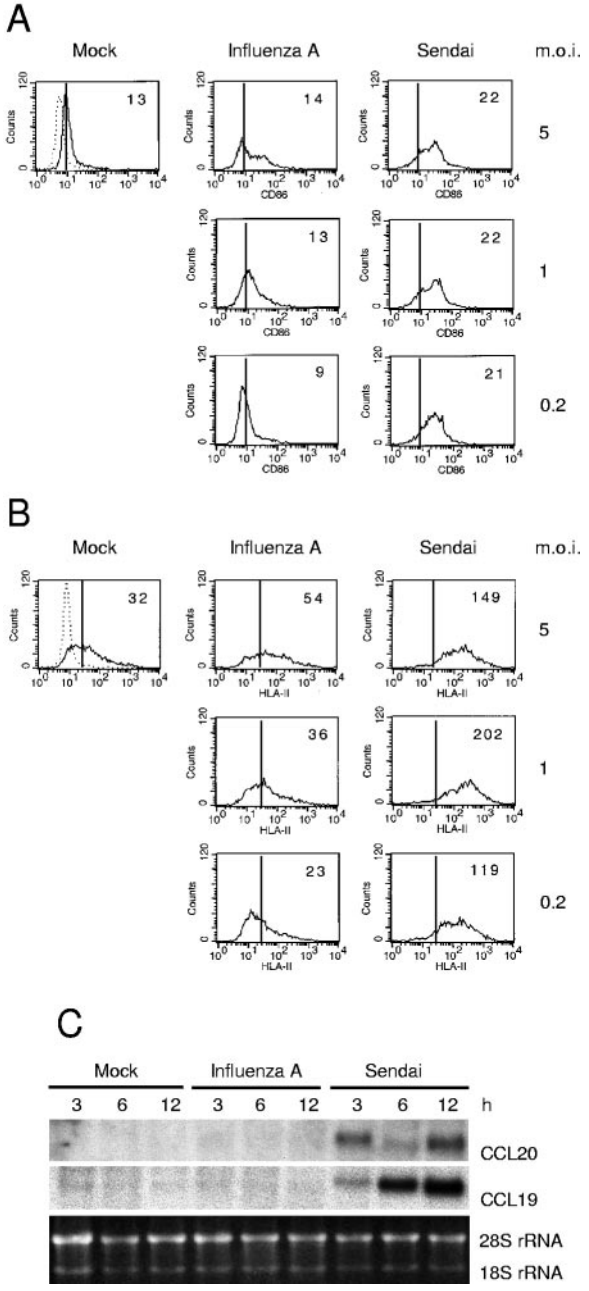

FIG. 2. Maturation of influenza A and Sendai virus-infected mDCs. (A and B) mDCs from three separate blood donors were infected with different doses of influenza A or Sendai viruses. After 18 h of virus infection mDCs were collected, pooled, and fixed with 1% paraformaldehyde. The cells were stained, and the expression of CD86 (panel A) and HLA class II (panel B) was analyzed by FACS. Values within the insets represent the geometric mean fluorescence intensities. Dotted lines indicate respective isotype controls. The results from one representative experiment of three are shown. (C) Virus-induced expression of CCL20 and CCL19 mRNAs. mDCs obtained from three blood donors were infected with influenza A virus or Sendai virus at an MOI of 5. Cells were collected at different times as indicated in the figure, and the total cellular RNA was collected and analyzed by Northern blotting with CCL20 and CCL19 cDNA probes. Ethidium bromide staining was used to control equal sample loading.

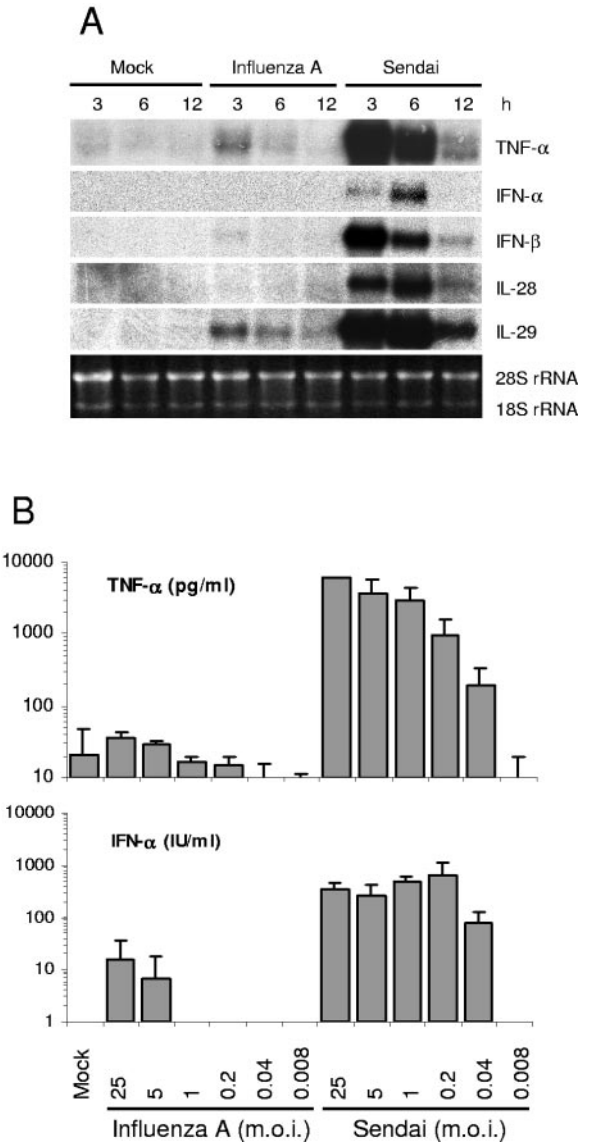

FIG. 3. Virus-induced expression of TNF-α, IFN-α/β, IL-28, and IL-29 genes. (A) mDCs from three blood donors were infected with influenza A (MOI = 5) or Sendai (MOI = 5) viruses for different periods of time. Cells were collected, total cellular RNA was isolated and processed for Northern blotting. The filters were probed with TNF-α, IFN-α (IFN-α1 probe), IFN-β, IL-28, and IL-29 specific cDNA probes. Ethidium bromide staining was used to control equal sample loading. (B) Virus dose-dependent production of TNF-α and IFN-α/β. mDCs were infected with different doses of influenza A or Sendai viruses for 20 h, and the cell culture supernatants were collected. TNF-α levels were measured by ELISA, and IFN-α/β levels were determined by a biological IFN assay. The results are the means (±1 standard deviation) of four different experiments, each performed with cells from three blood donors. Virus doses (MOI) as indicated in the figure.

sion was seen, and it was induced with a rapid kinetics (Fig. 3A). Sendai virus infection, instead, induced high levels of TNF-α, IFN-α/β, IL-28, and IL-29 mRNA expression. Although the peak mRNA levels for TNF-α, IFN-β, and IL-29 were seen at 3 h after Sendai virus infection, IFN-α and IL-28 mRNAs peaked somewhat later, at 6 h postinfection (Fig. 3A).

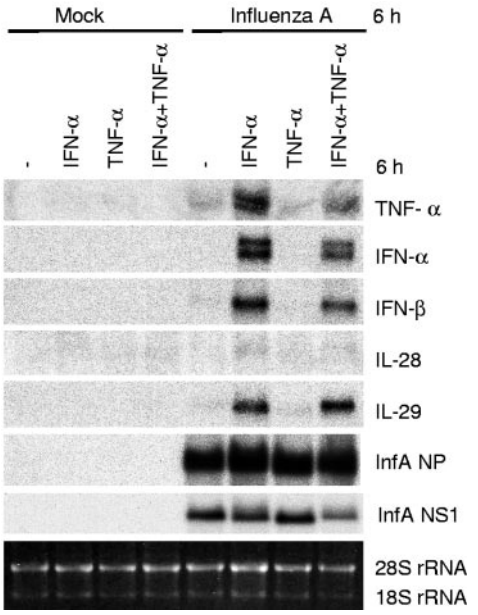

FIG. 4. Effect of IFN-α and TNF-α priming on influenza A virus-induced cytokine gene expression. mDCs were pretreated with IFN-α (100 IU/ml), TNF-α (5 ng/ml), or both for 6 h, followed by infection with influenza A virus (MOI = 5) for 6 h. Total cellular RNA was collected and analyzed by Northern blotting for TNF-α, IFN-α (IFN-α1 probe), IFN-β, IL-28, IL-29, and influenza A virus NP and NS1 mRNA expression. Ethidium bromide staining was used to control equal sample loading.

Virus dose-dependent production of TNF-α and IFN-α/βs into mDC culture supernatants was evaluated at 20 h after infection. As determined by ELISA, low or undetectable levels of TNF-α were observed in influenza A virus-infected cell culture supernatants. In contrast, Sendai virus induced TNF-α production in a dose-dependent manner from virus-infected mDCs and up to 6 ng of TNF-α/ml was produced with the highest virus doses (Fig. 3B). Virus-induced IFN-α/β production, as measured by a biological IFN-α/β assay, was low (15 IU/ml) in influenza A virus-infected mDCs, whereas up to 1,000 IU of IFN-α/β/ml was detected in Sendai virus-infected cell culture supernatants (Fig. 3B).

Effect of IFN-α and TNF-α pretreatment on influenza A virus-induced cytokine production in mDC. TNF-α and IFNs have an important role in the maturation of DCs and in the antiviral response in DCs (8). IFN-α/β also has a strong positive feedback effect on virus-induced IFN-α/β gene expression via IRF7 (29). We were thus interested in whether pretreatment of mDCs with TNF-α and/or IFN-α would enhance influenza A virus-induced cytokine gene expression. The cells were treated with IFN-α (100 IU/ml), TNF-α (5 ng/ml), or both for 6 h (or 16 h [results not shown]) prior to influenza A virus infection, and the induction of TNF-α, IFN-α/β, IL-28, and IL-29 mRNA expression was analyzed by Northern blotting. Priming the cells with IFN-α (alone or in combination with TNF-α) dramatically enhanced influenza A virus-induced expression of TNF-α, IFN-α, IFN-β, and IL-29 genes (Fig. 4). TNF-α priming alone had no effect on influenza A virus-induced cytokine mRNA expression. IFN-α or TNF-α doses used did not show marked inhibition of influenza A virus mRNA expression (Fig. 4).

Expression and functions of IFN-α/β-specific signaling molecules in DCs. TLRs, TLR-associated adapter molecules, IκB kinases and their downstream IRF, and NF-κB transcription factors play a key role in regulating host cell IFN response during virus infection. TLR3 and TLR7/TLR8, which recognize viral dsRNA and ssRNA, respectively (12, 15, 28, 29), are involved in host cell IFN-α/β production. TLR3 functions via TRIF and TLR7/8 via MyD88 adapter molecules (16, 54). Virus replication also activates specific IKKs, IKKε and TBK1 (13, 48), which regulate the activation of IRF3, IRF7, and NF-κB. In order to understand the molecular basis of enhanced virus-induced cytokine production in IFN-α-pretreated cells, the expression of these signaling molecules was analyzed by Northern and Western blotting. In unstimulated cells the basal mRNA expression of TLR3, TLR7, MyD88, and IRF7 was low, but these genes were readily induced by IFN-α (Fig. 5A). TLR8 and TRIF mRNA expression was seen in unprimed cells, but their expression was also enhanced by IFN-α to some extent. IKKε and TBK1 mRNA levels, in contrast, remained unchanged during cytokine priming (results not shown). Western blot analyses of cytokine-primed mDCs revealed that IKKε, TBK1, IRF3, NF-κB, p50, and p65 protein expression was detectable in DCs, and their levels remained unchanged during cytokine priming (Fig. 5B). In accordance with enhanced mRNA levels, IRF7 protein expression was enhanced by IFN-α (Fig. 5B).

To characterize the role of IRFs and NF-κB in IFN-α/β and IL-29 gene expression, oligonucleotide precipitation experiments were carried out. mDCs were pretreated with IFN-α, TNF-α, or their combinations for 16 h, followed by infection with influenza A virus. The proteins in nuclear extracts were precipitated by using IFN-α14, IFN-β, and IL-29 gene promoter ISRE and NF-κB site oligonucleotides. Especially, in IFN-α-primed cells influenza A virus infection (6 h) enhanced IRF3 binding to IFN-α14, IFN-β, and IL-29 promoter ISRE elements (Fig. 6). High-molecular-weight, hyperphosphorylated forms of IRF3 (P-IRF3) were clearly detectable, and immunoblot analysis with IRF3 S396-specific antibody (Fig. 6, P-IRF3 S396 panel) confirmed that influenza A virus-stimulated IRF3 phosphorylation took place at the C-terminal domain of IRF3. Binding of IRF7 to IFN-α14, IFN-β, or IL-29 ISRE elements was also observed, although binding was also detected in uninfected cells, and appeared to correlate with enhanced expression level of IRF7 in IFN-α-primed cells. Enhanced binding of p50/p65 complexes to the NF-κB sites of IFN-β and IL-29 promoters was also clearly seen in the nuclear extracts of influenza A virus-infected cells. TNF-α pretreatment resulted in enhanced NF-κB binding to these elements also in the absence of virus infection (Fig. 6), but further enhanced binding was seen in influenza A virus-infected cells.

Antiviral effect of IFN-α/β and IL-29 in mDC. mDCs were found to express IFN-α/β, IL-28, and IL-29 genes in response to virus infection. In order to assess the contribution of IFN-α, IFN-β, and IL-29 in host resistance to virus infection, the antiviral activity of these cytokines against influenza A virus was determined. IFN-γ was also included in antiviral analyzes. The antiviral effect of different IFN types and IL-29 was assessed by analyzing the expression of viral envelope glycopro-

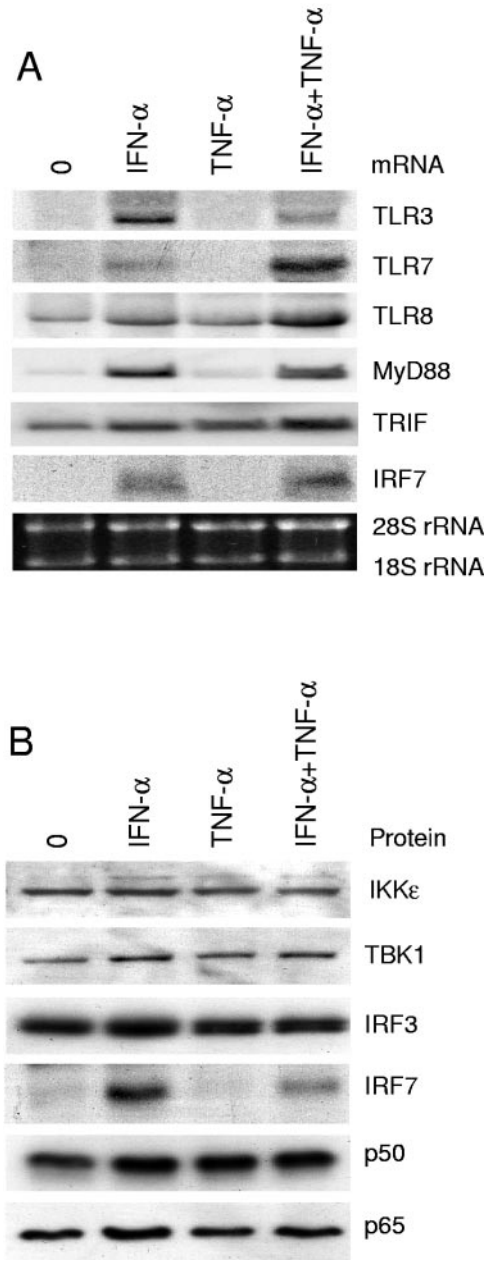

FIG. 5. Expression of cytokine-specific signaling molecules in DC. (A) Monocyte-derived DCs were stimulated with IFN-α (100 IU/ml) and/or TNF-α (5 ng/ml) for 6 h, and total cellular RNA was isolated. The expression of TLR3, TLR7, TLR8, MyD88, TRIF, and IRF7 genes was analyzed by Northern blotting. Ethidium bromide staining was used to control equal sample loading. (B) The protein levels of IKKε, TBK1, IRF3, IRF7, p50, and p65 in IFN-α (100 IU/ml)- and/or TNF-α (5 ng/ml)-stimulated mDCs (16 h stimulation) was analyzed by Western blotting.

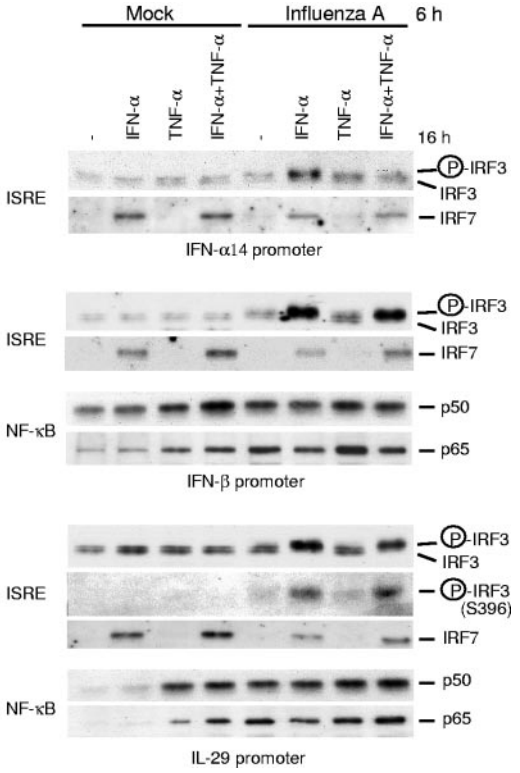

FIG. 6. Effect of IFN-α and TNF-α priming on influenza A virus-induced IRF3, IRF7, and NF-κB DNA binding to the ISRE and NF-κB sites of IFN-α, IFN-β, and IL-29 promoters. mDCs were pretreated with IFN-α (100 IU/ml), TNF-α (5 ng/ml), or both for 16 h, followed by infection with influenza A virus (MOI = 5) for 6 h. Cells were collected, and proteins from nuclear extracts were precipitated with IFN-α (α14), IFN-β, and IL-29 ISRE and NF-κB oligonucleotides as indicated in the figure. Oligonucleotide-bound proteins were analyzed by Western blotting with anti-IRF3, anti-IRF3 S396, anti-IRF7, anti-p50, or anti-p65 antibodies as indicated in the figure.

teins on the surface of IFN- and/or IL-29-pretreated influenza A virus-infected mDCs. To induce antiviral activity in mDCs, the cells were stimulated with natural IFN-α, recombinant IFN-α2b, recombinant IFN-β, and natural IFN-γ at concentrations of 10 and 100 IU/ml and with recombinant IL-29 at concentrations of 1 and 10 ng/ml. First, the ability of different IFN preparations or IL-29 to induce the expression of antiviral MxA protein was analyzed by FACS (Fig. 7A). IFN-α/β induced a significant, IFN-dose-dependent increase in MxA protein expression, whereas IL-29 and especially IFN-γ showed a weaker MxA protein-inducing activity. FACS analysis of viral protein expression in IFN- or IL-29-pretreated cells indicated that IFN-β, natural IFN-α, and recombinant IFN-α2b, in this rank order, showed significant antiviral activity against influenza A virus (Fig. 7). IL-29 also showed significant antiviral activity in mDCs, although it was weaker than those of IFN-α and IFN-β (Fig. 7). In the concentrations tested, IFN-γ had only marginal antiviral activity. Some additive MxA protein-inducing activity and antiviral effect was seen when IL-29 was combined with IFNs (Fig. 7B).

DISCUSSION

The clearance of virus infection depends on the activation of effective innate and adaptive immune responses. DCs play a crucial role as a link between innate and adaptive immune responses. By producing chemokines and cytokines, DCs activate innate immune responses and initiate the development of adaptive virus-specific immune responses. IFN-α and -β are the key players in host antiviral defense, since they exhibit

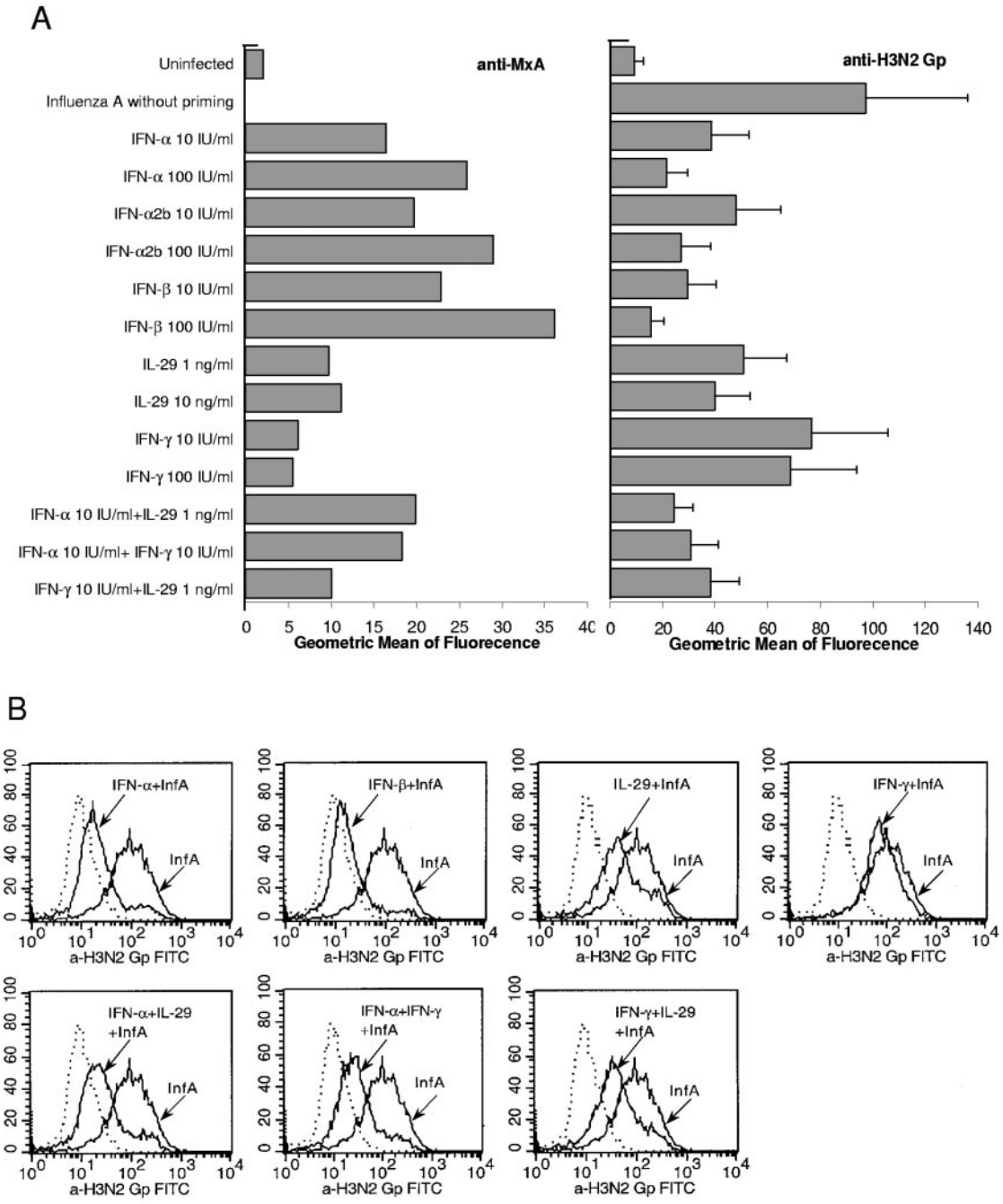

FIG. 7. Antiviral effect of IFN-α/β, IFN-γ, and IL-29 in DCs. mDCs from four individual blood donors were pretreated alone or in various combinations (as indicated) with doses of 10 or 100 IU of natural leukocyte IFN-α (IFN-α)/ml, recombinant IFN-α2b, recombinant IFN-β, or natural leukocyte IFN-γ and 1 or 10 ng of recombinant IL-29/ml for 24 h, followed by influenza A virus infection (MOI = 1) for 18 h. (A) Prior to virus infection part of the cells was collected, pooled, fixed, and permeabilized with 0.1% Triton X-100 for 5 min, followed by staining with anti-MxA and FITC-labeled secondary antibodies. MxA protein expression is shown as geometric mean fluorescence intensities. IFN- or IL-29-pretreated and virus-infected cells were collected separately, fixed, and stained for cell surface expression of influenza A virus glycoproteins. The results are the means (±1 standard deviation) from four different donors. IFN and IL-29 doses were as indicated in the figure. (B) FACS analysis of the expression of viral proteins on the surface of IFN- or IL-29-pretreated cells. Dotted lines represent uninfected cells. Solid lines represent influenza A virus glycoprotein-specific expression in virus-infected (InfA) or in cytokine-pretreated and virus-infected cells (cytokine + InfA) as indicated in the figure. The results from the cells of one representative blood donor (out of four) are shown.

direct antiviral activity and enhance the development Th1-type immune responses (39, 44, 45). Recently described IFN-like cytokines IL-28 and IL-29 are functionally similar to IFN-α/β and, although they use a different receptor system (23, 49), it is likely that IL-28 and IL-29 also contribute to the development of antiviral response. We and others have previously observed that the host cytokine response in influenza A virus-infected epithelial cells is restricted, and low levels of IFN-α/β and

other cytokines are produced (19, 40). Macrophages, in contrast, produce significant levels of chemokines, IFN-α/β, and other proinflammatory cytokines, including IL-18 in response to influenza A virus infection (31, 37, 38, 45). Here we have extended these analyses to mDCs and have examined the molecular mechanisms of influenza A virus- and Sendai virus-induced cytokine gene expression in human monocyte-derived DCs.

In the present study we show that influenza A virus infection does not efficiently induce mDC maturation. In addition, influenza A virus-induced antiviral cytokine production is low compared to that induced by Sendai virus. This difference was not due to differential infectivity, since both viruses infected mDCs equally well (Fig. 1). An interesting difference between the viruses was that whereas Sendai virus induced the expression of CCL20 and CCL19, which regulate the chemotaxis of immature and mature DCs, respectively (43), influenza A virus was unable to turn on the expression of these genes. The maturation of DCs is essential for the stimulation of antigen-specific T-cell responses (5, 8) and induction of cytokines, including that of IFN-α/β (26). It is also of note that influenza A virus induced TNF-α production very poorly. Since TNF-α has been shown to be one of the key cytokines regulating DC maturation (3, 27), the failure of influenza A virus to induce efficient TNF-α production may account for the lack of full DC maturation. We observed certain differences in the kinetics of IFN-α/β and IL-28/IL-29 mRNA expression. Previously, it has been shown that in virus infection initial IFN-β production is followed by a delayed induction of IFN-α genes (29). In Sendai virus-infected cells the expression of TNF-α, IFN-β, and IL-29 seemed to be induced very rapidly, whereas IFN-α and IL-28 genes were induced with a delay of a few hours (Fig. 3). In influenza A virus-infected DCs, early-stage cytokine (IFN-β and IL-29) induction was observed although it was weak, whereas efficient late-stage IFNs (IFN-α and IL-28) gene induction was not detected. One of the reasons for this phenomenon is likely to be influenza A virus nonstructural protein 1 (NS1). NS1 protein has been shown to interfere with the synthesis of host cell antiviral mRNAs, apparently by blocking the activation and subsequent nuclear translocation of IRF3 (52) and/or by interfering with the 3′-end processing of host cell pre-mRNAs, including those of IFN-α/β mRNAs (35). However, priming of mDCs with IFN-α can overcome the possible inhibitory effects of NS1 protein, since influenza A virus-induced IFN-α/β and IL-28/29 mRNA expression took place very well after IFN-α priming (Fig. 4). Plasmacytoid DC, which are considered to function as the major IFN-α/β-producing cells at early times of infection (17), are likely to be resistant to NS1-mediated IFN antagonism, since pDC can produce high levels of IFN-α/β in response to influenza A virus infection (4). It seems that after IFN-α priming, the capacity of mDCs to produce IFN-α/βs and IL-28/29 is increased (Fig. 4), and they have become functionally more closely related to pDCs.

In viral infections the induction of IFN-α/βs is regulated by IRF3 and IRF7, the latter of which is also upregulated by IFN-α/β providing a strong positive feedback signal for IFN production (29). In accordance with this we observed that IFN-α but not TNF-α priming increased influenza A virus-induced expression of IFN-α/β genes, as well as those of IL-28, IL-29, and TNF-α. Although IFN-α pretreatment induced the expression and, surprisingly, also the DNA binding of IRF7 (Fig. 6) virus-specific signals were required for the enhanced IFN-α/β and IL-28/29 gene expression. In addition to enhanced IRF7 expression, IFN-α pretreatment also resulted in enhanced expression of TLR3, TLR7, TLR8, MyD88, and TRIF genes. Enhanced TLR7/8 and MyD88 expression is likely to contribute to the positive feedback effect, especially in our DC model system, since TLR7/8 has recently been demonstrated to mediate cytokine gene induction in response to RNA viruses, including that of influenza A virus (12). Virus-activated kinases, IKKε, and TBK1, which regulate the phosphorylation of both IRF3 and IRF7, were expressed in mDCs at clearly detectable levels. The activation of these kinases have been shown to take place by viral proteins and the genetic material of viruses (ds- and ssRNA) (35). Recently, RIG-I, an RNA helicase, was identified to mediate dsRNA-stimulated activation of IRF3 and induction of IFN mRNA synthesis in response to Newcastle disease virus, another model of negative-stranded RNA viruses (12). IRF3 activation was also shown to occur via the phosphatidylinositol 3-kinase/Akt pathway in response to TLR3 stimulation by dsRNA (46). It will be of interest to determine the roles of these two recently described signal transduction pathways in influenza A virus infection.

In oligonucleotide precipitation experiments with ISRE elements from IFN-α14, IFN-β, and IL-29 gene promoters, we observed a clear enhancement in influenza A virus-induced IRF3 binding to these sites. In the case of IL-29 ISRE site, the use of IRF-3 S396-specific anti-phospho antibody (12) confirmed that the DNA-bound form of IRF3 was C terminally phosphorylated. It is worth noting that in mDCs influenza A virus infection was able to activate at least some IRF3, since already in unprimed cells IRF3 phosphorylation was evident (Fig. 6). These data are consistent with recent observations by other investigators (22). In oligonucleotide precipitation experiments the role of IRF7 was more difficult to judge, since the mere upregulation of IRF7 protein level led to some increase in IRF7 binding to IFN-α/β and IL-29 promoter elements. It is, however, well established that IRF7 is one of the important molecules regulating IFN-α-induced positive feedback mechanisms of IFN-α/β gene expression during viral infections (22, 25), and it is able to form heterodimers with IRF3 (2), which is likely to further enhance IFN-α/β gene expression. Influenza A virus infection was also able to stimulate NF-κB binding to IFN-β NF-κB binding site and to the putative IL-29 NF-κB binding site. Our results suggest that in virus infection IL-29 gene expression is regulated by functional ISRE and NF-κB promoter elements.

Since influenza A or Sendai viruses were able to induce IFN-α/β, IL-28, and IL-29 gene expression in mDCs, we carried out antiviral experiments with the respective proteins. Pretreatment of mDCs with IFN-α/β lead to an IFN-dose-dependent increase in cytoplasmic MxA protein expression and to a corresponding decrease in influenza A virus protein synthesis. IL-29 also induced MxA protein expression and showed a clear antiviral effect in mDCs, although not at the same level as the classical IFN-α/β. Our results suggest that both IFN-α/β, as well as IL-29 (and likely IL-28) significantly contribute to the host antiviral response. Our present work, as well as a recent reverse transcription-PCR analysis by Coccia

et al. (11), suggests that human pDC and mDCs are able to produce high levels of IL-28 and IL-29. Our data on the antiviral activity of IL-29 are consistent with the original publications describing the structural features and antiviral activity of IL-28A/B and IL-29 in other cell types than DCs (23). IFN-γ had only a marginal ability to enhance MxA expression, which correlated with a weak antiviral activity against influenza A virus in mDC. However, the combination of IFN-γ and IFN-α or IL-29 showed some additive antiviral activity. Our results show that IFN-α, IFN-β, and IL-29 have significant antiviral activity against influenza A virus in human mDCs.

In conclusion, our results demonstrate that human pathogenic influenza A virus is a relatively weak activator of DC maturation and antiviral cytokine gene expression in myeloid DCs. However, pretreatment of the cells with IFN-α/β leads to a dramatically enhanced activation of IFN-α/β, IL-28, IL-29, and TNF-α genes in response to influenza A virus infection. IFN-α priming increased the expression of TLR3, TLR7, TLR8, MyD88, TRIF, and IRF7 genes, which was likely to contribute to positive feedback signals. IFN-α priming also leads to enhanced binding of IRF3 to IL-29 gene promoter ISRE site, directly demonstrating an important role of IRF transcription factors in the regulation of this novel IFN-like gene. Finally, antiviral experiments carried out with IFN-α/β and IL-29 suggest that IL-29 (and likely IL-28A/B) significantly contributes to the host antiviral defense system.

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