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Essential Impact of NF-κB Signaling on the H5N1 Influenza A Virus-Induced Transcriptome[1]

Mirco Schmolke,* Dorothee Viemann,† Johannes Roth,† and Stephan Ludwig[2]*

Systemic infections of humans and birds with highly pathogenic avian influenza A viruses of the H5N1 subtype are characterized by inner bleedings and a massive overproduction of cytokines known as cytokine storm. Growing evidence supports the role of endothelial cells in these processes. The aim of this study was to elucidate determinants of this strong response in endothelial cells with a focus on the transcription factor NF-κB. This factor is known as a major regulator of inflammatory response; however, its role in influenza virus replication and virus-induced immune responses is controversially discussed. By global mRNA profiling of infected cells in the presence or absence of a dominant negative mutant of IκB kinase 2 that specifically blocks the pathway, we could show that almost all H5N1 virus-induced genes depend on functional NF-κB signaling. In particular, activation of NF-κB is a bottleneck for the expression of IFN-β and thus influences the expression of IFN-dependent genes indirectly in the primary innate immune response against H5N1 influenza virus. Control experiments with a low pathogenic influenza strain revealed a much weaker and less NF-κB-dependent host cell response. *The Journal of Immunology,* **2009, 183: 5180–5189.**

Since 1997, when the first cases of directly transmitted highly pathogenic avian influenza viruses (HPAIV)[3] from poultry to humans in East Asia were reported (1), a growing concern arose that these viruses might become the source of a new influenza pandemic. So far direct transmissions of HPAIV are rare events (2), need high doses of infectious particles, and did not lead to any unlimited spread. In poultry, HPAIV of the H5 subtype show high transmissibility and mortality accompanied by systemic infection of the host and severe inner bleedings (3). Infections with HPAIV are characterized by a hyperreaction of the host immune response accompanied by massive production of cytokines and chemokines (4, 5). This overproduction of cytokines and chemokines, known as cytokine storm is a cell intrinsic phenomenon (6). Growing evidence supports the contribution of endothelial cells in this process. This cell type is not only a major source of cytokines and chemokines (7) but is also extremely relevant for systemic viral dissemination, supported by the fact that endotheliotropsim has been demonstrated for HPAIV in infected birds (8, 9) and humans (10, 11). In this study, we used primary human endothelial cells that are highly permissive for and responsive to influenza A virus infections and show no defects in the innate immune response as widely observed for many transformed cell lines.

The innate host cell response is the first line of defense to viral infections. In the case of influenza virus infections, it is triggered by accumulation of viral 5′-triphosphate vRNA. This pathogen pattern is mainly sensed by the cytosolic helicase retinoic acid-induced gene I (RIG-I) (12). Activation of RIG-I leads to phosphorylation and nuclear translocation of the constitutively expressed transcription factor IFN regulatory factor (IRF) 3 via TANK-binding kinase 1 (13–15) and activation of NF-κB (p50/p65) via IκB kinase 2 (IKK2)-mediated phosphorylation/degradation of the inhibitor of κ B (IκBα) (16). NF-κB and IRF3 are known to regulate the expression of many cytokines and chemokines including IFN-β, a major mediator of the innate antiviral response. Both factors bind to adjacent promoter regions and as part of the so-called IFN-β enhanceosome (17, 18). IFN-β is secreted by the infected cell and can act in an autocrine or paracrine fashion by binding to the type 1 IFN receptor (IFNAR). Subsequently, activation of the JAK/STAT pathway initiates the formation of the IFN-stimulated gene factor 3 (ISGF3), consisting of STAT1, STAT2, and IRF9. ISGF3 binds to IFN-sensitive regulatory elements (ISRE) and regulates the expression of IFN-stimulated genes (ISG), including IRF7, myxovirus resistance A (MxA), 2–5-oligoadenylate synthetase 1, RIG-I, and IFN-β itself (19–23), which can directly or indirectly interfere with the replication of viruses.

The function of IRF3 and AP-1 in enhancing type I IFN production upon influenza A virus infection are undeniable (24). However, the role of NF-κB in influenza virus infected cells is the subject of ongoing discussions. Recent reports either claim no involvement of NF-κB in the antiviral gene expression profile induced by influenza viruses (25) or even present data that support an inhibitory function of NF-κB in this context (26). This is in contrast to earlier studies that clearly underline the necessity of initial NF-κB binding to the IFN-β enhanceosome to allow efficient production of IFN-β and thus promote expression of ISGs (27).

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[3] Abbreviations used in this paper: HPAIV, highly pathogenic avian influenza virus; IRF, IFN regulatory factor; IKK2, IκB kinase 2; ISRE, IFN-sensitive regulatory element; ISGF3, IFN-stimulated gene factor 3; ISG, IFN-stimulated gene; MxA, myxovirus resistance A; RIG-I, retinoic acid-induced gene I; EGFP, enhanced GFP; MOI, multiplicity of infection; GO, Gene Ontology; IP-10, IFN-γ-inducible protein 10; NS1, nonstructural protein 1; HMEC-1, human microvascular endothelial cell 1; p.i., postinfection.

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To dissect the function of NF-κB signaling in the network of activated pathways triggering the antiviral response in systemic infections with H5N1 HPAIV, we specifically interfered with NF-κB signaling by means of expression of a dominant negative form of IKK2. This approach has been successfully used previously to efficiently blunt NF-κB activation (28–31). By global gene expression profiling, we demonstrate the essential impact of NF-κB signaling on the antiviral gene response against HPAIV of the H5N1 subtype.

Materials and Methods

Reagents and plasmids

Recombinant human IFN-β was purchased from PBL. BAY11-7085 was purchased from Sigma-Aldrich. Cells were preincubated with 5 μM of the incubator for 30 min before infection. The retroviral expression plasmids pCFG5-IEGZ HA and pCFG5-IEGZ IKK2KD were described previously (28–31).

Viruses and cells

The HPAIV strain A/Thailand/KAN-1/2004 (H5N1) isolated form a fatal human case was used with permission from Dr. P. Puthavathana (Bangkok, Thailand). The low pathogenic human influenza A virus strain A/WSN/33 was taken from the virus collection of the Institute of Molecular Virology (Muenster, Germany). Viruses were propagated on Madin-Darby canine kidney (MDCKII) cells. MDCKII were cultured in MEM (PAA) containing 10% v/v FCS and 100 U/ml penicillin/0.1 mg/ml streptomycin (1× penicillin/streptomycin; Life Technologies). Primary HUVEC (Cambrex) were cultured as described previously and used in passages five to seven. Human microvascular endothelial cell 1 (HMEC-1) were cultured in MCDB-131 containing 10% FCS, 1 μg/ml hydrocortisone, 10 ng/ml recombinant human epidermal growth factor (R&D Systems), 10 mM L-glutamine (Life Technologies), and 50 mg/ml gentamicin. Phoenix packaging cells (Orbigen) and HEK293T (American Type Culture Collection) were cultured in DMEM (PAA) containing 10% v/v FCS and 1× penicillin/streptomycin.

Plaque assay

PFU of a given virus suspension were determined as described earlier (32).

Retroviral gene transfer

Fifteen micrograms of the empty retroviral pCFG5-IEGZ HA vector or pCFG5-IEGZ IKK2KD expressing dominant negative IKK2 (29) was transfected into 3×10^6 Phoenix packaging cells with polyethyleneimine as described previously for HEK293 (33) and selected with 250 μg/ml Zeocin (Invitrogen) for 2 wk to gain stable producer cells. Three $\times 10^6$ stable producer cells were seeded on 100-mm dishes 48 h before transduction. Twenty-four hours before transduction, medium was changed to HUVEC culture medium (6 ml/dish) and retrovirus-containing supernatants were harvested 24 h later. HUVEC and HMEC-1 were infected with retroviral supernatants as described previously (29). Retrovirally transduced HMEC-1 were selected with 250 μg/ml Zeocin (Invitrogen) for 2 wk to gain stable cell lines.

The efficiency of retroviral gene transfer of HUVEC and HMEC-1 was measured by flow cytometric detection of recombinant enhanced GFP (EGFP) that was coexpressed with the gene of interest from a bicistronic mRNA with a FACSCalibur cytometer (BD Biosciences) 48 h after transduction. Transduction rates ranged from 90 to 100%. Expression of transgenes was measured by Western blot.

Western blot and flow cytometry

Cells were lysed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (34). Radioimmunoprecipitation assay protein lysates were mixed with 5× Laemmli buffer, separated by SDS-PAGE, and blotted onto nitrocellulose membranes. Antisera directed against ERK2, IκBα, and IKK2 were purchased form Santa Cruz Biotechnology and Abs against STAT1 and phospho-STAT1 Tyr^{701} were obtained from BD Biosciences. A murine mAb against influenza A virus M1 was purchased from Serotec. A murine mAb against influenza A virus nonstructural protein 1 (NS1) was generated by V. Wixler in the Institute of Molecular Virology.

Flow cytometric measurement of intracellular cytokines was performed as described earlier (28). Mouse mAb against human IL-8 and MCP-1 were purchased from BD Biosciences. Goat anti-mouse Cy5-labeled secondary Abs was a gift from V. Wixler (Institute of Molecular Virology). At least 10^4 cells were analyzed in a FACSCalibur cytometer (BD Biosciences) using CellQuest Pro analysis software.

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated from 100-mm dishes with a 80–90% confluency-grown HUVEC monolayer (~1.5–2 $\times 10^6$ cells) using the RNeasy Kit (Qiagen) according to the manufacturer's instruction. One microgram of total RNA was reverse transcribed with MBI Revert AID Reverse Transcriptase (MBI Fermentas) and oligo(dT) primer according to the manufacturer's instruction. The cDNA was diluted 1/10 and 0.5 μl was used for real-time PCR analysis. Primer sequences are included in the supplementary material (supplemental Table S1).[4] Real-time PCR was performed with a Stratagene MX3005P cycler and Brilliant SYBR Green Mastermix (Stratagene) according to the manufacturer's instruction. Relative changes in expression level (*n*-fold) are calculated according to the $2^{-\Delta\Delta CT}$ method (35).

DNA microarray and statistical data analysis

Total cellular RNA was isolated from three independent experiments with wild-type HUVEC that were infected for 5 h with a multiplicity of infection (MOI) of 5 of the H5N1 virus using a RNeasy kit (Qiagen). Three additional independent H5N1 infections were performed for microarray analyses with empty retroviral expression vector- or IKK2KD-expressing HUVEC. Samples were processed for microarray hybridization using Affymetrix Human Genome 133 Plus 2.0 Gene Arrays according to the manufacturer's instructions. Fluorescent signals were detected by the GeneChip Scanner 3000 and recorded and computed by GeneChip Operating Software version 1.4 (Affymetrix). Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/Access code: GSE13637).

For a more sophisticated data analysis, we used the Expressionist Suite software package from GeneData (Basel, Switzerland) as described elsewhere (29). Only genes with a fold change of >2.0 or <2.0 and a $p \leq 0.05$ (paired *t* test) in multiple independent experiments were considered as regulated genes. "On/off"-regulated genes were evaluated as described previously (29). We considered genes with on:off ratios of 0:3, 0:2, 1:3, 3:0, 2:0, and 3:1, respectively. From this group of on/off-regulated genes, we only included regulations with a high fold change of ≥5 and a $p < 0.05$ in the list of regulated genes to differentiate on/off phenomenas occurring around the background threshold from significant on/off phenomenas. We applied principal component analyses to reduce mathematically the dimensionality of the entire spectrums of gene expression values of a microarray experiment to three components (36).

To identify functional categories of genes that are overrepresented in the data sets of regulated genes, we first assigned Gene Ontology (GO) annotations to every probe set spotted on the Affymetrix 133 Plus 2.0 Array and compared it with the distribution of GO annotations in the gene group of interest applying Fisher's exact test. In the case of genes that are represented by two or more probe sets, only one transcript was taken into account to avoid potential bias.

Interaction networks showing direct and indirect relations of given gene products were generated with the Ingenuity Pathway Analysis 7.1 software (Ingenuity Systems). The significance of the generated networks was determined by comparison of the interaction of a similar number of random gene products. The resulting score is a numerical value used to rank networks according to their degree of relevance to the network eligible molecules in your data set. The score takes into account the number of network-eligible molecules in the network and its size, as well as the total number of network-legible molecules analyzed and the total number of molecules in Ingenuity's knowledge base that could potentially be included in networks. The score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact Test. The score is the negative log of this *p* value. It must be noted that the score is not an indication of the quality or biological relevance of the network; it simply calculates the approximate "fit" between each network and your network-eligible molecules. Networks showing the involvement of a given group of mRNAs in certain biological processes were generated with Gene Spring GX 10.0 software (Agilent).

Promoter analysis

For the promoter analyses, we took advantage of a computational method for transcriptional regulatory network inference, CARRIE (Computational

[4] The online version of this article contains supplemental material.

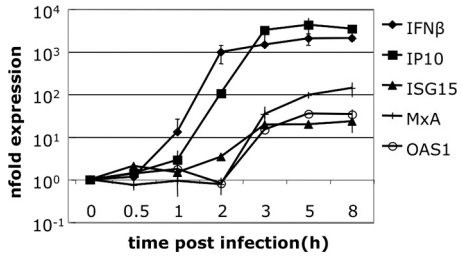

FIGURE 1. Time course of H5N1-induced genes. HUVEC were infected with 5 MOI of H5N1 influenza A virus for the indicated time points. Expressional changes of mRNAs of IFN-β (♦), IP-10 (■), ISG15 (▲), MxA (†), and OAS1 (○) were detected by real-time RT-PCR and are depicted as mean *n*-fold (±SD) of three independent experiments normalized to controls.

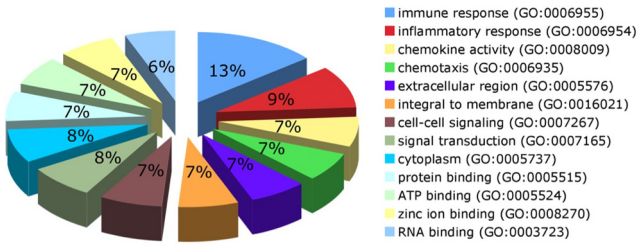

FIGURE 2. Clustering of H5N1-induced genes according to their GO annotated function. H5N1 influenza A virus-induced mRNAs (up-regulated and switched on) were grouped according to their predominant GO annotation. Relative distribution of mRNAs (percent) per GO cluster is indicated. GO clusters with <5 mRNAs per cluster are not depicted.

Ascertainment of Regulatory Relationships Inferred from Expression) described by Haverty et al. (37). Briefly, microarray data and promoter sequence data derived from the TRANSFAC database 7.0 are conflated. The promoter regions of the group of unregulated genes are compared with the promoters of regulated genes to compute the relative overabundance of *cis*-elements in the group of regulated genes. Thereby transcription factor binding sites are detected which potentially contribute to the regulation of a given group of genes according to their significance of overrepresentation.

Results

To gain a global overview on the H5N1 virus-induced gene expression program in endothelial cells, HUVEC were infected with a MOI of 5 (MOI = 5) to achieve infection rates of >80%, as detected by immune fluorescence staining of the viral nuclear protein 8 h after infection (data not shown). HUVEC support efficient and productive replication of influenza A viruses as shown by plaque assay of supernatants from infected endothelial cell cultures in comparison to A549, human lung epithelial cells, which are a standard cell culture model for influenza A virus infection (supplemental Fig. 1). Before initiation of the microarray analysis, we tested by real-time RT-PCR at which time point postinfection transcription of mRNAs of prototype genes, such as IFN-β and different ISGs, could be detected. Fig. 1 shows the *n*-fold mRNA expression levels normalized to uninfected cells. IFN-β mRNA expression reaches a maximum after 2 h while prototype ISGs, such as IFN-γ-inducible protein 10 (IP-10), OAS1, or MxA, are expressed in slightly delayed kinetics as expected (Fig. 1). Proinflammatory marker mRNAs like IL-8 or ICAM are induced in a similar kinetic (data not shown) although to a lesser extent. To minimize the effects of secondary infections, we decided to use a time point of 5 h after infection to harvest mRNA, which is well within the primary replication cycle of influenza viruses. We also decided to consider only those mRNAs to be regulated that showed at least a significant ($p \leq 0{,}05$) 2-fold change in expression levels compared with the control group. In former studies, this threshold guaranteed reliable results and avoided too much false positive results due to low signal-to-noise ratio (29, 30).

Analysis of the gene expression profile reveals that within 5 h after infection of HUVEC with the human H5N1 isolate A/Thailand/KAN-1/2004, 111 mRNAs were up-regulated and 38 genes are switched on compared with mock-infected control cells. Interestingly, >600 mRNAs were down-regulated or switched off upon H5N1 virus infection (data not shown). Although we cannot rule out a specific down-regulation of individual host mRNAs, it is more likely that the majority of mRNAs are down-regulated in consequence of unspecific 5′ cap snatching (38) or interference with processing of cellular RNAs by the viral NS1 (39).

To further characterize the genes induced upon infection with the H5N1 isolate, we grouped these genes into different functional clusters according to GO classification (www.geneontology.org/). About one-third of the depicted mRNAs belong to the GO clusters of inflammatory/immune response and chemotaxis (Fig. 2). This pattern matches perfectly with recent findings on infections of humans with HPAIV of the H5N1 subtype, which are accompanied by a massive systemic production of cytokines and chemokines, the so-called cytokine storm. Supplemental Table II shows a representative subset of mRNAs induced by H5N1 virus infection (noncharacterized gene products were not listed). Hits were grouped according to their GO annotated function and ranked according to their fold change expression. Consistently with the GO clustering, a major subset of H5N1-induced mRNAs are described to be associated either with the antiviral type 1 IFN response (e.g., OAS1, MxA) or inflammatory processes (IL-8, ICAM-1). The cytokine storm is a characteristic feature of systemic infections with highly pathogenic influenza viruses of the H5 subtype. To test whether the induction of proinflammatory cytokines and ISGs as well as the NF-κB dependence of this cellular response is limited to H5N1 isolates, we analyzed HUVEC infected with a low pathogenic isolate, WSN. Infection of HUVEC with 5 MOI resulted in moderate gene induction after 5 h compared with H5N1-infected HUVEC. Induction of IFN-β was ~20 times lower after WSN infection, which consequently results in lower induction of ISGs like Mx1 and OAS1, as demonstrated by real-time RT-PCR (supplemental Fig. 2). Likewise, production of proinflammatory cytokines and chemokines or endothelial surface markers was less pronounced or missing (e.g., IL-6, IL-8, or E-Selectin) in WSN-infected HUVEC in comparison to H5N1-infected HUVEC.

NF-κB is a crucial factor for expression of antiviral and immune modulatory cytokines and chemokines that are known to contribute to the antiviral status of a tissue and are involved in induction of cell survival/apoptosis (40, 41). To dissect the function of NF-κB in the primary host gene response, we introduced a dominant negative mutant of the inhibitor of κ B kinase 2 (IKK2KD) into HUVEC by retroviral gene transfer. This tool was already successfully used for specific and efficient inhibition of stimulus-induced NF-κB signaling in these cells (29, 30). Successful transduction of the transgene was verified by FACS measurement of coexpressed eGFP that was translated from the same bicistronic mRNA. Expression of the IKK2KD transgene (Fig. 3*A*) was further detected in Western blots from total cell lysates (Fig. 3*B*). TNF-α-induced IκBα degradation was markedly inhibited although not completely abolished in IKK2KD-expressing HUVEC (Fig. 3*B*).

The functional block of NF-κB signaling was verified by impaired expression of the NF-κB-dependent gene products IL-8 and MCP-1 16 h after stimulation with 2 ng/ml TNF-α (Fig. 3*C*). To

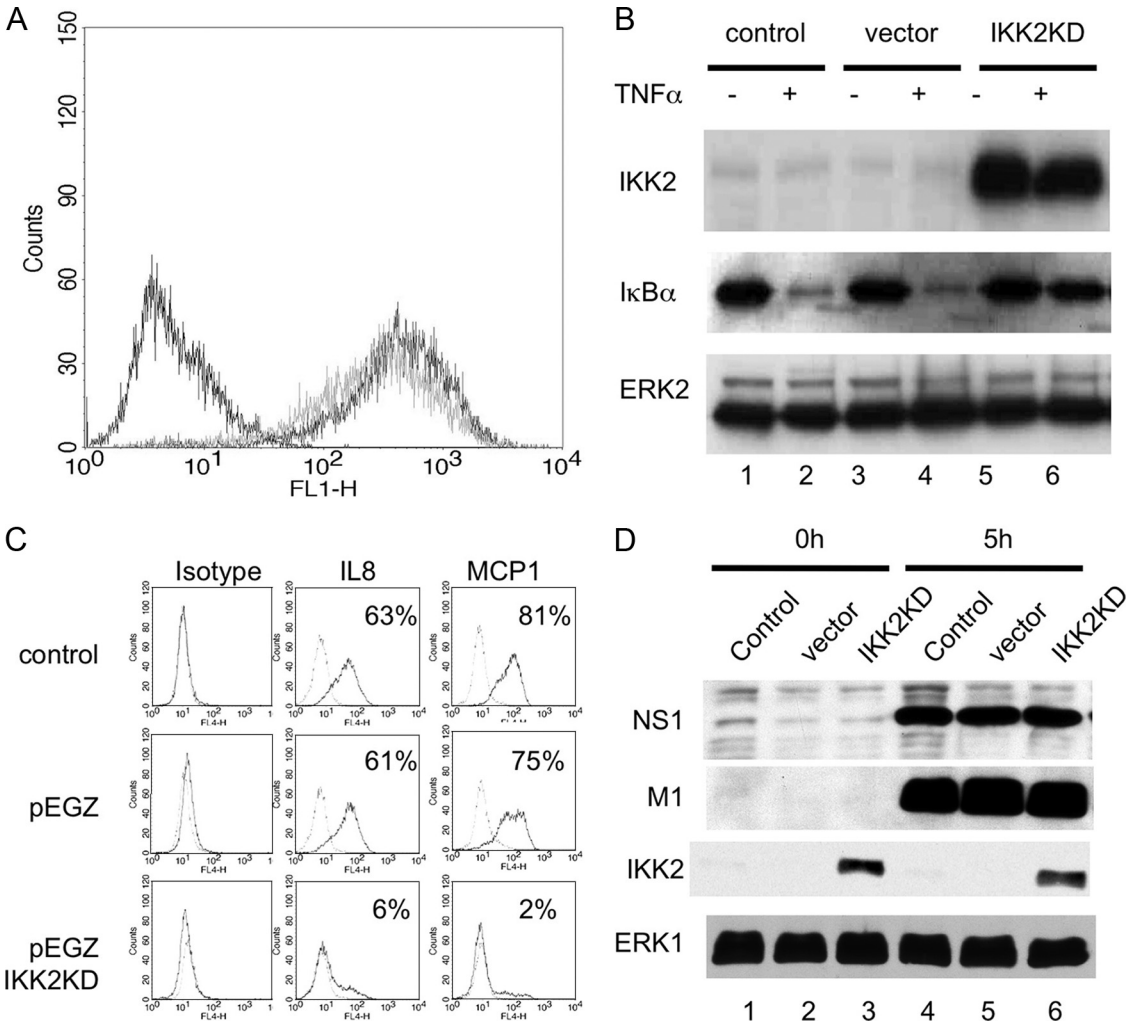

FIGURE 3. Block of NF-κB signaling by expression of a dominant negative IKK2 mutant. *A*, Retroviral transduction of HUVEC: eGFP reporter gene expression was measured by cytometric analysis. Fluorescence histograms of control cells, vector-transduced cells, and IKK2KD-expressing cells are depicted. Representative data are shown. *B*, Expression of IKK2KD was confirmed in Western Blot analysis (*upper panel*, *lanes 5* and *6*). TNF-α (2 ng/ml) induced degradation of IκBα (*middle panel*, *lanes 2*, *4*, and *6*) is blocked in the presence of IKK2KD. Equal loading was confirmed by detection of ERK2 (*lower panel*). *C*, Expression of IKK2KD blocks TNF-α-induced expression of NF-κB-dependent gene products. HUVEC were stimulated with 2 ng/ml TNF-α for 16 h in the presence of 25 mM monensin for 16 h. Accumulation of IL-8 and MCP-1 was detected by specific Abs and quantified by FACS analysis. Gray curves indicate untreated cells and black curves indicate TNF-α-treated cells. *D*, Western blot analysis of viral M1 and NS1 5 h p.i. in control cells (*lanes 1* and *4*), vector-transduced cells (*lanes 2* and *5*), and IKK2KD-expressing cells (*lanes 3* and *6*). IKK2KD expression was confirmed with specific Abs and equal loading was verified with Abs against ERK1.

exclude impaired virus propagation by NF-κB blockage in early stages of the replication cycle, we determined the expression of the viral NS1 and the matrix protein M1 5 h after infection, respectively. Viral protein levels were not altered in infected IKK2KD-expressing HUVEC (Fig. 3*D*), which is in accordance with earlier findings in other cell types (31, 42).

To determine the impact of NF-κB on the overall gene expression program induced by H5N1 infection, we performed mRNA array analysis of HUVEC infected with the H5N1 virus (MOI = 5) in the presence or absence of IKK2KD. As an initial comparative analysis of global gene expression profiles in wild-type, vector-transduced, or IKK2KD-expressing cells, we performed principal component analysis. This is a bioinformatic tool for efficient data reduction without loss of information (43). For that purpose, each gene profile is transformed to a vector according to the expression data of each gene in all experimental conditions used. This approach helps to illustrate the global variance by which different data sets correlate. The principle components of each individual infection experiment are depicted as vector clouds in a three-dimensional vector space (Fig. 4*A*). The clouds of each group of up-regulated mRNAs (e.g., control or H5N1 virus infected) perfectly localized together, indicating reliable reproduction of data within the triplicates. Moreover, a clear separation of the groups representing H5N1 virus-infected cells vs uninfected as well as IKK2KD-transduced cells is visible. The induced gene entities of vector-transduced cells perfectly match with the data from wild-type control cells. Comparison of vector, control, and IKK2KD-expressing HUVEC clearly shows that blocking of NF-κB signaling reduces the variance of H5N1-mediated expression changes compared with the gene profiles of uninfected control. However, inhibition only partially reverts the influenza A virus-induced gene spectrum (Fig. 4*A*), suggesting that either all genes are only partially dependent on NF-κB or that there is a more variable degree of NF-κB dependence, implying that there might be a distinct gene subset with no or only a slight requirement for the factor. A closer look on the gene patterns revealed that almost 90% of H5N1-induced mRNAs were NF-κB dependent (Fig. 4*B*), however, to a variable extent. Among these genes,

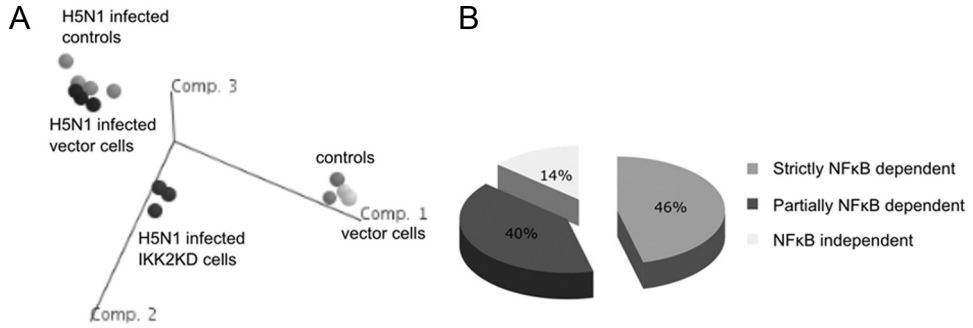

FIGURE 4. Influence of NF-κB block on H5N1 influenza virus-induced mRNAs: *A*, Principle component analysis of at least three individual experiments per settings. Vector clouds of control HUVEC, vector-transduced HUVEC, H5N1-infected control HUVEC, H5N1-infected vector-transduced HUVEC, and H5N1-infected IKK2KD-expressing HUVEC are depicted as indicated. Each vector cloud represents the up-regulated/switched on mRNAs of an independent experiment. *B*, Relative distribution of H5N1-induced mRNAs (percent) according to the NF-κB dependence determined by microarray analysis. Strictly NF-κB-dependent mRNAs show expression levels below 2-fold in presence of IKK2KD. A more detailed analysis is listed in supplemental Table S1.

46% were strictly NF-κB dependent (switched off in the presence of IKK2KD), while 40% were partially NF-κB dependent. In contrast, the expression of IKK2KD has no significant effect on the genes that are down-regulated or switched off upon influenza virus infection (data not shown). Strikingly, IKK2KD expression most severely influenced those mRNAs that were massively induced or switched on by infection (supplemental Table S2). According to the microarray data, IFN-β itself belongs to those mRNAs that strictly need intact NF-κB signaling for their transcription (supplemental Table S2). To validate the microarray data, we performed real-time RT-PCR analysis for a subset of H5N1-induced mRNAs in the presence or absence of IKK2KD. All tested H5N1-induced mRNAs are at least partially down-regulated upon expression of IKK2KD as shown in supplemental Table S1. In parallel, we used a chemical inhibitor of the NF-κB pathway to validate the results obtained with the dominant negative IKK2 mutant in HUVEC. Like IKK2KD-transduced HUVEC, the BAY11-7085-treated HUVEC show massively impaired production of proinflammatory cytokines and chemokines upon H5N1 infection. Similar the expression of IFN-β was reduced 5-fold, which consequently resulted in impaired ISG levels (supplemental Fig. 3). This further underlined the importance of NF-κB for the primary gene expression response to influenza virus infection. Although HUVEC are a well-established and widely accepted model for studies of endothelial cell function, we validated our results in HMEC-1. In accordance with our findings in HUVEC, infection of HMEC-1 with H5N1 influenza A virus resulted in massive up-regulation of proinflammatory cytokines and ISGs (supplemental Fig. S4, *C* and *D*).

Since we proposed a distinct impact of NF-κB signaling in H5N1 influenza A virus-infected HUVEC, we were curious about the effects of IKK2KD expression on the gene profile induced by the low pathogenic control strain. Interestingly, we observed a reduced impact of NF-κB blockade on the already weak response to WSN infection, either by introduction of the dominant negative IKK2 or by addition of the pharmacological inhibitor BAY 11-7085. Although IFN-β mRNA induction was reduced by 5-fold in the presence of IKK2KD after H5N1 infection, we did not observe a significant reduction in WSN-infected HUVEC. In HMEC-1, we saw a similarly reduced dependence of the WSN-induced host response on NF-κB signaling (supplemental Figs. S2, *A* and *B*, S4, *C* and *D*, and S5*B*). A more direct comparison including statistical analysis of the two cell models and the NF-κB dependence of the host responses to the high pathogenic and low pathogenic isolate, respectively, are shown in supplemental Table IV). By Western blot, we could show that the low pathogenic WSN seems to replicate slower in HUVEC, which might explain the overall reduced host response (supplemental Fig. S5*A*). Taken together, these findings point to specific dependence of the H5N1-induced antiviral response on NF-κB signaling.

The observation that IFN-β is strongly dependent on NF-κB in H5N1-infected HUVEC prompted us to ask whether a portion of the identified NF-κB-regulated genes may be rather indirectly controlled by the factor via induction through the IFN-β signaling pathway. STAT1 phosphorylation is an immediate hallmark response to type I IFN. To test whether the IFN-β or other STAT1-activating cytokines are actually released as proteins from infected cells and reduced in IKK2KD-expressing cells, we performed conditioned medium experiments. Wild-type HUVEC or cells that were vector transduced or IKK2KD transduced were infected for 5 or 8 h, respectively. Supernatants were sterile filtered and transferred to untreated HUVEC for 15 min. Fig. 5 shows that the STAT1 phosphorylation at Tyr^{701} can be readily observed in HUVEC after addition of the conditioned supernatants from H5N1-infected wild-type cells (Fig. 5, *lanes 2*, *4*, and *6*, *upper panel*). Conditioned supernatants from uninfected HUVEC had no effect on STAT1 phosphorylation (Fig. 5, *lanes 1*, *3*, and *5*, *upper panel*). The specific inhibition of NF-κB signaling clearly results in reduced levels of phosphorylated STAT1 (Fig. 5, *lane 6*), which suggests a reduced production of type I IFNs in these cells. Thus, the secondary, autocrine type I IFN response is indirectly influenced by blocking NF-κB signaling.

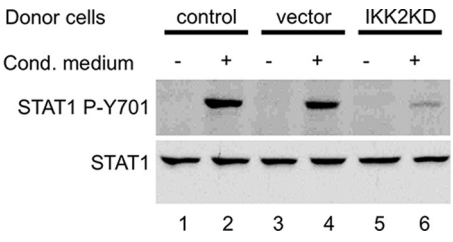

FIGURE 5. Influence of NF-κB block on H5N1 influenza virus-induced IFN expression: Western blot analysis of total lysates of HUVEC treated with conditioned medium from mock-infected control cells (*lanes 1*, *3*, and *5*) and H5N1-infected cells (5 MOI, 5 h) (*lanes 2*, *4*, and *6*). Donor cells were left untreated (*lanes 1* and *2*), transduced with empty vector (*lanes 3* and *4*), or pCFG5-IEGZ IKK2KD (*lanes 5* and *6*). STAT1 Tyr^{701} phosphorylation was detected 15 min after treatment with conditioned medium (*upper panel*). Equal loading was verified by detection of total STAT1 (*lower panel*).

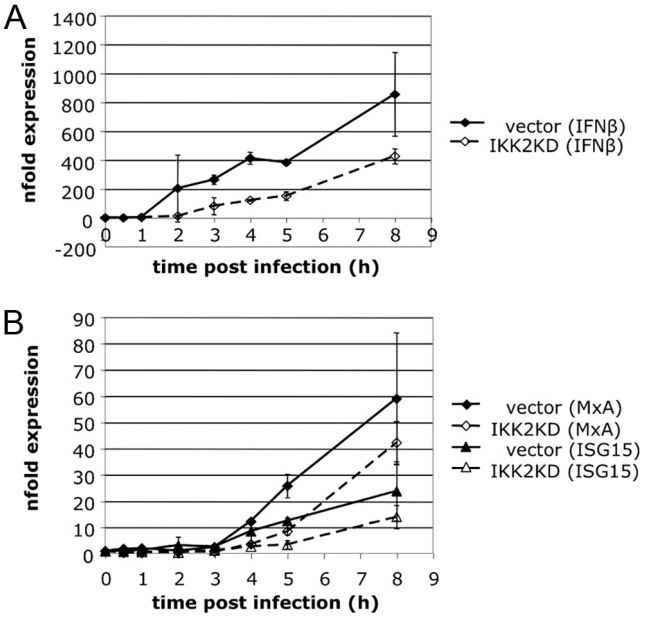

FIGURE 6. Time course of H5N1-induced mRNAs in the presence of IKK2KD. Induction of (*A*) IFN-β (♦ and ◇), (*B*) MxA (♦ and ◇), and ISG15 (▲ and △) mRNAs were measured after H5N1 infection in a time-dependent manner by highly specific real-time RT-PCR. Filled symbols/constant lines indicate vector-transduced cells and open symbols/dashed lines indicate IKK2KD-expressing cells. Mean *n*-fold expression (±SD) of three independent experiments normalized to controls is depicted.

Next, we addressed the question whether the observed effects of IKK2KD on ISG expression are exclusively due to indirect mechanisms via regulating IFN-β or additionally controlled by direct coregulatory functions of NF-κB in ISGF3- mediated transcription. In a time course of infection, transcriptional induction of IFN-β and ISGs is decreased in IKK2KD-expressing cells at any given time point (Fig. 6). This was a first indication that an initial block of IFN-β production is the main NF-κB-controlled event.

To further test whether NF-κB mainly functions as the inducer of IFN-β in concert with IRF3 or directly influences the expression of ISGs, we stimulated IKK2KD-expressing endothelial cells with IFN-β for 3 h. This time point was chosen to mimic the peak activity of virus-induced IFN-β production. IFN-β-induced expression of MxA and OAS1 mRNA, as prototype ISGs, are not influenced in the presence of the dominant negative IKK2 mutant, which means that the induction of distinct genes generally known as ISGs via IFNAR1 and JAK/STAT signaling does not necessarily depend on NF-κB (Fig. 7*A*). This experiment however does not mimic the overall spectrum of cytokines and chemokines that are released in parallel to IFN-β during an influenza infection and does not fully exclude a coregulatory role of NF-κB. To simulate a more realistic stimulation with the complete set of influenza virus-induced cytokines and chemokines, we again performed conditioned medium experiments. This time the untreated donor cells were infected for 3 h and conditioned supernatants of mock-treated or H5N1 influenza virus-infected cells were transferred to the vector- or IKK2KD-expressing reporter cells for 2 h. A potential transfer of infectious viruses was monitored by highly sensitive real-time PCR (44), which did not detect any viral matrix gene vRNA or mRNA in the reporter cells after 2 h (data not shown). Three hours after infection, the donor cells clearly produced IFN-β (Fig. 7*B*) on mRNA and protein level as shown by real-time PCR and detection of phosphorylated STAT1 in a Western blot (Fig. 7*C*) and indicated by the synthesis of ISGs, such as MxA and IP-10 (Fig. 7*B*). Interestingly, the reporter cells did not show a drastic increase in IFN-β mRNA levels (mean *n*-fold below 2-fold) although ISGs were readily transcribed. ISGs like Mx1 or OAS1 were induced by

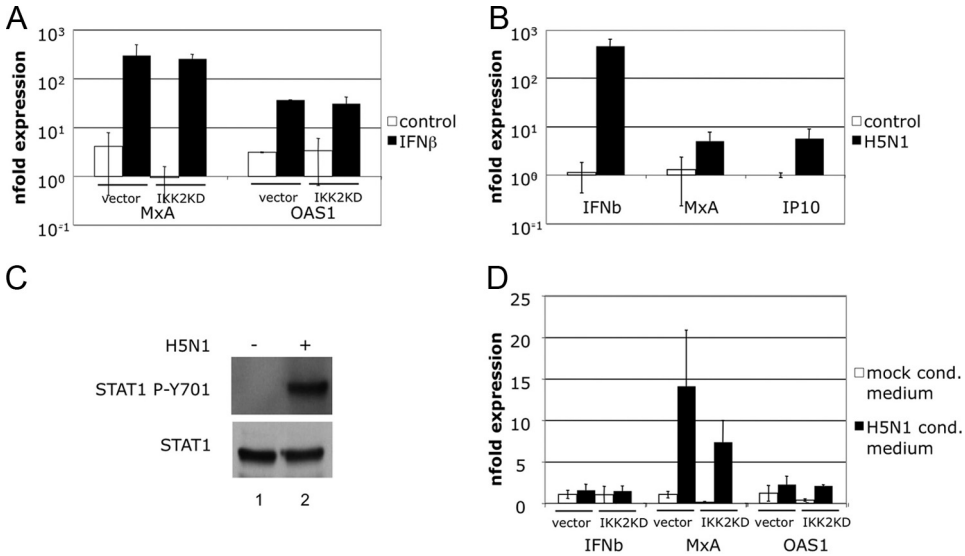

FIGURE 7. Influence of NF-κB block on IFN-β and H5N1-conditioned medium-mediated signaling. *A*, Vector-transduced HUVEC (*columns 1* and *2*) and IKK2KD-expressing HUVEC (*columns 3* and *4*) were treated for 3 h with 100 U/ml recombinant human IFN-β (■) or left untreated (□). Expression of MxA and OAS1 mRNA was analyzed by real-time RT-PCR. Mean *n*-fold expression (±SD) of three independent experiments normalized to controls are depicted. *B*, Donor cells for production of conditioned medium were mock infected (□) or infected with 5 MOI of H5N1 for 3 h (■). Levels of IFN-β, MxA, and IP-10 mRNA were detected by real-time PCR. Mean *n*-fold expression (±SD) of three independent experiments normalized to controls is depicted. *C*, STAT1 Tyr^{701} phosphorylation was detected from total lysates of mock-infected donor cells (*lane 1*, *upper panel*) or H5N1-infected donor cells (*lane 2, upper panel*). Equal loading was verified by detection of total STAT1 (*lower panel*). *D*, HUVEC were transduced with empty vector (*columns 1* and *2* of each panel) or pCFG5-IEGZ IKK2KD (*columns 3* and *4* of each panel) and treated with conditioned medium of mock-infected cells (□) or H5N1-infected cells (■) for 2 h. Levels of IFN-β, MxA, and OAS mRNAs were detected by real-time RT-PCR. Mean *n*-fold expression (±SD) of three independent experiments normalized to controls is depicted.

H5N1 virus-conditioned medium (Fig. 7*D*). However, this induction was much weaker compared with ISG induction 5 h postinfection (p.i.) with influenza A virus. After transfer of H5N1 virus-conditioned medium, the block of NF-κB signaling did not significantly alter the expression of OAS1 and Mx1. This underlines that NF-κB has no coregulatory function in induced expression of these prototype ISGs by IFN-β or other STAT-activating cytokines.

Pathway analysis of the complete H5N1-induced gene profile (supplemental Fig. 6) using Ingenuity Pathway Analysis 7.1 software generated significant interaction networks with p values up to 10^{-59} (only the most significant network is shown). It strengthened our initial findings that NF-κB and IFN-β harbor central positions in the H5N1 influenza virus-induced gene profile. Next, we wanted to define biological processes that might be differentially controlled by strictly or partially NF-κB-dependent gene products induced by H5N1 influenza virus. As shown in supplemental Fig. 7, the strictly NF-κB subset of mRNAs belongs to signaling pathways that open up to biological processes like IFN type I production (IFN-α and IFN-β) and activation of leukocytes and monocytes. In contrast, the remaining partially dependent mRNAs control biological processes like T cell activation. However, there is some overlap of biological functions belonging to the innate antiviral response in which both groups participate (supplemental Fig. 7). This is not surprising, since the initial antiviral response is a highly cross-linked process, with several self-enhancing signaling loops (45, 46).

Finally, we asked which transcription factors are additionally involved in regulation of H5N1-induced genes that were only partially blocked by IKK2KD expression. The promoter regions of up-regulated genes were examined by CARRIE analysis as described in *Materials and Methods*. Supplemental Table S3 depicts only those transcription factor binding sites which were found in at least four of six analyses with independent control groups of promoter regions of unregulated genes. Strikingly, the transcription factors that regulate type I IFN gene expression and that are subsequently involved in expression of ISGs (IRF1, IRF7, IRF8 (also known as ICSBP) and IRF9 (component of the ISRE-binding complex)), NF-κB, and to a lesser extent AP-1 ($p = 0.03$) are overrepresented in this set. It should be noted that IRF3 itself is considered within the group of the general IRF binding site, because the consensus site is overlapping to that of other IRF members. Although this in silico approach does not clarify which of these binding sites are functionally active, it provides a good overview of factors that may principally be involved.

The expression of several influenza virus-induced mRNAs was only partially blocked by expression of IKK2KD. This gave rise to the question which other factors besides NF-κB are relevant for the H5N1 virus-induced gene spectrum. AP-1, IRF3, and IRF7 also have been shown to be components of the IFN-β enhanceosome (18). IRF3 is constitutively present in the cytosol and migrates to the nucleus upon TBK/IKKε-dependent activation (15) while IRF7 is induced in response to IFN1R activation via the JAK/STAT pathway (47). Computational analysis of the promoter regions of partially NF-κB-dependent genes unraveled that binding sites for members of the IRF family, namely, IRF1, 7, 8 (ICSBP), 9 (enclosed in the ISRE element), and a general IRF binding site covering IRF3 are highly overrepresented (supplemental Table S2). This indicates that IRFs may drive the residual virus-induced gene expression observed upon blockade of NF-κB activity.

Discussion

Systemic infections with HPAIV of the H5N1 subtype are associated with a massive production of cytokines and chemokines. Endothelial cells have been shown to contribute to this hyperactivation of the immune system in human cases (11). It is still uncertain whether this so-called cytokine storm contributes to the pathogenesis of the virus (48) and so far it remains unsolved why H5N1 influenza A viruses allow the production of such a broad spectrum of antiviral-acting ISGs despite their efficient antagonizing strategies. Moreover, it is still an open question which signaling pathways are involved in the regulation of the H5N1 influenza virus-induced overexpression of cytokine and chemokine genes.

By global mRNA profiling, we could show here that infection of primary endothelial cells with highly pathogenic influenza A viruses of the H5N1 subtype has a severe impact on the transcriptome of the host cell. This is to our knowledge the first report on H5N1-induced gene expression in a human primary cell culture model. Moreover, it is the first study of highly pathogenic influenza A virus-infected endothelial cells. A recent study could show virus replication with a H3N2 influenza A virus isolate in HUVEC (49). Since endothelial cells line all blood vessels in mammalian organisms and are a major source of cytokines and chemokines, it is highly likely that these cells are involved in a systemic infection with HPAIV and contribute to the global host response. Case reports from human and animal infections with HPAIV strengthen this hypothesis (11, 50). These cells replicate the virus efficiently in cell culture and show a fast and profound host response upon infection. About 10 times more mRNAs were down than up-regulated. It is highly likely that the massive down-regulation of mRNAs is at least partially initiated by 5′ cap snatching and subsequent degradation of cellular mRNAs (38). Although further studies have to be performed to examine whether there is a specific down-regulation of antiviral-acting genes by H5N1 viruses, in this study we exclusively focused on the up-regulated mRNAs. The induced profile indeed shows a broad overlap to mRNA profiles from IFN-β-stimulated HUVEC (51). However, there are certain published ISGs which were not induced upon IFN type I stimulation in the study by Indraccolo et al., (51) but are expressed in the case of influenza A virus infection (e.g., IP-10, IP9, Mig, RANTES). A recent study underlined that certain genes, classified as ISGs, are expressed independently of, although often in parallel to type I IFNs (52).

In comparison to a previously performed transcriptional profile of an influenza A virus-infected lung epithelial cell line (53), we detect a much broader spectrum of induced mRNAs in endothelial cells including a broad spectrum of cytokines and chemokines. Interestingly, Geiss et al (53) did not detect IFN-β mRNA itself among the up-regulated mRNAs at 4 or 8 h after infection. This implicates a cell-type-specific response to influenza virus infection. Infection of HUVEC with a low pathogenic control isolate (WSN) resulted in a less pronounced antiviral and proinflammatory response.

It is well known that influenza virus infection induces activation of NF-κB signaling via different mechanisms (54, 55), although the function of NF-κB activation in the signaling network is discussed highly controversially. We and others could show that influenza A virus infection is associated with expression of NF-κB-dependent gene products such as proinflammatory cytokines in cell culture (6, 53, 56–58) and in vivo models (59–61). Work from our group and others revealed that influenza A virus replication induces and depends on activation of NF-κB (31, 42, 62, 63), most presumably by subsequent activation of caspases that enhance the passive release of viral ribonucleoproteins from the nucleus in late phases of viral replication. During preparation of this manuscript, Kumar et al. (64) additionally claimed that NF-κB signaling is important for the synthesis of viral RNA in influenza virus-infected cells. In contrast, studies in NF-κB knockout cells in recent

years showed that activation of NF-κB is either obsolete for production of ISGs upon virus infection (25) or even negatively regulates production of ISGs and suppresses viral replication (26). In contrast, the importance of NF-κB for the formation of the IFN-β enhanceosome is undeniable (24). These contradictory findings prompted us to examine which role NF-κB signaling plays in influenza A virus-induced cellular gene expression responses, with a focus on the induction by HPAIV of the H5N1 subtype, that are characterized by massive induction of cytokines and chemokines in vivo.

To block NF-κB signaling, we used a dominant negative mutant of IKK2. This approach has been successfully used previously to efficiently block NF-κB signaling for gene expression analysis (28–30). Selective inhibition of NF-κB signaling by the dominant negative mutant IKK2KD reverts at least partially the H5N1 virus-induced gene expression profile. The expression of 46% of the H5N1-induced mRNAs was completely blocked in the presence of the dominant negative IKK2 mutant, among them IFN-β mRNA itself. Among all microarray studies performed so far in this field, to our knowledge our analysis shows for the first time directly the obligatory dependence of influenza A virus-induced IFN-β production on functional NF-κB signaling. Approximately 43% of H5N1-induced mRNAs were partially regulated by NF-κB.

Surprisingly, the antiviral host response induced by the low pathogenic influenza isolate WSN did not show a similar dependence on NF-κB. This points to a distinct function of NF-κB signaling in the antiviral response against highly pathogenic influenza strains. Ongoing studies address the question if this could be a molecular reason for the observed cytokine storm in vivo.

In case of the H5N1-induced gene profile, almost all genes that were previously described to be ISGs were found among NF-κB-regulated genes. This suggests, that the inhibitory function of IKK2KD on the H5N1 virus-induced gene profile is to a large extent mediated indirectly via reduced expression levels of IFN-β. By analyzing the nature of the regulatory binding sites within the promoter regions of virus-induced genes, we could show that NF-κB is less significantly overrepresented than different IRFs. In combination with the drastic effect of IKK2KD expression exhibited on the overall gene expression profile, it is highly likely that NF-κB is essential for the direct regulation of a few crucial signaling molecules regulating the antiviral gene program. We could strengthen this hypothesis by analyzing the time course of IFN-β and ISG induction in the presence of IKK2KD. At any time point of the kinetic up to 8 h of infection, the H5N1 virus-induced production of IFN-β was reduced in cells expressing the dominant negative IKK2. In consequence, the production of ISGs was delayed in a similar fashion. Moreover, stimulation of IKK2KD-expressing HUVEC with IFN-β or treatment with virus-conditioned medium did not reveal a direct NF-κB dependence in ISG expression. Thus, functional NF-κB signaling in HUVEC is a critical bottleneck for an efficient antiviral response against H5N1 influenza viruses and indirectly affects ISG expression by initially controlling expression of IFN-β. This most likely occurs by enhancing the accessibility of the IFN-β promoter for IRF3 and AP-1 as the initial step for formation of the enhanceosome and subsequent IFN-β transcription (65). In support of that hypothesis, we could demonstrate by conditioned medium transfer experiments that the initial gene products induced by virus infection are capable of mediating an IKK2-independent ISG response. Furthermore, release of the factors appeared to be time dependent and to boost the antiviral response in cells infected for 5 h, in comparison to cells only treated for 2 h with conditioned medium (harvested 3 h p.i. from infected cells).

In contrast to our findings, studies in cells of NF-κB-deficient mice revealed an inhibitory function of NF-κB in IFN-mediated signaling (26). This would implicate that ISGs are expressed to a higher extent in IKK2KD-expressing cells compared with vector-transduced cells upon stimulation. With our approach, we could neither confirm this for IFN-β-induced ISGs nor for influenza A virus-induced ISGs. One reason for this discrepancy might be that there are compensatory effects in the knockout situation of mouse cells. In knockout cells, NF-κB signaling is completely blunted, while in our approach it is not. We manipulated the NF-κB pathway by introduction of a dominant negative isoform of IKK2, which allows residual signaling via the remaining endogenous IKK2 to a certain extent.

In contrast to NF-κB, IRFs were shown to be essential and sufficient for activation of the IFN-β promoter. Previous studies by Grandvoux et al. (66) used a constitutively active IRF3 mutant (IRF3–5D) to induce certain ISGs, such as OAS1 or ISG15. It would be of great interest to see to what extent the coexpression of a dominant negative IKK2 mutant reverts the effects. Our results would imply that indeed a basal activity of NF-κB is essential for IFN-β expression and consequently for the IRF3–5D-induced gene profile. This cooperation of NF-κB and IRF3 was described in a virus-independent model by Schafer et al. (67).

Recently, Lee et al. (6) showed in a macrophage model that p38 MAPK signaling is of major importance for the hypercytokinemia in H5N1 influenza virus-infected individuals (6). The p38 MAPK signaling pathway triggers a broad spectrum of transcription factors, among them activating transcription factor 2. Together with c-Jun, this transcription factor forms the AP-1 that binds to the IFN-β enhanceosome. By computational analysis, we found that AP-1 binding sites in H5N1-induced genes are much less prominent compared with the representation of IRF or NF-κB binding sites within the total spectrum of H5N1-activated genes. Moreover, the presence of AP-1 binding sites is enhanced in partially NF-κB-dependent mRNAs, indicating an NF-κB-independent regulatory function of AP-1.

Experimental approaches have to reveal whether p38 MAPK signaling like NF-κB is a bottleneck for the expression of essential regulatory molecules like AP-1 in the primary innate immune response against highly pathogenic influenza viruses, which would mean that NF-κB and AP-1 are essential and equivalent partners in backing up the IRF-mediated production of IFN-β.

In conclusion we could demonstrate here by pathway specific transcriptome analysis that the gene profile induced by highly pathogenic influenza A viruses largely depends on functional NF-κB signaling. Future studies will have to reveal whether this dependence on NF-κB signaling is the molecular reason for the massive production of cytokines and chemokines *in vivo*.

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Disclosures

The authors have no financial conflict of interest.

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