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RIG-I Signaling Is Essential For Influenza B Virus-Induced Rapid Interferon Gene Expression

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ABSTRACT

Influenza B virus causes annual epidemics and along with influenza A virus accounts for substantial disease and economic burden throughout the world. Influenza B infects only humans and some marine mammals and is not responsible for pandemics possibly due to a very low frequency of reassortation and a lower evolutionary rate compared to influenza A virus. Influenza B virus has been less studied than influenza A and thus a comparison of influenza A and B virus infection mechanisms may provide new insight into virus-host interactions. Here we analyzed the early events in influenza B virus infection and interferon (IFN) gene expression in human monocyte-derived macrophages and dendritic cells. We show that influenza B virus induces IRF3 activation and IFN-λ1 gene expression with a faster kinetics compared to influenza A virus without a requirement for viral protein synthesis or replication. Influenza B virus-induced activation of IRF3 required the fusion of viral and endosomal membranes and nuclear accumulation of IRF3 and viral NP occurred concurrently. In comparison, immediate early IRF3 activation was not observed in influenza A virus-infected macrophages. Experiments with RIG-I, MDA5 and RIG-I/MDA5 deficient mouse fibroblasts showed that RIG-I is the critical pattern recognition receptor needed for the influenza B virus-induced activation of IRF3. Our results show that innate immune mechanisms are activated immediately after influenza B virus entry through the endocytic pathway, whereas influenza A virus avoids the early IRF3 activation and IFN gene induction.

IMPORTANCE

Recently, a great deal of interest has been paid on identifying the ligands for RIG-I under a natural infection as many of the previous studies have been based on transfecting the cells with different types of viral or synthetic RNA structures. We shed light on this question by analyzing the earliest step in
innate immune recognition of influenza B virus by human macrophages. We show that influenza B virus induces IRF3 activation leading to the IFN gene expression after the viral vRNPs are released to the cytosol and are recognized by RIG-I receptor meaning that already the incoming influenza B virus is able to activate IFN gene expression. In contrast, influenza A (H3N2) virus failed to activate IRF3 at very early times of infection suggesting that there are differences in the innate immune recognition between influenza A and B viruses.

**INTRODUCTION**

Influenza A and B viruses are important respiratory pathogens and cause seasonal epidemics with an estimated 250 000 - 500 000 annual deaths. Influenza A and B viruses are structurally similar; they are negative sense RNA viruses with a single-stranded segmented genome. The genome is structured in eight viral ribonucleoprotein (vRNP) complexes where the ssRNA is associated with multiple nucleoprotein (NP) molecules and a polymerase complex consisting of PB1, PB2 and PA proteins (1). The vRNP complexes are packaged in a matrix protein shell surrounded by a host-derived lipid envelope in which the viral glycoproteins hemagglutinin (HA) and neuraminidase (NA) are embedded. Influenza viruses bind to sialic acids on cell surface glycoproteins and enter the cells mainly via a clathrin-mediated endocytosis but also by macropinocytosis and clathrin-independent entry pathways (2, 3). Influenza viruses take advantage of the host endocytic pathway; a reduction of pH during the maturation of endosomes induces a conformational change in viral HA molecules and triggers the fusion between viral and endosomal membranes. The fusion is followed by the uncoating of the capsid by M1 dissociation due to acidification of the virion via M2 ion channel protein. This results in the release of vRNPs into the cytosol. The influenza virus genome is then imported into the nucleus for transcription and replication of viral genes. Primary transcription of the viral genome is triggered by the
virion-associated polymerase protein complex which leads to the translation of early viral proteins in
the cell cytoplasm. Newly synthesized polymerase, NP and NS1 proteins are transported into the
nucleus, where they initiate and regulate the replication and synthesis of complementary (cRNA) and
viral RNA (vRNA) molecules followed by secondary rounds of transcription. At later stages of the
infection, new vRNP complexes are packaged in the nucleus followed by M1 and NEP-regulated
export of vRNPs into the cytoplasm. Here, they associate with viral envelope glycoproteins HA and
NA on the plasma membrane leading to budding of the newly formed viral particles (4).

Host cells respond to influenza virus infection by producing interferons (IFN) and antiviral proteins
thus establishing an antiviral cellular state to restrict the spread of the infection. The most important
cellular sensors for RNA viruses are cytosolic retinoic acid-inducible gene-I (RIG-I) -like receptors
(RLRs), RIG-I and melanoma differentiation-associated protein 5 (MDA5), which recognize and bind
virus-derived ssRNA and dsRNA structures (5-7). Endosomal Toll-like receptors (TLRs), such as
TLR3 and TLR7/8 also recognize viral dsRNAs and ssRNAs, respectively (8-11). RLRs and TLRs
regulate IFN and other proinflammatory cytokine responses during influenza infection in certain cell
types. However, it remains unclear at what point in the influenza virus entry and/or replication cycle
viral RNA is sensed and IFN gene expression is induced. One study suggested that influenza A virus
RNA synthesis and nuclear export but not viral replication triggers IFN gene expression (12). Other
studies have proposed that RIG-I can recognize the incoming negative sense RNA virus via its 5'-
triphasphorylated genomic RNA even though it is bound by NP proteins and the viral RNA polymerase
complex (13-15). It has also been suggested that the incoming influenza A virus carries certain viral
structures that inhibit RIG-I activation and IFN induction even though the viral RNA is recognized by
RIG-I at the time of entry into the cytosol (16).
Upon binding of ssRNA or some dsRNA molecules RIG-I oligomerizes and associates with the adaptor protein mitochondrial antiviral-signaling protein (MAVS) on mitochondrial-associated membrane (MAM) and the outer membrane of mitochondria (17, 18). This activates a signaling cascade, which leads to serine/threonine-protein kinase TBK1/IKKɛ-mediated phosphorylation and nuclear translocation of the transcription factor interferon regulatory factor 3 (IRF3) and induction of IFN genes (18, 19). RIG-I signaling also can induce the activation of classical kinases IKKα and IKKβ, which promote signaling by the nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways (19). To counteract virus-induced activation of IFN gene expression, influenza A and B viruses have evolved mechanisms to inhibit the activation of innate immunity. The best known conserved virulence factor of influenza A and B viruses is the multifunctional NS1 protein which can inhibit RIG-I signaling and interfere with the processing of host cell pre-mRNA molecules (20). Influenza A virus also encodes additional virulence factors (PB1-F2, PB1-N40 and PA-X proteins) that antagonize innate immune responses (21-23).

Most information on influenza virus lifecycle and immune restriction has been obtained from influenza A virus research and it has been presumed that influenza B virus behaves in a similar fashion. However, we suggest that there may be differences between the viruses regarding the activation mechanisms of IFN gene expression. We have shown previously that influenza B virus induces a very rapid IFN response in human monocyte-derived dendritic cells (moDC) that does not depend on transcription or replication of the virus (24). Herein, we show that the early influenza B virus-induced IFN gene expression is RIG-I dependent and takes place after the vRNPs are released into the cytosol. These conclusions are supported by detailed gene expression analyses, cell biological approaches and the use of RIG-I and MDA5 knockout (KO) mouse cell lines.
MATERIALS AND METHODS

Ethics Statement. Adult human blood was obtained from anonymous healthy blood donors through the Finnish Red Cross Blood Transfusion Service (permission no 29/2014, renewed annually). Animal immunizations related to this study were approved by the Ethical Committee of National Institute for Health and Welfare (permission KTL 2008-02).

Cell cultures. Monocytes were purified from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki) as described previously (25). Human peripheral blood mononuclear cells were isolated by density gradient centrifugation over a Ficoll-Paque gradient (Amersham Biosciences). To obtain monocytes for macrophage differentiation, mononuclear cells were allowed to adhere to plates (Falcon, Becton Dickinson) for 1 h at +37°C in RPMI-1640 (Sigma-Aldrich) supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine, and 20 mM HEPES. Non-adherent cells were removed by washing with cold PBS and remaining monocytes were cultured in macrophage/serum-free medium (Life Technologies) supplemented with antibiotics and human rGM-CSF, 10 ng/ml (Nordic Biosite). The cells were differentiated into macrophages for 7 days changing fresh culture media every 2 days.

In each experiment, 3 to 4 different donors were grown and used separately for infection experiments.

To obtain monocyte-derived dendritic cells (moDCs), Ficoll-Paque gradient centrifugation was followed by Percoll gradient (Amersham Biosciences) centrifugation. The top layer containing monocytes was collected, and the remaining T and B cells were depleted using anti-CD3 and anti-CD19 magnetic beads (Dynal). Monocytes were allowed to adhere to plates for 1 h at +37°C in RPMI-1640 supplemented as above. Adherent monocytes were washed with PBS and immature moDCs were generated by cultivating cells in RPMI-1640 medium supplemented with 10% FCS (Integro), 10 ng/ml
human rGM-CSF, 20 ng/ml human rIL-4 (R&D Systems) and antibiotics. The cells were cultivated for 6 days and fresh media was added every 2 days.

Primary wild type (wt) and Irf3−/−Irf7−/− mouse embryonic fibroblasts (MEFs) and Rela−/−Rel−/−Nfκb1−/− (NF-κB KO) MEF cell line were kindly provided by Dr. A. Hoffmann, Signaling Systems Lab, Los Angeles. Primary wild type (Rig-I+/+), Rig-I−/−, Mda5−/− and Rig-I−/−Mda5−/− MEFs were obtained from mouse embryos and they were immortalized by rigorous passaging protocol to obtain wt and KO cell lines. The MEF cell lines were cultured in DMEM-medium supplemented with 0.6 µg/ml penicillin, 60 µg/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES and 10% FCS.

Viruses and infection experiments. Human influenza A virus A/Beijing/353/89 (H3N2) and influenza B virus B/Shangdong/7/97 and Sendai virus (strain Cantell) were grown from a 10−5 dilution of stock virus in allantoic cavities of 11-day-old embryonated chicken eggs at +34°C for 3 days. Influenza stock virus titers were determined by a plaque assay on MDCK cells and were as follows: 6x10^7 PFU/ml for A/Beijing and 18x10^7 PFU/ml for B/Shangdong. The multiplicity of infection (MOI) was based on the titers determined in MDCK cells. The Sendai virus stock titer (6x10^9 PFU/ml) was previously determined in moDCs by flow cytometry (26).

MoDCs, macrophages and MEFs were infected with influenza viruses for different times, as indicated in the figures. To obtain a synchronized infection for immunofluorescence assays, the viruses were adhered to cells on ice for 1 h after which the medium was replaced with warm medium (37°C) to allow the viruses to enter the cell. For infectivity experiments in macrophages the cells were washed and media replaced 1h after infection. If inhibitors were used, they were added onto the cells 30 min before infection. Inhibitors were purchased from Sigma Aldrich and the pretitrated, functional concentrations used were 10 nM BafilomycinA1, 80 µM Dynasore (27, 28) and cycloheximide 10
8 μg/ml. In LPS stimulation 100 ng of E.coli serotype O111:B4 LPS (Sigma Aldrich) was used/well. Cells were harvested and samples for qRT-PCR, immunoblotting or immunofluorescence were prepared.

Quantitative reverse transcriptase polymerase chain reaction, qRT-PCR. Cells from different blood donors were harvested and pooled after virus infection, and total cellular RNA was isolated using the RNEasy Mini kit (Qiagen) including DNase digestion (RNase-free DNase kit, Qiagen). One μg of total cellular RNA was reverse transcribed into cDNA in TaqMan RT buffer with 5.5 mM MgCl₂, 500 μM dNTPs, 2.5 μM Random hexamers, 0.4 U/μL RNase inhibitor and 1.25 U/μL MultiScribe reverse transcriptase (Applied Biosystems). cDNA samples were then amplified in TaqMan universal PCR master mix buffer (Applied Biosystems) with Gene Expression system assay mix oligonucleotides (Applied Biosystems) to analyze mRNA levels for human IFN-β1 (Hs00277188_s1), IFN-λ1 (Hs00601677_g1), RIG-I (Hs00225561_m1), IFITM3 (Hs01922752_s1) and mouse IFN-β1 (Mm00439552_s1), CXCL10 (Mm00445235_m1), TNF-α (Mm00443260_g1), and IAV or IBV NP and NS1 (24). Each cDNA sample was amplified in duplicate or triplicate with Mx3500P (Agilent Technologies). The mRNA levels were normalized against human 18S rRNA or mouse GAPD with TaqMan Endogenous Control kits (Applied Biosystems) and the amounts of cytokine mRNAs relative to unstimulated cells were calculated with the ΔΔCt –method as instructed by the manufacturer.

Immunoblotting. For protein analyses the cells from different blood donors were collected, washed with PBS and pooled, and whole cell lysates were prepared in passive lysis buffer of Dual Luciferase Assay Kit (Promega) containing 1mM Na₃VO₄ and Complete protease inhibitor (Roche). MEF cells were lysed with RIPA buffer (1%Triton-X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2) containing 1mM Na₃VO₄ and Complete protease inhibitor (Roche). Total cellular proteins were denatured in the Laemmli sample buffer and boiled. Equal amounts of
proteins were separated on SDS-PAGE and transferred to Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were blocked for 1 h at RT with 5% milk in PBS (blocking buffer). The rabbit antibodies against human IRF3, influenza A NS1 were prepared as described previously (26, 29). The antibody against influenza A virus NP was prepared in rabbits by immunizing the animals four times at 4-week intervals with an *Escherichia coli*-expressed glutathione-Sepharose-purified (Amersham Biosciences) glutathione S-transferase (GST)-NP fusion protein (100 μg/immunization/animal). The antibody against influenza B virus NP and NS1 were prepared similarly except 35 μg of baculovirus-expressed, histidine-tagged antigen purified with preparative SDS-page and 20 μg of baculovirus-expressed preparative SDS-page purified antigen with Freund’s adjuvant were used, respectively. The staining was performed in blocking buffer at room temperature for 1 h. Antibodies for human Ser396 phosphorylated IRF3 (p-IRF3, #4947), IRF3 (#4302), IκBα (#9242), and GAPDH (#2118) were from Cell Signaling Technology, and all these antibodies recognize also mouse proteins. The staining was performed in PBS containing 5% BSA at 4°C overnight. Antibody against IFITM3 (AP1153a) was from Abgent and against actin (sc-10731) from Santa Cruz Biotechnology. HRP-conjugated antibodies (Dako) were used in the secondary staining at RT for 1 h. Protein bands were visualized on HyperMax films using an ECL plus system (GE Healthcare).

**Immunofluorescence microscopy.** Macrophages were differentiated on glass coverslips and infected with influenza viruses at an MOI of 30, incubated on ice for 1 h, after which the virus inoculum was removed and replaced with warm media to allow virus entry to the cells at 37°C 5% CO2. After indicated times the cells were fixed with 3% paraformaldehyde at room temperature for 20 min, washed with PBS, permeabilized with 0.1% Triton X-100 for 5 min, washed and blocked with 0.5% BSA in PBS for 30 min. The cells were stained for 45 min in PBS containing 0.5% BSA at 37°C with
guinea pig anti-NP antibodies (1:400 dilution) prepared similarly as the corresponding rabbit antibodies described in immunoblotting method, and rabbit anti-IRF3 (1:50 dilution) or mouse anti-LAMP1 (sc-20011, Santa Cruz Biotechnology) or mouse anti-EEA1 (610456, BD Biosciences). Secondary antibodies were FITC-labelled goat anti-guinea pig, Rhodamine Red-X-labelled goat anti-rabbit and Rhodamine-labelled goat anti-mouse diluted at 1:100 (Jackson ImmunoResearch Laboratories). In infectivity experiments NucBlue® Fixed Cell ReadyProbes® Reagent (Life Technologies) was added to Alexa Fluor 488 (Life Technologies) secondary antibody to stain the nucleus. The coverslips were washed with PBS containing 0.05% Tween 20, then with distilled water, and mounted in 25% Mowiol (Polysciences) in 25 mM Tris-HCl, pH 7.5, 50% glycerol, and 2.5% 1,4-diazabicyclo[2.2.2.]octane. The cells were visualized under a Leica TCS SPE confocal microscope with 63x NA 1.40 oil objective with 1 Airy unit pinhole maintaining the same image acquisition settings for all acquired images. The cells in the infectivity experiments were analyzed with Zeiss Stallion fluorescence microscope with Hamamatsu ORCA-Flash 4.0 LT sCMOS camera and 20x NA 0.4 objective using Slidebook 6-software (Inteligent Imaging Innovations).

RESULTS

Influenza A and B viruses induce IFN gene expression with different kinetics in human macrophages. We have shown previously that influenza B virus induces IFN gene expression more rapidly than influenza A in human moDCs and A549 cells (24). To extend these studies further, we analyzed IFN responses in human monocyte-derived macrophages. These primary cells were infected with influenza A/Beijing/353/89 and B/Shangdong/7/97 viruses at a multiplicity of infection (MOI) of 5, based on the titers determined with a plaque assay in MDCK cells. The expression levels of IFN-λ1,
IFN-β, viral NP and NS1, RIG-I and IFITM3 mRNA were analyzed by qRT-PCR at different time points. In these human macrophages, influenza B virus induced maximal IFN-λ1 and IFN-β mRNA expression within 2 to 4 hours whereas the maximum IFN mRNA levels after influenza A virus infection were seen at 16 to 24 hours (Fig. 1A). No substantial differences were observed in viral NP gene expression suggesting that both viruses replicated equivalently in macrophages. The kinetics of expression of IFN-inducible genes RIG-I and IFITM3 after influenza A and B virus infection mirrored the kinetics of IFN mRNA expression. The effect of virus dose on the kinetics of IFN-λ1 and NP gene expression was studied with MOIs of 1, 5, 25 and 125 in macrophages (Fig. 1B). Even though the increasing virus amount did accelerate the early IFN-λ1 gene expression, influenza B virus was clearly more efficient in inducing IFN-λ1 in the 2 hour time point. The MOI values are presented as titers determined in MDCK cells throughout the study to be able to better compare the virus amount used in different cell types. We also confirmed the infectivity in macrophages of four different donors with immunofluorescence assay (Fig. 1C). Based on qRT-PCR data influenza A and B viruses appeared to replicate equally well in the human macrophages (Fig. 1B, NP panel), while influenza B showed apparently 5-fold better infectivity compared to influenza A virus, as judged by immunofluorescence assay (Fig. 1C).

**Influenza-induced early IFN gene expression correlates with the activation of IRF3.** IRF3 is a key transcription factor regulating early IFN gene expression, with NF-κB having a contributing role. To analyze whether there were differences in the kinetics and extent of activation of these transcription factors in influenza A and B virus-infected macrophages, we examined the phosphorylation status of IRF3 and the degradation of NF-κB inhibitor α (IκBα) by Western blotting (WB) at different time points of infection (Fig.2). In addition, the protein expression of IFN-inducible MxA and viral NP and NS1 was studied. Also the nuclear accumulation of IRF3 in macrophages was visualized by...
immunofluorescence microscopy (Fig. 3). The phosphorylation of IRF3 correlated with the kinetics of IFN-λ1 and IFN-β gene expression (see Fig. 1A), as the phosphorylation of IRF3 was apparent at 1h and peaked at 2h time point in influenza B virus-infected macrophages (Fig. 2). In comparison, influenza A virus-induced IRF3 phosphorylation was visible at 4 hours and was further enhanced at the 8 and 24 h time points. The degradation of IκBα, which precedes the activation of NF-κB, was weakly detectable at the 2h time point for influenza B and at the 8 and 24h time points in influenza A virus-infected cells.

In immunofluorescence microscopy experiments macrophages were infected with influenza A and B viruses at a high MOI of 30 and placed on ice for 1h to obtain a synchronized infection. The high MOI was used to be able to detect the incoming virus with the NP antibody and to confirm that all the cells were infected. The infection and internalization of viruses was triggered by increasing the temperature to 37°C and nuclear accumulation of viral NP and IRF3 into the nucleus was monitored over a 3h time course. At 1h after warming of cells, IRF3 was observed in the nucleus in influenza B virus-infected macrophages (Fig. 3A) whereas in influenza A virus-infected cells IRF3 nuclear translocation was not detected during the early phase (Fig. 3B). By 3 h after synchronized infection influenza A and B virus replication occurred, since viral NP accumulated into the nucleus.

**IRF3 and IRF7 transcription factors are indispensable for the influenza B virus-induced IFN-β and proinflammatory cytokine gene expression.** To define the signaling pathway(s) responsible for influenza B virus-induced IFN/cytokine gene expression we took advantage of a cell line generated from mouse embryonic fibroblast (MEF) cells lacking functional IRF3 and IRF7 or NF-κB genes. Wild type (wt) and IRF3/7 double KO cells were infected with influenza A and B viruses with an MOI of 15 for different times and cytokine gene expression was analyzed by qRT-PCR. A higher MOI of 15 compared to the MOI of 5 in macrophages was used because the mouse cells appeared to be more
resistant to human influenza viruses. In wt mouse fibroblasts, influenza B virus induced a maximal IFN-β gene expression by 3 hours whereas in influenza A virus-infected cells IFN-β gene expression did not peak until 24 hours after infection (Fig. 4A). As expected, neither influenza B nor A virus induced IFN-β gene expression in IRF3/7 KO cells although both viruses infected wt and KO cells with almost equal kinetics and efficacy as analyzed by monitoring viral NP and NS1 expression by WB and qRT-PCR (Fig. 4A and 4B). We also analyzed the expression of CXCL10 and TNF-α mRNAs and found that in influenza B virus-infected IRF3/7 KO cells the expression of both of these genes was also absent. However, influenza A virus infection readily induced the expression of CXCL10 and TNF-α mRNA in both wt and IRF3/7 KO cells. We also analyzed the phosphorylation of IRF3 in mouse cells by WB; although we found weaker IRF3 phosphorylation in general, there was a clear kinetic difference between influenza A and B viruses (Fig. 4B), as seen in human macrophages. In wt MEFs IκBα degradation occurred at 1h time point in influenza A virus-infected cells whereas no clear degradation of IκBα was observed in influenza B virus-infected cells. We also analyzed IFN-β gene expression in triple KO MEFs lacking the NF-κB components p65, c-Rel and p105. Early IFN-β gene expression was reduced to some extent (10 to 40-fold) in influenza B virus-infected NF-κB KO cells (Fig. 4C).

IRF3 is activated after influenza B virus vRNPs are released into the cytosol from the endosomal pathway. To correlate the stage in the viral lifecycle with IRF3 activation by influenza B virus we analyzed influenza B virus-infected macrophages at 15-minute intervals and stained for viral NP protein (green) to visualize virus entry and IRF3 (red) to define its nuclear accumulation (Fig 5). Viruses were allowed to attach the cells on ice for 1 h followed by a change of media (37°C) and chase for up to 3 h. In confocal images at time point 0’ the NP was localized at the plasma membrane. Within 15 min of incubation at 37°C we detected green granules inside the cells and between 30 and 45 min
some NP localized in the nucleus. At one hour viral NP accumulated in the nucleus and at 3 hours newly synthesized NP was seen. We confirmed that the NP at the 3 h time point was nascently generated since enhanced accumulation of NP in the nucleus was not seen in cycloheximide (CHX) treated cells (Fig. 5, compare IBV DMSO vs. CHX). The activation and nuclear import of IRF3 occurred within 45 to 60 min and correlated with the entry of viral vRNPs into the nucleus. This suggested that IRF3 is activated after influenza B vRNPs are released into the cytoplasm. To confirm this hypothesis we used a specific inhibitor of vacuolar H+ ATPase, Bafilomycin A1 (BafA1), which prevents the acidification of the endosomes required for influenza virus entry into the cytosol. In the presence of BafA1, nuclear accumulation of viral NP or IRF3 was not detected (Fig. 5, lower panels).

To link these findings to the innate immune signaling phenotypes, we analyzed IFN-λ1 gene expression in BafA1-treated macrophages (Fig. 6A) and moDCs (Fig. 6B). BafA1 treatment reduced the early phase of influenza B virus-induced IFN-λ1 gene expression. The phosphorylation of IRF3 also was reduced in BafA1-treated cells at the 2h and 3h time points in moDCs (Fig. 6B left panel), consistent with the observed inhibition of nuclear translocation of IRF3 in macrophages (Fig. 5). A dynamin-inhibitor, Dynasore, which prevents clathrin-mediated endocytosis, also blocked the early phase of influenza B virus-induced IFN-λ1 gene expression and IRF3 phosphorylation in moDCs (Fig. 6B, left panel). As a control we treated cells with LPS, which is recognized by TLR4 and does not need endosomal acidification for signaling. As expected, BafA1 did not inhibit LPS induced IFN-λ1 gene expression in moDCs (Fig. 6B right panel). However, TLR4 receptor is sensitive to Dynasore since it needs to be endocytosed before signaling via TRIF adaptor leading to IFN gene expression (30). As another control we used Sendai virus, which fuses directly at the plasma membrane. As expected, BafA1 did not inhibit IFN-λ1 gene expression in Sendai virus-infected macrophages (Fig. 6A, right columns).
Although the kinetics of the different phases of influenza A virus entry have been described previously (31, 32), such an information for influenza B virus is limited. In our experiments the entry of influenza B virus seemed to be rapid, as in less than 1 hour the viral NP entered the nucleus. To define this further, we performed immunofluorescence microscopy experiments to determine whether influenza B virus also uses early and late endosomes for its entry. We co-stained with EEA1 for early endosome or LAMP1 for late endosome/lysosome, and influenza B virus NP. Colocalization of EEA1 with influenza B virus NP was detected within 15 to 30 min and some colocalization with LAMP1 and NP was seen at 30 min (Fig. 7, see insets in lower panels). In BafA1-treated macrophages influenza B virus NP did not enter the nucleus and the colocalization with the endosomal markers was sustained for 1 to 2 hours with EEA1 and even to 4 hours with LAMP1. We also analyzed IRF3 activation in the same experiment and found it occurred at 1 h time point, after colocalization of viral NP with the endosomal markers (Fig. 7, upper 2 panels).

**RIG-I is essential for the influenza B virus-induced early IRF3 activation.** To elucidate which of the RLR receptors was responsible for the early innate immune recognition of influenza B virus infection we used MEFs lacking RIG-I, MDA5 or both proteins (7, 33). We infected wt and single and double receptor KO MEFs with influenza B and influenza A viruses at an MOI of 30 and analyzed the expression of IFN-β and viral NS1 RNA by qRT-PCR (Fig. 8A) and the phosphorylation of IRF3 and expression of NP and NS1 by WB (Fig. 8B). As the phosphorylation of IRF3 was weak in mouse cells (Fig. 4B) a high amount of virus was used to better visualize the activation of IRF3. In RIG-I−/− cells, influenza B virus induced IFN-β gene expression was completely absent; whereas in influenza A virus-infected cells primarily the early IFN gene expression (2 and 4 h) was missing or reduced (Fig. 8A). The early activation of IRF3 was RIG-I-dependent since influenza B virus-induced IRF3
phosphorylation did not take place in RIG-I−/− MEFs (Fig. 8B). In contrast, in MDA5 KO cells, IRF3 phosphorylation was induced normally by influenza B virus infection. The experiment also revealed differences between influenza A and B viruses. Unlike influenza B virus, influenza A virus showed some residual IFN gene expression activity also in RIG-I KO cells and IRF3 phosphorylation was reduced both in RIG-I or MDA5 single KO cells.

DISCUSSION

In the present study we analyzed the mechanisms of activation of innate immune responses in human monocyte-derived macrophages and dendritic cells after influenza A and B virus infections. We show that in human macrophages influenza B virus activates IRF3 phosphorylation and induces its nuclear accumulation soon after infection. This is followed by activation of IFN-λ1 and IFN-β gene expression and induction of IFN-stimulated genes (ISG), including RIG-I and IFITM3. In influenza A virus infected cells, these genes were induced with slower kinetics. IRF3 activation after influenza B virus infection occurred in the presence of protein synthesis inhibitor, CHX indicating that its activation and IFN gene expression is triggered directly by the incoming virus. Blocking influenza B virus vRNP release from endosomes with pharmacological inhibitors prevented the early activation of innate immune response. Finally, by using KO MEFs, we demonstrate that IRF3 and RIG-I play a crucial role in influenza B virus induced early IFN gene expression.

The present study was carried out mainly in human macrophages, in contrast to previously used moDCs (24). Tissue resident alveolar macrophages are the first line innate immune cells to fight influenza infection and also are possible targets of infection in the lungs (34). Analogous to studies in moDCs and A549 cells (24), influenza B virus induced IRF3 phosphorylation and IFN and ISG gene
expression in macrophages at early times after infection. Influenza B virus does not readily infect mouse cells and this species specificity depends partially on the viral NS1 protein which binds and inhibits the antiviral action of human but not mouse ISG15 (35). In MEFs, influenza B virus has previously been shown to induce IFIT2 and ISG15 in a RIG-I dependent manner (7) and in agreement with this data, we also detected RIG-I and IRF3/7 dependent IFN-β gene expression and IRF3 activation in MEFs.

Previously we showed that UV-treated, inactivated influenza B virus still induced IFN gene expression in human moDCs whereas UV-treated influenza A virus did not (24). UV irradiation of the virus is thought to block the primary transcription possibly due to the uracil dimers generated by the radiation. A recent study analyzed influenza A virus-induced IRF3 activation in the presence of chemical inhibitors, including actinomycin D, which blocks the primary transcription of the virus (12). This paper showed that the primary transcription but not the replication of influenza A was needed to activate IRF3. Another recent study carried out with several negative sense RNA viruses suggested that the incoming viral nucleocapsid containing a 5’-triphosphate genome can activate RIG-I signaling without transcription of viral genes (13). A more recent study showed that RIG-I is activated by the incoming influenza A virus 5´ triphosphate dsRNA panhandle (14). Our data supports this latter model well, at least for influenza B virus infection, which triggers IRF3 activation at the earliest phase of infection when NP starts to accumulate into the nucleus and before any viral protein synthesis or replication takes place. However, we did not observe IRF3 activation after the entry of influenza A virus which is consistent with our previous data that UV-treated influenza A virus is incapable of inducing IFN gene expression (24). In the study by Killip and coworkers, innate immune responses and IRF3 activation were analyzed at a relatively late time point (8 h) (12). This time point may reflect detection of newly synthesized viral RNAs or replication intermediates. However, we clearly show that
influenza B virus activates IRF3 at 1 h post infection in a RIG-I-dependent manner, which means that RIG-I must sense the incoming vRNA structures. We hypothesize that even if the incoming influenza A nucleocapsids were recognized by RIG-I, the virus could have some RIG-I and/or MAVS signaling inhibitors that prevent IRF3 activation. Supporting this theory, Liedmann and coworkers described motifs in the polymerase proteins PB1 and PA of influenza A that seemed to inhibit IRF3 activation. When these sites were mutated, the mutant but not the wild type virus activated IRF3 at an 8 h time point (16). To further complicate things, Weber and coworkers showed that RIG-I itself could function as an antiviral protein by binding and inhibiting the incoming influenza A nucleocapsids, independently of IFN induction, and the mammalian adapted influenza A viruses with PB2-627K are more resistant to the direct antiviral action of RIG-I than avian influenza A viruses with PB2-627E sequences. It remains to be investigated whether influenza B virus has evolved similar RIG-I counteracting strategies. Regardless, our results indicate that in the human macrophages and moDCs IFN signaling is activated by the incoming influenza B virus. Perhaps influenza B virus polymerase complex is not as tightly associated with the viral genome in the vRNP complex compared with human adapted influenza A viruses and thus the influenza B virus panhandle structures are more accessible for RIG-I recognition.

Banerjee and coworkers have recently developed an image-based assay to study the precise kinetics of influenza virus entry (31). The steps from endocytosis to HA acidification and from acidification to membrane fusion were rapid, but after membrane fusion a 45 min lag period was estimated to occur between membrane fusion and vRNP uncoating. We observed influenza B NP in the nucleus already at 1 h post infection and speculate that in the case of influenza B virus, viral and endosomal membrane fusion and uncoating may occur more rapidly than for influenza A virus resulting in faster entry into the nucleus. For this phenomenon there may be a couple of explanations: (a) influenza B virus HA fusion peptide is more exposed, which may lead to membrane fusion at higher pH and accelerated
fusion kinetics compared to influenza A virus (32); (b) the uncoating of influenza A virus takes place when the virion gets acidified via viral M2 ion channel leading to dissociation of M1 protein. As influenza B virus has an additional ion channel NB, the virion may become acidified more efficiently and thus the vRNPs may be released into the cytoplasm earlier. However, NB ion channel is likely not the only contributing factor as mutating this ion channel leads to reduced IRF3 activation compared to wt virus but still the activation of IRF3 is more efficient than with influenza A virus (data not shown). As viral vRNPs are released earlier from the endosome and uncoated, they may be recognized sooner as foreign RNA structures via cytosolic PRRs.

Innate immunity receptors known to recognize viral RNA structures are RIG-I, MDA5, TLR3, TLR7 and TLR8. Macrophages and dendritic cells are equipped with a range of innate immune receptors to recognize foreign microbial structures. As influenza viruses enter the cells via an endosomal pathway, where TLR3, 7 and 8 are localized, viral entry could be recognized in the endosomes. This occurs in plasmacytoid DCs (pDCs), which express low levels of RLRs (36) but high levels of endosomal TLR7 and IRF7 and produce high amounts of IFN-α during influenza A virus infection (11). It is possible that in pDCs some viruses cannot escape the endosomal pathway before degradation and thus viral RNA structures are released from the virion to be recognized by endosomal TLRs. Our experiments with BafA1 that result in the retention of influenza viruses in the endosomal pathway suggest that the incoming influenza B virus RNA is not recognized by TLRs in macrophages or moDCs. However, BafA1 treatment may affect the maturation and signaling of TLR receptors (37), so we cannot fully exclude their role. Nevertheless, experiments in knock-out MEFs suggested that the early influenza B virus-induced innate immune responses are mediated by RIG-I in the cytosol. In summary, we have demonstrated significant differences between influenza A and B virus infection in their ability to activate early innate immune responses in primary human immune cells. A better understanding of the
early entry and immune recognition events may help to explain the pathogenesis of influenza A and B virus infections and provide us the means to develop efficient intracellular viral inhibitors.

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REFERENCES


FIGURE LEGENDS

Figure 1. Kinetics of influenza-induced IFN gene expression in human macrophages. (A) Human monocyte-derived macrophages were infected with influenza virus strains A/Beijing/353/89 (IAV) and B/Shangdong/7/97 (IBV) at an MOI of 5 for different times as indicated in the figure. Cells from three donors were pooled, total cellular RNA was isolated, and IFN, viral NP and NS1, RIG-I and IFITM3 gene expression was measured by qRT-PCR and fold induction over the mock-infected sample or 1 h infected sample (NP and NS1) was calculated. (B) Macrophages from four donors were infected as above with an MOI of 1, 5, 25 or 125 for different times and IFN-λ1 and viral NP gene expression was analyzed with qRT-PCR. The results are presented as the means (+1 standard deviation of the means) of two to three technical replicates and are from a representative experiment out of three (A) or two (B). (C) Macrophages were grown on coverslips and infected with viruses as above with MOIs from 25 to 0.04 for 16 h. The coverslips were prepared for immunofluorescence and stained with guinea pig anti-influenza A or B NP and nuclear stain using Alexa Fluor 488 conjugated anti-guinea pig secondary antibody. The mean percentage of NP positive cells from four donors is presented with one SD unit and altogether 8 donors were analyzed.

Figure 2. IRF3 activation correlates with an early influenza B virus-induced IFN response. Macrophages were infected with influenza A/Beijing/353/89 and B/Shangdong/7/97 viruses (MOI of 5) and cells from three donors were collected at different time points and pooled. Whole-cell lysates were prepared for SDS-PAGE and immunoblot analysis with antibodies against p-IRF3, IκBα, IRF3, influenza A and B virus NP and NS1 and MxA was carried out. Actin and GAPDH protein levels were analyzed to control equal loading of the samples. The data are representative of two independent experiments.
Figure 3. IRF3 and influenza B virus NP accumulate in the nucleus simultaneously in macrophages. Human macrophages were differentiated on coverslips followed by infection with (A) influenza B/Shangdong/7/97 and (B) influenza A/Beijing/353/89 viruses with an MOI of 30 on ice for 1 h. Subsequently, virus entry proceeded after warming to 37°C and cells were fixed with PFA after indicated incubation times. Guinea-pig anti-NP antibodies were used to detect influenza A or B virus NP (green) and rabbit anti-human IRF3 was used to stain for IRF3 (red), secondary antibodies were FITC-labelled donkey anti-guinea-pig and Rhodamine Red-X-labeled donkey anti-rabbit immunoglobulins. Second row of images shows insets of NP stainings indicated by white rectangular. Data shown is representative of four independent experiments on cells from 11 donors.

Figure 4. IRF3 and IRF7 transcription factors are essential for influenza virus induced IFN gene expression. (A and B) Wt MEFs and MEFs lacking IRF3 and IRF7 transcription factors (IRF3/7) were infected with influenza A/Beijing/353/89 and B/Shangdong/7/97 viruses with an MOI of 15 for indicated times. RNA samples were collected for analyzing the gene expression of IFN-β, viral NP and NS1, TNF-α and CXCL10 genes by qRT-PCR (A) and protein samples were prepared for immunoblot analysis with antibodies against p-IRF3, IRF3, IκBα and influenza A and B virus NP and NS1 (B). GAPDH protein levels were analyzed to control equal loading. (C) Wt MEFs and MEFs lacking the NF-κB components p65, c-Rel, and p105 (NF-κB) were infected with influenza A/Beijing/353/89 and B/Shangdong/7/97 viruses with an MOI of 15 for indicated times. RNA samples were collected for analyzing the gene expression of IFN-β and viral NP genes by qRT-PCR. The qRT-PCR data is presented as relative gene expression levels for viral NP gene in relation to that of the 30 min or 1h sample and for cytokine genes in relation to those of the mock-infected samples. The results are presented as the means from triplicate measurements with 1 SD units and representative experiments out of two are shown.
Figure 5. Endosomal acidification but not de novo protein synthesis is needed for influenza B virus induced IRF3 activation. Human macrophages were differentiated on coverslips followed by infection with influenza B/Shangdong/7/97 virus with an MOI of 30 on ice for 1 h. Subsequently, virus entry proceeded after warming to 37°C and cells were fixed with PFA after indicated incubation times. The inhibitors, BafA1 and CHX or DMSO vehicle were added to the cells 30 min before infection. Guinea-pig anti-NP antibodies were used to detect influenza B virus NP (green) and rabbit anti-human IRF3 was used to stain IRF3 (red), secondary antibodies were FITC-labelled donkey anti-guinea-pig and Rhodamine Red-X-labelled donkey anti-rabbit immunoglobulins. Under each frame an inset of one cell marked with a white rectangular is presented. Data shown is representative of two independent experiments on cells from 6 donors.

Figure 6. Inhibition of endosomal acidification decreases early influenza B virus-induced IFN gene expression in human monocyte-derived macrophages and dendritic cells. (A) Macrophages were treated with BafA1 or vehicle control followed by infection with influenza A/Beijing/353/89, B/Shangdong/7/97 or Sendai viruses with an MOI of 5 with times indicated in the figure. The cells from 4 donors were pooled for isolation of total cellular RNA and qRT-PCR analysis of IFN-λ1 gene expression. The data is presented as fold induction over mock-infected sample as the means of three technical replicates with 1 SD unit. (B) MoDCs were treated with BafA1, Dynasore or vehicle control followed by infection with influenza B/Shangdong/7/97 (MOI of 5) (left panel) or stimulated with LPS (right panel) for different times. The cells from 4 donors were pooled for qRT-PCR analysis of IFN-λ1 gene expression and WB analysis for p-IRF3, IκBα, IRF3, and influenza B virus NP expression. GAPDH protein levels were analyzed to control for equal protein loading. The data is representative of two to three independent experiments.
Figure 7. Influenza B NP colocalizes with classical markers of the endosomal pathway. Human macrophages were differentiated on glass coverslips and infected with influenza B/Shangdong/7/97 virus with an MOI of 30 on ice for 1 h. Virus entry proceeded after warming to 37°C and cells were fixed with PFA after indicated incubation times. BafA1 or DMSO was added to the cells 30 min before infection. Primary antibodies used were guinea-pig anti-NP IBV, rabbit anti-human IRF3, mouse anti-EEA1 and mouse anti-LAMP1. Secondary antibodies were FITC-labelled donkey anti-guinea-pig and Rhodamine Red-X-labelled donkey anti-rabbit and rhodamine-labelled goat anti-mouse immunoglobulins. Insets of 15, 30 and 60 minute time points are visualized in the lower panel and marked with white rectangles in the upper panels. One representative experiment out of two is shown.

Figure 8. RIG-I receptor is responsible for the early influenza B virus-induced IRF3 phosphorylation and activation. (A) Wt (RIG-I+/+) MEFs and MEFs lacking RIG-I, MDA5 or both receptors were infected with influenza A/Beijing/353/89 and B/Shangdong/7/97 viruses with an MOI of 30 for indicated times. Cells were collected and total cellular RNA was isolated for qRT-PCR analysis of IFN-β and viral NS1 gene expression. The qRT-PCR data is presented as fold induction over mock-infected samples and the means with 1 SD units from triplicate measurements are shown. (B) Protein samples were isolated for the analysis of p-IRF3, viral NP and NS1 and GAPDH by WB. A representative experiment out of three is shown.