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Inhibition of influenza virus-induced NF-kappaB and Raf/MEK/ERK activation can reduce both virus titers and cytokine expression simultaneously *in vitro* and *in vivo*

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ABSTRACT

Influenza virus (IV) infection can cause severe pneumonia and death. Therapeutic actions are limited to vaccines and a few anti-viral drugs. These target viral functions thereby selecting resistant variants. During replication IV activates the Raf/MEK/ERK-cascade and the transcription factor NF-kappaB. Both result in virus supportive and anti-viral effects by promoting viral genome transport for virus assembly and by inducing expression of pro-inflammatory host factors. Apart from tissue damage caused by the virus lytic replication, an imbalanced overproduction of anti-viral cytokines can cause severe lung damage as observed in human H5-type IV infections. Recently we showed that inhibition of NF-kappaB activity reduces the virus titer *in vitro* and *in vivo*. We have now analyzed whether inhibition of these pathways, allows simultaneous reduction of virus titers and virus-induced cytokines. The results show that inhibition of either pathway indeed leads to decreased virus titers and cytokine expression. This was not only true for infected permanent cells or primary mouse alveolar epithelial cells, but also in infected mice. Hereby we demonstrate for the first time *in vitro* and *in vivo* that virus titers and pro-inflammatory cytokine expression can be modulated simultaneously. This could provide a new rationale of future therapeutic strategies to treat IV pneumonia.

1. Introduction

Influenza viruses (IV) are important human pathogens worldwide, causing annual epidemics and sometimes even pandemic outbreaks (Webster et al., 1992). In humans they normally infect the upper and lower respiratory tract. This can lead to severe pneumonia, acute respiratory distress syndrome (ARDS) and often to death (Chan et al., 2005; Cheung et al., 2002; Cox and Subbarao, 1999; Lee et al., 2007). The primary targets of IV in humans are epithelial cells of the respiratory organ, but macrophages and recruited leukocytes can also be infected (Julkunen et al., 2000). IV belongs to the family *Orthomyxoviridae* consisting of three genera A, B and C of which type A is the most pathogenic in humans. Type-A IV possesses a genome of eight single-stranded RNA segments of negative polarity. The three subunits (PB1, PB2, and PA) of the viral polymerase and the nucleoprotein (NP) together with the viral RNA form the replication and transcription active ribonucleoprotein (RNP) complexes (Lamb and Krug, 2001; Webster et al., 1992). The viral genome is replicated in the nucleus of the infected cell and therefore the RNPs have to undergo nuclear export to be packaged and produce infectious progeny virions. Among the viral proteins, the viral non-structural protein 1 (NS1) is a multifunctional protein playing a key role in the pathogenesis and virulence of IV (for review see Hale et al., 2008). By use of a recombinant IV with a *NS1* gene deletion (delNS1) it was demonstrated that NS1 suppresses virus-induced type-I interferon (IFN-alpha/beta) expression and thus greatly increases viral replication efficiency (Garcia-Sastre et al., 1998).

Upon IV infection of the respiratory tract, epithelial cells and leukocytes, the primary viral targets, activate transcription factors that will induce both innate and adaptive immune responses with production of cytokines and chemokines such as IFN, RANTES,

Abbreviations: PR8, A/PR/8/34 (H1N1); FPV, A/FPV/Bratislava/79 (H7N7); IV, influenza A virus; FFU, foci forming units; MOI, multiplicity of infection; A549, human alveolar epithelial cells.

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MCP-1, IL-8, MIP-1alpha, MIP-1beta, MIP-3alpha and IP-10 (Droebner et al., 2008; Hofmann et al., 1997; Julkunen et al., 2000; Ludwig et al., 2006; Matikainen et al., 2006; Osterlund et al., 2005). Even though the production of these factors can vary depending on the cell type they are important for rapid migration of leukocytes from the blood stream through the vascular endothelium to the site of inflammation. For example, IL-8 is associated with induction of neutrophil migration, MCP-1 with monocyte/macrophage recruitment and RANTES with macrophage and T cell recruitment (Julkunen et al., 2000; Matsukura et al., 1998), whereas mediators such as IFN-alpha/beta, IL-6 and TNF-alpha act as pro-inflammatory factors (Julkunen et al., 2000). IFN-alpha/beta is one of the most important cytokines in viral infections and affects many functions resulting in further recruitment of monocytes/macrophages, natural killer (NK) cells and T cells. (Garcia-Sastre, 2006; Julkunen et al., 2000; Samuel, 2001).

Recent reports demonstrated that not only the virus-induced tissue damage itself, but also the unbalanced production of antiviral cytokines (“cytokine burst”) can potentiate lung tissue damage and impair gas exchange as observed in severe infections with human IV and with highly pathogenic avian IV (HPAIV) of the H5N1 subtype (Beigel et al., 2005; Chan et al., 2005; Cheung et al., 2002; de Jong et al., 2006).

Mitogen-activated protein kinase (MAPK) signalling pathways transduce extra-cellular signals converting them into several cellular responses that affect cell growth, differentiation and development as well as inflammation and apoptosis (Zhang and Liu, 2002). We and others have shown that IV activates several signalling pathways in the infected cell, such as the Raf/MEK/ERK-, the JNK-, the p38-pathway and the nuclear transcription factor kappa B (NF-kappaB) (Flory et al., 2000; Kujime et al., 2000; Ludwig et al., 2001; Ludwig et al., 2006; Pahl and Baeuerle, 1995; Pleschka et al., 2001). Other pathways, such as PI3K and transcription factors IRF3/7 have also recently been found to be activated by IV (Ehrhardt et al., 2006; Hale et al., 2006). In the Raf/MEK/ERK pathway the kinase Raf becomes activated via PKC-alpha or Ras upon extra-cellular stimuli. By consecutive phosphorylation/activation the signal is transmitted via MEK (mitogen-activated protein kinase/ERK kinase) (Zhang and Liu, 2002), to ERK (extra-cellular-signal-regulated kinases) (Chang et al., 2003; Zhang and Liu, 2002). Activated ERK enters the nucleus and phosphorylates transcription factors, thereby altering gene expression (Chang et al., 2003). This pathway is activated late during the IV replication cycle and is essential for an efficient nuclear RNP export (Ludwig et al., 2004; Marjuki et al., 2006; Pleschka et al., 2001). We have also shown that specific MEK inhibitors, or dominant negative mutants of ERK and Raf, lead to impaired influenza A- and B virus replication in permanent cell lines (Ludwig et al., 2004; Pleschka et al., 2001). Besides its virus supportive function in the context of IV replication (Marjuki et al., 2006; Pleschka et al., 2001) the pathway has also been shown modulate IL-8 production (Kuderer et al., 2003).

Activation of NF-kappaB is one of the hallmarks of host cell response to invasion by different pathogens, including IV. NF-kappaB is activated upon transient expression of IV proteins, such as haemagglutinin (HA), nucleoprotein (NP) and matrix protein (M1) and accumulation of viral RNA species (Flory et al., 2000; Pahl and Baeuerle, 1995). One of the critical requirements of the canonical NF-kappaB activation is the activation of the IkappaB kinase (IKK) complex, which results in the phosphorylation of the NF-kappaB inhibitor IkappaBalpha, leading to its ubiquitination and subsequent degradation. The liberated NF-kappaB translocates into the nucleus to transactivate responsive genes (Schmitz et al., 2004) resulting in the expression of various anti-viral cytokines/chemokines, as well as other important genes related to host defence mechanisms (Hiscott et al., 2001). In IV infection NF-kappaB acts by inducing pro-apoptotic factors (Wurzer et al., 2004), and by activating caspases (Wurzer et al., 2003) leading to enhanced nuclear export of viral RNPs. NF-kappaB has also been shown to be responsible for an increase in the suppressor of cytokine signaling-3 (SOCS-3) which counteracts type I IFN induced gene expression, early in the viral replication cycle (Pauli et al., 2008), and to directly suppress IFN-stimulated gene (ISG) promoter regions (Wei et al., 2006), leading to an impaired antiviral response. NF-kappaB p65 has been shown to differentially regulate viral RNA synthesis (Kumar et al., 2008), and in studies with H5N1 subtype HPAIV, almost all virus-induced genes responsible for the overrepresented cytokines were NF-kappaB-dependent (Schmolke et al., 2009; Viemann et al., 2011). Previously we and others (Mazur et al., 2007; Nimmerjahn et al., 2004; Wurzer et al., 2004) demonstrated that NF-kappaB activity is essential for efficient IV propagation, as inhibition of NF-kappaB activation results in impaired nuclear RNP export and therefore in reduced virus titers (Mazur et al., 2007).

Specific inhibitors that block NF-kappaB activation (Mazur et al., 2007) or affect the Raf/MEK/ERK cascade via MEK inhibition (Favata et al., 1998) not only impair IV propagation but as they target cellular and not viral functions, do not lead to emergence of resistant virus variants (Ludwig et al., 2004; Mazur et al., 2007; Pleschka et al., 2001).

The aim of this study envisioned targeting ERK- and NF-kappaB-activation with the purpose of limiting viral spread and release of host defence mediators at the same time. Comparing results from different model systems, by use of a permanent human lung epithelial cell line (A549), primary murine alveolar epithelial cells (AEC), as well as a mouse *in vivo* IV infection model, and also using different human and avian IV strains (A/PR/8/34, H1N1 and A/FPV/Bratislava/79, H7N7), we show that virus titers and cytokine expression can be reduced simultaneously by inhibiting virus-induced NF-kappaB- or Raf/MEK/ERK pathway activity. Targeting these mechanisms may therefore limit both direct cytopathic effects due to viral replication as well as host defence-mediated immune responses, shown to be linked to the severe outcome of infections with human IV and HPAIV strains.

2. Materials and methods

2.1. Inhibitors, antibodies and reagents

Bay 11-7082 and U0126 (Calbiochem) were dissolved in DMSO, both to a stock concentration of 100 mM. Phospho-specific anti P-ERK antibody, anti-ERK2 sera, HRP-conjugated anti-mouse and anti-rabbit IgGs were all purchased from Santa Cruz Biotechnology. Antibody against influenza virus NP was obtained from Biodesign International. Texas-Red labelled anti-mouse IgG was bought from Sigma. The NF-kappaB p65 Transcription Factor Assay Kit (TransAM) was acquired from Active Motif.

2.2. Cells and viruses

Avian influenza virus A/FPV/Bratislava/79 (H7N7; FPV) and the human prototype strain A/Puerto-Rico/8/34 (H1N1; PR8) were obtained from the virus strain collection of the Institute of Medical Virology, Giessen, Germany. The PR8 mutant lacking the *NS1* gene (delNS1) was a kind gift from T. Wolff, Robert-Koch-Institute, Berlin, Germany.

Both, MDCK (Madine Darby Canine Kidney) cells and A549 (human alveolar epithelial carcinoma) cells were grown in DMEM (Dulbeco’s Modified Essential Medium) supplemented with 10% heat-inactivated foetal calf serum (FCS) and antibiotics (P/S: 100 U/ml penicillin and 0.1 mg/ml streptomycin) – complete DMEM (DMEM/FCS/P/S).

2.3. Mice

C57BL/6 (weight 18–21 g) were purchased from Charles River Laboratories (Sulzfeld, Germany). All experiments were approved by the local government committee of Giessen.

2.4. Virus infection and titration

For infection, cells were washed with PBS++ (PBS containing 1 mM $MgCl_2$, 0.9 mM $CaCl_2$), and incubated for 1 h at room temperature (RT) with the indicated multiplicity of infection (MOI) in PBS/BA/P/S (PBS++ containing 0.2% BSA, BA; 100 U/ml penicillin and 0.1 mg/ml streptomycin, P/S). After 1 h incubation, inoculum was aspirated and cells were incubated with DMEM/BA/P/S (DMEM, 0.2% bovine albumin and antibiotics) at 37 °C/5% CO_2. For the PR8 infection the DMEM/BA/P/S was supplemented with 1 μg/ml of trypsin (PAA). At the indicated time points post infection (p.i.) supernatants were collected to determine virus titers and cytokines, and cell lysis was performed to analyze specific protein production/activity.

For virus titration MDCK cells were grown in 96-well plates to 90% confluency, washed with PBS++ and infected with 10-fold dilutions of the respective supernatants in PBS/BA/P/S for 1 h at RT. The inoculum was aspirated and the cells were then incubated with methylcellulose (MC, Sigma) media (MEM supplemented with 1% P/S, 0.3% BA, 0.3% $NaHCO_3$, 1.5% MC, 0.01% DEAE Dextran) for 30–48 h. Then, plates were washed with PBS to remove the MC media, and incubated overnight in fixing solution (1% Triton X-100, 4% paraformaldehyde (PFA) in PBS). The plates were then washed 3× with PBS and incubated with primary antibody (mouse-anti-NP, 1:6000) for 1 h at RT. Again, plates were washed 3× and incubated with secondary antibody (anti-mouse IgG-HRP, 1:1000), 1 h at RT. The plates were washed again with PBS, as before, and stained with AEC staining kit (Sigma), air dried and scanned for counting.

2.5. Primary cell isolation

Briefly, mouse primary alveolar epithelial cells (AECs) were isolated as previously described (Corti et al., 1996), but with some modifications. C57/BL6 mice were euthanized by an overdose of isofluorane (Abbott, Wiesbaden, Germany) and exsanguinated by cutting the inferior vena cava. Lungs were then perfused with 20 ml sterile HBSS (Hanks BSS, PAA) via the right ventricle until they were optically free of blood. A small incision was made into the exposed trachea to insert a shortened 21-gauge cannula which was then firmly fixed. About 1.5 ml of sterile dispase (BD Biosciences) followed by 500 μl of sterile 1% low melting agarose in PBS (Sigma–Aldrich) were administered into the lungs. After 2 min of incubation, the lungs were removed and placed into a culture tube containing 2 ml of dispase for 40 min, at RT. The lungs were then transferred into a culture dish containing DMEM/2.5% HEPES buffer/0.01% DNAse (Serva), and the lung tissue was carefully dissected from the airways and large vessels. The cell suspension was successively filtered, resuspended in 10 ml of DMEM supplemented with 10% FCS and antibiotics, and incubated with biotinylated rat anti-mouse CD16/32, rat anti-mouse CD31 and rat anti-mouse CD45 mAbs (BD Pharmingen) for 30 min at 37 °C. Cells were then washed and incubated with streptavidin-linked MagneSphere Paramagnetic Particles (Promega) for 30 min at RT with gentle rocking, followed by magnetic separation of contaminating leukocytes and endothelial cells for 15 min. The purity of freshly isolated AECs contained in the supernatant was always >95%, as assessed by immunofluorescence staining with rabbit anti-mouse widespread cytokeratin mAb (Dako), and viability was consistently >90% (analyzed by trypan blue staining). The cells were plated on 24-well cell culture plates at a density of 4×10^5 cells/well and grown to 90% confluency for 2 days with DMEM supplemented with 10% FCS and antibiotics. On day 2 the cells were washed and serum starved with 0.2% FCS and left until day 3, upon which they were submitted to virus infection, as described above.

2.6. Viability assay

This was performed in two ways. A549 cells were grown on 96-well plates and left untreated or incubated with 150 μl/well medium containing inhibitors (25 μM of Bay 11-7082 or 50 μM of U0126) or 0.5 μl/ml DMSO (diluted in DMEM/BA), for 8, 10, 24, 48 and 72 h. This medium was then replaced by fresh medium for 1 h, to allow cell proliferation. After this, the medium was aspirated and 200 μl of MTT-mix (DMEM supplemented with 10% FCS and antibiotics containing 175 μg/ml tetrazolium bromide, Sigma) was added to each well. Cells were incubated further for 90 min at 37 °C and subsequently fixed with 4% PFA (in PBS) for 30 min at RT. Cells were dried and the tetrazolium crystals were dissolved by adding 200 μl of isopropanol to each well. The plates were shaken for 10 min and analyzed photometrically at 560 nm excitation in an enzyme-linked immunosorbent assay (ELISA) reader.

Otherwise, to analyze primary AEC viability, cells were submitted to WST-1 viability test (Roche). This assay can be performed in living cells, without any need of fixing the cells. Briefly, the medium was removed and stored in 96-well plates at RT and cells were washed with PBS and incubated for 1 h at 37 °C with media containing WST-1 reagent, according to the Manufacturer's instructions. Supernatant was replaced by the original medium and collected in 96-well plates for photometrically analysis at 450 nm excitation in an ELISA reader. This was repeated at all three time points (10, 24 and 32 h).

2.7. Western blots

Cell lysis and Western blots were performed as previously described (Marjuki et al., 2006). Briefly, cell lysate was cleared by centrifugation, and protein concentration was determined by Bradford assay before the protein was subjected to SDS–PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore Immobilon-P transfer membranes, Roth). Membranes were incubated with a phospho-specific anti P-ERK mAb (1:500) diluted in blocking buffer (5% non-fat dry milk in TBS/Tween (20 mM Tris–HCl pH 7.6, 140 mM NaCl, 0.05% Tween 20)). After stripping (Roth) bound antibodies and washing in TBS/Tween buffer, total ERK2 was detected using anti-ERK2 antisera (1:500) for 1 h at RT. Proteins recognized by primary antibodies were further analyzed with peroxidase-coupled, species-specific HRP-conjugated anti-mouse monoclonal secondary antibodies (1:1000) and a standard enhanced chemiluminescence reaction (Amersham Biosciences). Quantification of specific bands was done with the PC-BAS software package (Fuji).

2.8. NF-kappaB analysis

NF-kappaB activity was measured with an ELISA based high-throughput screening system (TransAM kit, ActiveMotif) in which immobilized oligonucleotides containing an NF-kappaB (5′-GGACT TTCC-3′) consensus binding sequence detects only the active form of NF-kappaB that will bind to this site. NF-kappaB dimers can only bind DNA after activation and translocation into the nucleus therefore, only NF-kappaB dimers that can bind the consensus sequence are measured in the DNA–protein binding assay and inactive cytosolic NF-kappaB will not cause any signal. The primary antibodies used then to detect bound NF-kappaB recognize an epitope on p65 that will only be accessible when NF-kappaB is active and

bound to the target DNA. A secondary HRP-conjugated antibody is used for colorimetric analysis via spectrophotometry.

In our study, lysates were prepared from infected and treated A549 cells, as mentioned before, and collected for further analysis. The assay was performed according to the Manufacturer's protocol and analyzed by ELISA reader at 450 nm wavelength.

2.9. Cytokine analysis

Supernatants from infected and control samples were collected at the respective times and analyzed for cytokine expression. Human cytokines (IL-8, IL-6, MCP-1, and RANTES) and mouse cytokines (KC, IL-6, MCP-1, and RANTES) were analyzed by multiplex cytokine array kits (BioRad, Invitrogen) according to the Manufacturer's instructions. For both human and mouse IFN-beta analysis the supernatants were measured with an ELISA kit (Invitrogen), according to the Manufacturer's instructions. Mouse TNF-alpha was measured in bronchoalveolar lavage (BAL) with the Opteia set mouse TNF-alpha kit (BD Biosciences).

2.10. Confocal laser scanning microscopy and immunofluorescence assay (IFA)

A549 cells or AECs were grown on glass cover-slips and infected as indicated, incubated in medium with or without Bay 11-7082 or U0126 as indicated, washed with PBS at the indicated time points p.i., and fixed with 4% paraformaldehyde (in PBS) overnight at 4 °C. Cells were then washed 3× with PBS and incubated with PBS, 1% Triton X-100 for 45 min at RT. Then the cells were incubated with the mouse anti-NP antibody (1:200). After additional washes cells were incubated with Texas Red-labelled goat anti-mouse IgG (1:200, Sigma) in PBS/3% bovine serum albumin for 1 h, and after three washes further incubated with DAPI (Roth) and mounted as described (Pleschka et al., 2001). Fluorescence was visualized with a TCS SP5 confocal laser scanning microscope (Leica).

2.11. In vivo experiments

Mice were infected by intra-tracheal administration of 500 PFU of PR8 diluted in sterile PBS in a total volume of 70 μl or mock-infected with 70 μl sterile PBS (controls) and treated intra-peritoneally with either the inhibitors (Bay 11-7082 or U0126) or solvent controls (DMSO or DMSO/Cremophor EL) 24 h before infection, on the day of infection and every 24 h thereafter. Cremophor EL (CremEL, Fluka) serves as a non-ionic solubilizer and emulsifier to solve hydrophobic drugs into aqueous solutions allowing the drug to be more readily absorbed and was used to minimize total amount of DMSO administration. U0126 dissolved in DMSO/CremEL (1:4 dilution) was used at a concentration of 30 mg/kg and applied via intra-peritoneal injection to mice. The IKK inhibitor, Bay 11-7082 dissolved in DMSO, was administered at a concentration of 8.2 mg/kg mouse in the same way as the U0126.

Mice were sacrificed on day 2 or 5 p.i. by an overdose of isofluorane. For the bronchoalveolar lavage (BAL), the trachea was exposed, and a small incision was made to insert a 21-gauge cannula which was then firmly fixed. The lungs were then washed with 1 ml (2 × 500 μl 2 mM EDTA in PBS) collected and analyzed for virus titers and cytokines. All samples were kept at −80 °C, until further analysis.

2.12. Statistical analysis

The results correspond to the mean ± SD of the indicated experiments. The statistical significance of differences between the indicated groups was tested using the unpaired Student's *t* test with a threshold of *p*: *<0.05; **<0.01; and ***<0.001.

3. Results

3.1. Inhibition of influenza virus-induced Raf/MEK/ERK signal cascade and of NF-kappaB activation decreases virus titers as well as cytokine/chemokine expression in A549 cells

Activation of both, Raf/MEK/ERK signalling and NF-kappaB leads to expression of several cytokines (Bernasconi et al., 2005; Kuderer et al., 2003; Osterlund et al., 2005). Since IV activates these pathways we investigated whether their inhibition would affect virus-induced cytokine production.

Firstly we analyzed the correlation between virus-induced signalling and virus-induced cytokine release in a permanent cell culture. To this point we determined NF-kappaB-activation, Raf/MEK/ERK cascade induction, virus titers and cytokine production in human alveolar epithelial (A549) cells infected with either FPV or PR8 (MOI = 1), with or without inhibitor treatment. NF-kappaB activity was monitored at 6 and 8 h post infection (p.i.). Viral NF-kappaB activation was found to be significantly decreased upon treatment with the irreversible inhibitor of IkappaBalpha phosphorylation, Bay 11-7082, in FPV- (∼60%, Fig. 1A) and PR8-infected cells (∼70%, Fig. 1B). As for the Raf/MEK/ERK pathway, a gradual increase in ERK activation over time induced by both viruses was dramatically reduced upon treatment with the specific MEK inhibitor U0126. For infection with FPV a reduction by 60–80% (Fig. 2A) and for infection with PR8 a reduction by 85–95% was observed (Fig. 2B). Prior to the application of the inhibitors in the individual experiments the concentrations used were proved to be non-toxic via MTT cell viability assay (data not shown).

Confirming earlier findings (Mazur et al., 2007; Nimmerjahn et al., 2004; Pleschka et al., 2001; Wurzer et al., 2004), inhibition of the aforementioned pathways concomitantly led to a drastic reduction of infectious virions. As depicted in Fig. 1C and D, NF-kappaB inhibition by Bay 11-7082 resulted in a decline of virus titers of FPV by 70–50% and of PR8 by 65–80%. MEK inhibition by U0126 effectively reduced titers of FPV by 75–60% (Fig. 2C) and of PR8 by 65% (Fig. 2D).

To elucidate the effect of the specific inhibitors on virus-induced cytokine/chemokine secretion, supernatants collected at 10 h p.i. from A549 cells infected with FPV and PR8 (MOI = 1) treated with or without inhibitors, were analyzed for IL-8, MCP-1, IL-6 and RANTES levels by multiplex immunoarrays. The rationale to choose these factors is based upon our own previous study showing that PR8 infection of primary murine alveolar epithelial cells (AEC) and resident murine alveolar macrophages strongly induced the release of monocyte chemoattractants MCP-1 and RANTES and elicits a basal-to-apical monocyte transepithelial migration (Herold et al., 2006). Others have shown that the neutrophil chemoattractant IL-8 is in part regulated by the Raf/MEK/ERK cascade in A549 cells (Kuderer et al., 2003) and IL-6 was shown to be up-regulated in IV infections (Guillot et al., 2005; Szretter et al., 2007) which seems to depend on the Raf/MEK/ERK pathway (Chakrabarty et al., 2007; Moon et al., 2008).

We found that the expression profiles of cytokines/chemokines at 10 h p.i. varied depending on the virus used. FPV infection had no effect on IL-8, MCP-1 and IL-6 release, but led to an increase in RANTES (Fig. 3A–D) production, whereas PR8 infection induced IL-8 and IL-6 expression. Consistent for all results, though, was that both inhibitors were able to lessen cytokine/chemokine production (Table 1). In the case of IL-8, U0126 was more effective than Bay 11-7082. IFN-beta secretion measured at 24 h p.i. could not be detected upon FPV- and PR8 infection. In contrast, the delNS1 virus (a PR8 virus with a deleted *NS1* gene) was able to efficiently stimulate secretion of this cytokine (2867 pg/ml – data not shown).

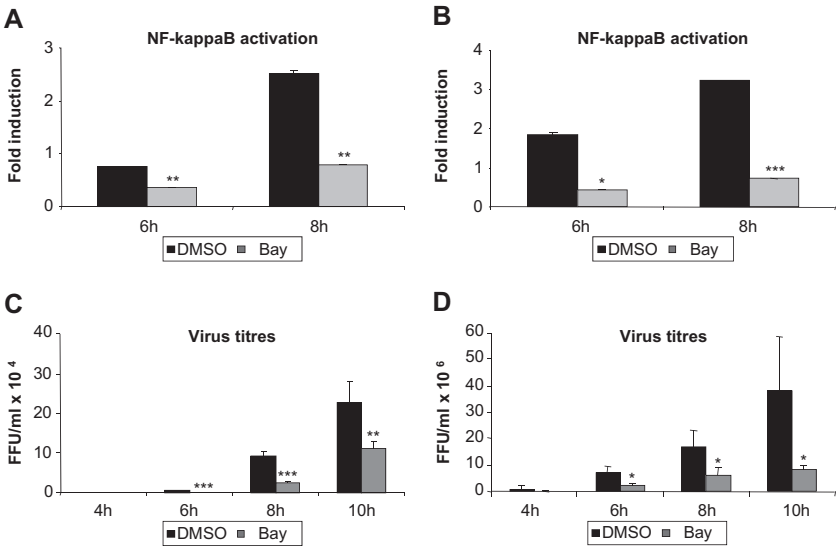

Fig. 1. Virus-induced NF-kappaB activity and virus titers are reduced by IKK inhibition. A549 cells were infected with FPV (A and C) or PR8 (B and D) (MOI = 1) and incubated with DMSO or Bay 11-7082 (25 μM). At time points indicated cell lysates were analyzed for NF-kappaB activity (TransAM). The supernatants collected at the indicated time points were analyzed for virus titers. The results are representative of three independent experiments. *p* Values (*<0.05; **<0.01; ***<0.001) are given in comparison to infected cells without inhibitor treatment.

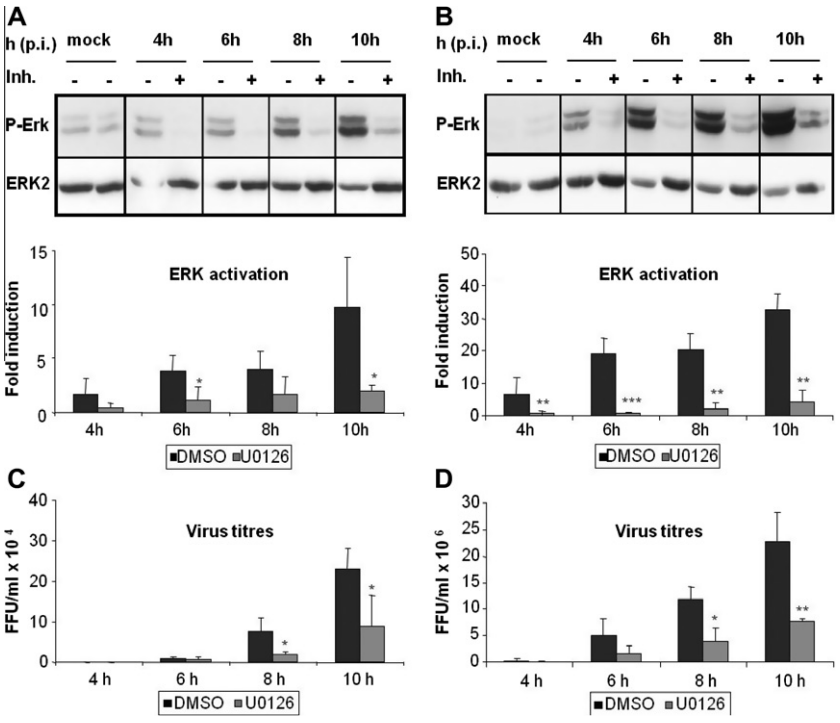

Fig. 2. Virus-induced ERK activity and virus titers are reduced by MEK inhibition. A549 cells were infected with FPV (A and C) or PR8 (B and D) (MOI = 1) and incubated with DMSO or with U0126 (50 μM). At the respective time points (4, 6, 8, and 10 h) cell lysates were analyzed for ERK activation (Western blot), using phospho-specific mAB (P-ERK). Respective bands of three independent experiments were quantified and relative ERK activation was calculated and normalized to the loading control (ERK2). The supernatants collected at the indicated time points were analyzed for virus titers. *p* Values (*<0.05; **<0.01; ***<0.001) are given in comparison to infected cells without inhibitor treatment.

3.2. Bay 11-7082 and U0126 decrease virus titers and cytokines/chemokines in mouse primary AECs

After we demonstrated that inhibition of virus-induced ERK- and NF-kappaB activity not only decreases virus titers, but at the same time reduced the amount of important pro-inflammatory cytokines/chemokines in a permanent cell line we aimed to confirm these findings in murine primary alveolar epithelial cells (AECs) representing target cells of IV lung infection in a relevant model. Therefore, AECs were infected with FPV (MOI = 0.1) or PR8 (MOI = 0.01) and treated at non-toxic concentrations (data not shown) with solvent (DMSO), Bay 11-7082 (15 μM) or U0126 (40 μM) for different time points. Supernatants were analyzed for virus titers 24 h and 32 h p.i. as well as cytokine/chemokine production (10 h p.i.). Confirming the results obtained from A549 cells, both inhibitors efficiently and significantly decreased virus titers

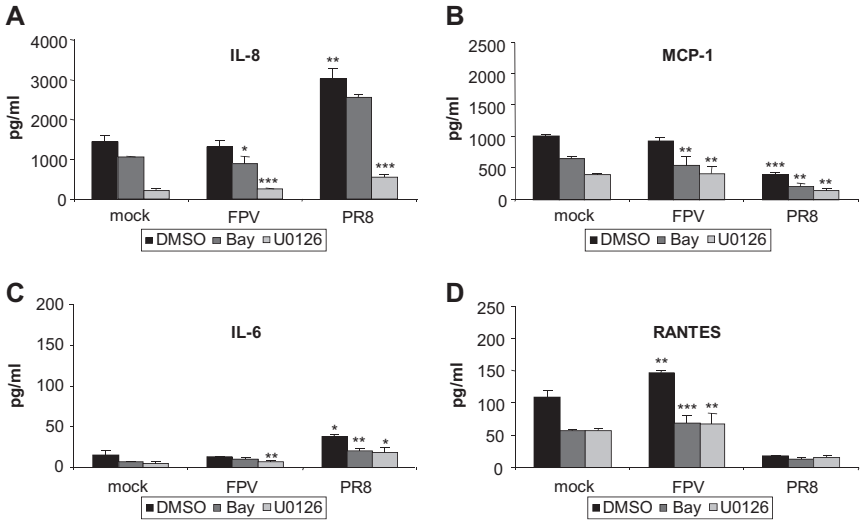

Fig. 3. Virus-induced cytokine release is decreased upon inhibitor treatment. A549 cells were infected with FPV, PR8 or delNS1 (MOI = 1) and treated with or without the MEK inhibitor (U0126) or the IKK inhibitor (Bay 11-7082). Supernatants sampled at the indicated time points were analyzed for cytokine/chemokine expression (multiplex immunoassay): IL-8 (A), MCP-1 (B), IL-6 (C) and RANTES (D). All groups were assayed in triplicates. *p* Values (*<0.05; **<0.01; ***<0.001) are given in comparison to mock vs. infected or infected (−) inhibitor vs. infected (+) inhibitor treatment.

Table 1
Summary of reduction of virus titers, cytokine/chemokine by the specific inhibitors.

System	Virus	Inhibitor	Titre	IL-8/KC	MCP-1	IL-6	RANTES	IFN-beta	TNF-alpha
A549 cells	FPV	Bay	++	+	++	∼	+++	ND	NA
		U0126	+	+++	++	++	++	ND	NA
	PR8	Bay	+	∼	++	++	∼	ND	NA
		U0126	++	+++	++	+	∼	ND	NA
Primary AECs	FPV	Bay	++	ND	++	+++	∼	+	NA
		U0126	++	ND	++	+++	∼	+	NA
	PR8	Bay	++	ND	++	++	∼	∼	NA
		U0126	++	ND	+	++	∼	∼	NA
C57BL/6 mice	PR8	Bay	+	∼	++	+	+	+	∼
		U0126	++	∼	∼	∼	+	∼	+

A549, AECs or C57BL/6 mice were infected with either FPV or PR8 virus and treated with inhibitors Bay or U0126. This table shows the summary of the effect of the applied inhibitors in the different *in vitro* or *in vivo* systems on virus titers as well as cytokine/chemokine release.
+++, Extremely effective; ++, very effective; +, effective; ∼, tendency; -, no effect; ND, not detected; NA, not analysed.

from AECs. Bay 11-7082 by 60–40% for FPV infection (Fig. 4A) and 75–85% for PR8 infection (Fig. 4B), and U0126 reduced FPV titers by 60% and PR8 titers by 85%. This demonstrates that activity of NF-kappaB as well as of the Raf/MEK/ERK pathway is also important for viral propagation in primary alveolar epithelial cells.

Whereas FPV infection slightly increased RANTES (Fig. 4C–F) production 10 h p.i., it reduced KC (the murine analogue of the human IL-8) and had no effect on MCP-1 or IL-6 secretion. PR8 had no effect on RANTES induction and led to a pronounced KC reduction, whereas MCP-1 was slightly increased. PR8 also significantly caused IL-6 release, as compared to mock-infected controls. Again, both inhibitors lowered cytokine production induced by infection with both viruses (Fig. 4A–F and Table 1). Evaluation of IFN-beta release from primary cells shown in Fig. 4G demonstrated that FPV infection led to significant IFN-beta production, whereas PR8 infection only slightly induced increase of this cytokine. Once more, the IFN-beta levels were reduced by inhibitor treatment. The control infection with delNS1 virus promoted very high IFN-beta secretion (data not shown).

3.3. IKK- and MEK-inhibition efficiently block FPV- and PR8-induced nuclear RNP export in AECs

Both pathway inhibitors had been previously shown to lead to nuclear RNP retention in different cells such as A549 cells and hence reduced virus titers (Mazur et al., 2007; Pleschka et al., 2001). To exclude that this effect was exceptional to permanent cell lines, we decided to confirm these findings in AECs. Primary mouse AECs were therefore infected with either FPV or PR8 (MOI = 0.2 and 0.02) and treated with either DMSO (solvent), Bay 11-7082 (15 μM) or U0126 (40 μM) for 10 h p.i. Immunofluorescence analysis of the intra-cellular RNP localization demonstrates nuclear RNPs export in AECs, but upon treatment with either inhibitor, viral RNPs were mainly observed in the nucleus (Fig. 5). Taken together, the results demonstrate that the effect of impaired nuclear RNP export and the resulting decrease in viral titers by inhibition of the Raf/MEK/ERK and NF-kappaB pathways also applies in primary alveolar epithelial cells and therefore likely reflects the potential mode of action in the infected lung.

3.4. Bay 11-7082 and U0126 decrease virus titers and alveolar cytokine release in C57BL/6 mice

Our results so far suggested that the hypothesis to decrease both virus titers and virus-induced cytokine secretion was valid in cell culture. We therefore aimed to test our approach in an *in vivo* "proof of concept" study. To this point we used intratracheal PR8 infection as a widely employed IV infection model resulting in severe pneumonia and acute lung injury in mice (Buchweitz et al., 2007; Herold et al., 2008; Pica et al., 2000). Mice

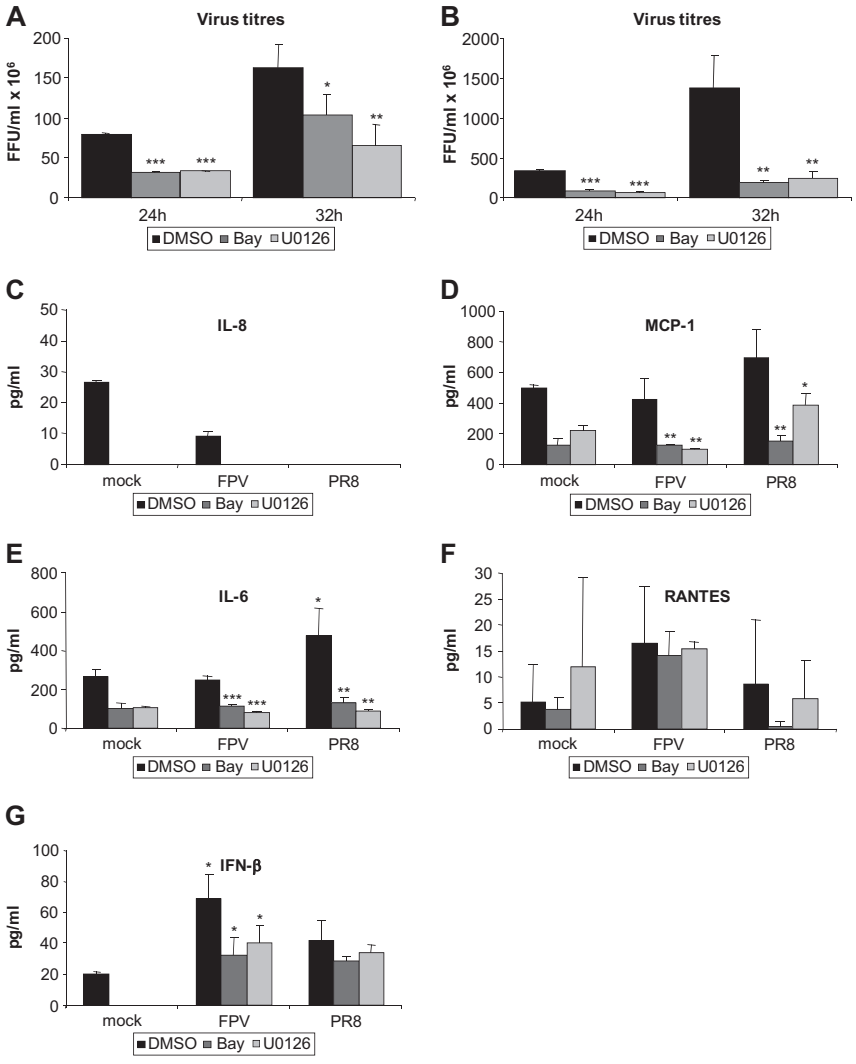

Fig. 4. Virus titers and cytokines levels in primary alveolar epithelial cells upon inhibitor treatment. Primary murine AECs were infected with either FPV (MOI = 0.1) or PR8 (MOI = 0.01), and treated with DMSO (solvent), Bay 11-7082 (15 μM) or U0126 (40 μM) and incubated for 10, 24 and 32 h. Supernatants collected at 24 h and 32 h p.i. were analyzed for virus titers (FPV (A) and PR8 (B)). All groups were assayed in triplicates. *p* Values (*<0.05; **<0.01; ***<0.001) are given in comparison to cells infected (−) inhibitor vs. infected (+) inhibitor treatment. Supernatants taken at 10 h p.i. were analyzed for KC (C), MCP-1 (D), IL-6 (E) and RANTES (F) (multiplex immunoassay). Supernatants taken at 24 h p.i. were additionally analyzed for IFN-beta (G) (ELISA). All groups were assayed in triplicates. *p* Values (*<0.05; **<0.01; ***<0.001) are given in comparison to mock vs. infected or infected (−) inhibitor vs. infected (+) inhibitor treatment.

were treated intra-peritoneally with either solvent or inhibitor (Bay 11-7082 or U0126) 1 day before infection and every 24 h for up to 5 days p.i.. Virus titers determined at day 2 and 5 p.i. in bronchoalveolar lavage fluid (BAL) recovered from infected mice showed a significant decrease upon inhibitor treatment (Fig. 6). PR8 propagation was effectively reduced by 60% at both time points upon Bay 11-7082 treatment and by 95% (day 2) and 50% (day 5) upon U0126 treatment. This confirms earlier reports for relevance of the NF-kappaB activity (Wurzer et al., 2004), and demonstrates for the first time the *in vivo* significance of the Raf/MEK/ERK cascade for IV replication. Therefore, both activities are crucial for effective IV propagation *in vivo*.

TNF-alpha is a major pro-inflammatory mediator released mainly from alveolar macrophages and an important cytokine induced during IV infections (Cheung et al., 2002; Lee et al., 2005). TNF-alpha was increased in the BAL fluid of PR8-infected, solvent treated mice as compared to mock-infected mice 2 days p.i. (Fig. 7A). Administration of both inhibitors to PR8-infected mice decreased TNF-alpha production (Table 1). When analyzing IFN-beta secretion (Fig. 7B and Table 1), the results showed a significant increase in infected, solvent treated mice as compared to control. Only Bay 11-7082-treated mice, however, had significantly lower amounts of this cytokine. Even though U0126 exerted no significant effect on IFN-beta release, a tendency in reducing IFN-beta amounts was seen.

KC (Fig. 7C) in BAL 5 days p.i. was increased in infected, solvent treated mice as compared to mock-infected mice. Although there was apparently no significant decrease upon inhibitor treatment in both groups (Bay 11-7082 and U0126) a tendency for reduction was noted. MCP-1, IL-6 and RANTES (Fig. 7D–F) were significantly increased in infected mice as compared to mock-infected groups. Bay 11-7082 inhibitor treatment effectively reduced this induction. However, whereas MCP-1, IL-6 and RANTES were significantly reduced by Bay 11-7082 only in the case of RANTES a significant effect of U0126 treatment was observed. Nevertheless, both inhibitors decreased the production of the cytokines analyzed. Taken together, NF-κB- and MEK inhibition significantly impaired IV replication in the lung and reduced PR8-induced alveolar cytokine release in infected C57BL/6 mice.

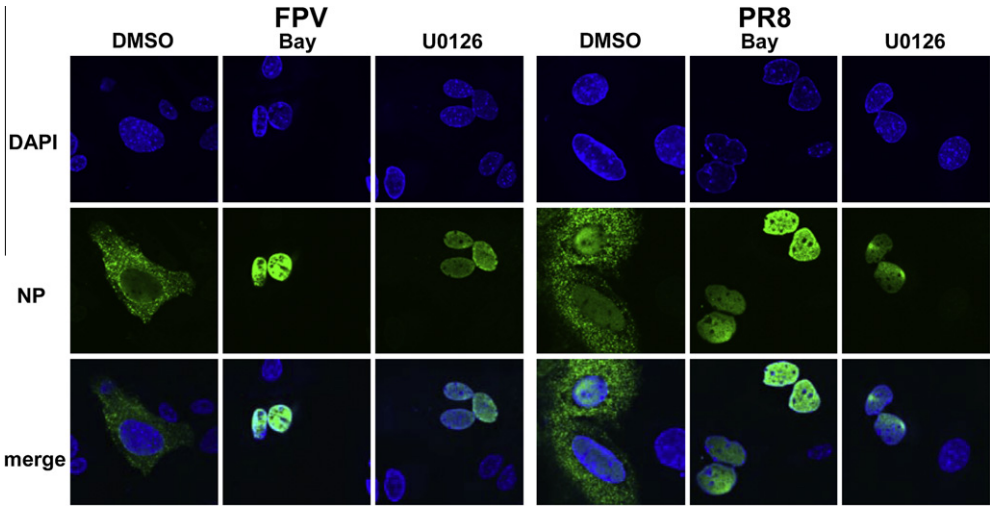

Fig. 5. Nuclear RNP export in primary alveolar epithelial cells. AECs were infected with FPV (MOI = 0.2) or PR8 (MOI = 0.02), and incubated for 10 h and treated with DMSO, Bay 11-7082 (15 μM) or U0126 (40 μM). At 10 h p.i. cells were fixed and analyzed for RNP localization using anti-NP specific mAb in immunofluorescence assay and confocal laser scanning microscopy. Representative pictures of three independent experiments are shown.

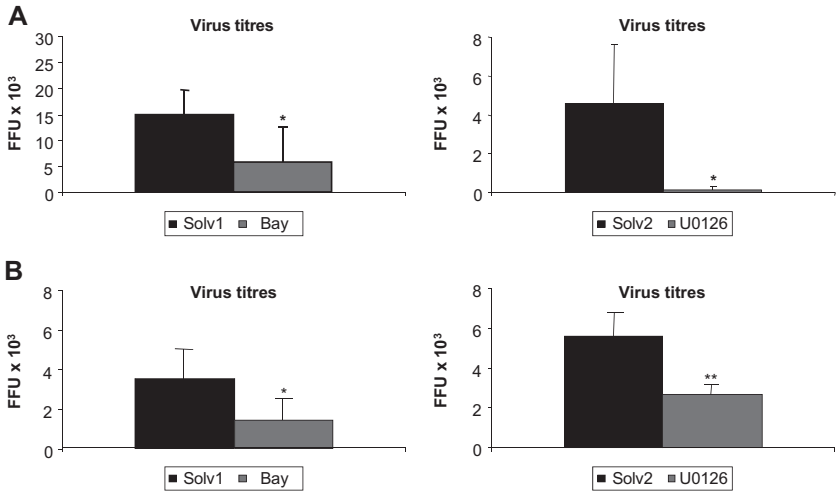

Fig. 6. Virus titers in bronchoalveolar lavage (BAL) fluid obtained from PR8 infected mice treated with U0126 or Bay 11-7082. Mice were infected intra-tracheally with PR8 (500 PFU in 70 μl PBS) or mock-infected (70 μl PBS). Solvent (DMSO or DMSO/CremEL), Bay 11-7082 or U0126 were administered intraperitoneally every 24 h starting 24 h before infection. Mice were sacrificed on day 2 (A) or day 5 (B) p.i., and BAL was performed and virus titers were determined. *p* Values (*<0.05; **<0.01; ***<0.001) of treated mice are given in comparison to solvent treated mice. All groups were assayed in quadruplicate (day 2 p.i.) or triplicate (day 5 p.i.).

4. Discussion

In the present study we intended to elucidate (i) the possibility of a simultaneous modulation of IV propagation and cytokine/chemokine production as well as (ii) estimating an *in vivo* relevance for a potential therapeutic approach. To this point, we were able to established for the first time that inhibition of the virus-induced Raf/MEK/ERK cascade and NF-kappaB activation, which both represent mechanisms required for efficient IV propagation, not only effectively impaired virus replication, but at the same time also lowered virus-induced pro-inflammatory cytokines/chemokines (IL-8/KC, MCP-1, IL-6, RANTES, IFN-beta and TNF-alpha) *in vitro* and *in vivo*. Notably, effects differed between the different *in vitro* and *in vivo* systems applied. This might be related to the complexity of the different models and should be considered when one draws conclusion from results in comparable settings. The principal ability to treat and protect mice against lethal IV infection by MEK inhibition through U0126 administration is demonstrated in our additional study (Droebner et al., submitted for publication).

As reported, IV activation of the classical Raf/MEK/ERK pathway is linked to efficient virus propagation (Ludwig et al., 2004; Olschläger et al., 2004; Pleschka et al., 2001). The present study not only confirms that avian and human IV infection leads to ERK activation in A549 cells, (Fig. 2A and B), and that MEK inhibition effectively reduces virus titers (Fig. 2C and D), but also that the results from infected and U0126-treated mouse primary AECs mirror the effect observed in A549 cells (Fig. 4A and B). Confirming our *in vitro* findings, PR8-infected C57BL/6 mice treated daily with U0126 exhibited similar reduction of virus titers (Fig. 6). *In vivo* application of U0126 has been employed before in a mouse asthma model and it was speculated that it could have a therapeutic potential for the treatment of airway inflammation (Duan et al., 2004). Dependency of IV-propagation on the Raf/MEK/ERK pathway has also been shown *in vivo* by using transgenic mice with a constitutively active form of Raf in alveolar epithelial cells. Here, IV almost exclusively replicated in alveolar cells carrying the transgene as opposed to the wild type animals (Olschläger et al., 2004).

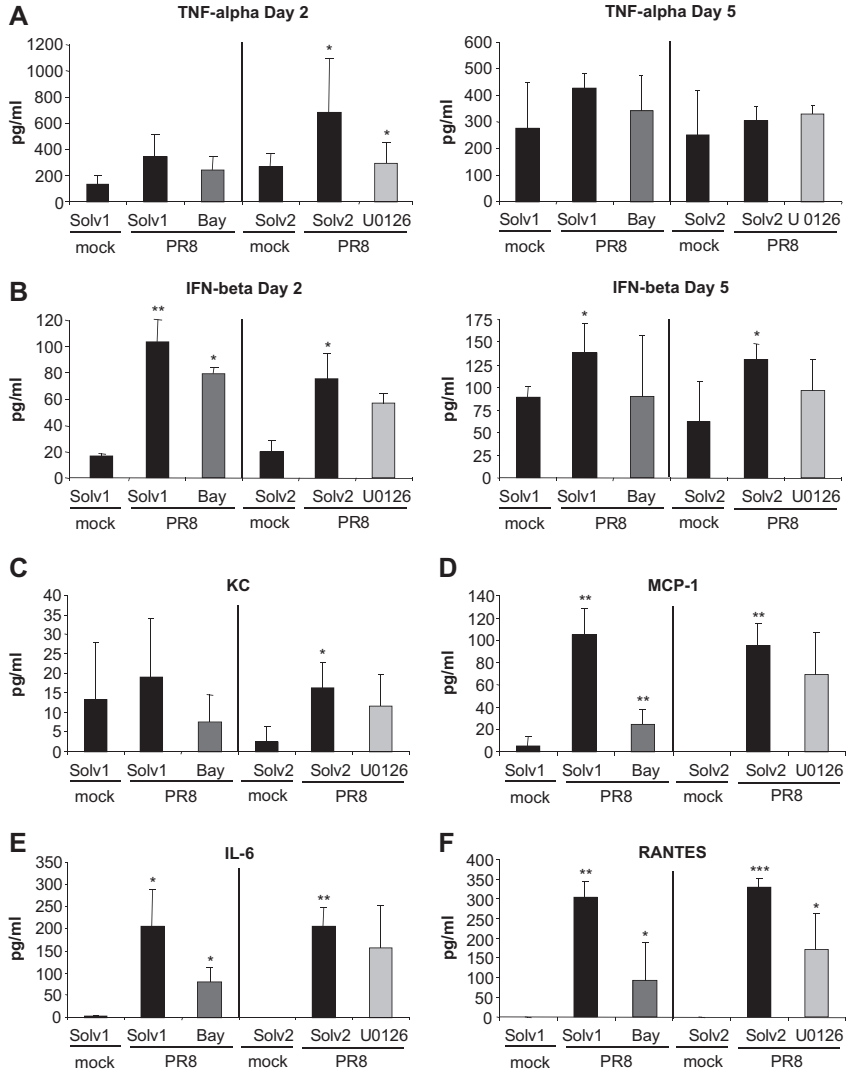

Fig. 7. Cytokine release in infected mice treated with U0126 or Bay 11-7082. Mice were infected intra-tracheally with PR8 (500 PFU in 70 μl PBS) or mock-infected (70 μl PBS). Solvent 1 (Solv1: DMSO) or solvent 2 (Solv2: DMSO/CremEL), Bay 11-7082 or U0126 were administered intraperitoneally, every 24 h starting 24 h before infection. Mice were sacrificed on day 2 or day 5 p.i. and BAL was performed. TNF-alpha (A) and IFN-beta (B) were determined (ELISA). Also KC (C), MCP-1 (D), IL-6 (E) and RANTES (F) were measured (multiplex immunoassay). *p* Values (*<0.05; **<0.01; ***<0.001) are given in comparison to mock vs. infected or infected (−) inhibitor vs. infected (+) inhibitor treatment. Bar graphs represent data from three mice with exception to mock groups at day 5, which include data from two mice per group.

During the short term treatment we found that treated mice were in slightly better condition concerning physiology and behavior. Nevertheless, addressing the effect of prolonged MEK inhibition on the survival of mice, an in-depth study of the anti-viral *in vivo* activity of U0126 demonstrates markedly improved survival of mice at 14 days p.i. (Droebner et al., submitted for publication).

Regarding inhibition of NF-kappaB activity and mouse survival in an antiviral approach, it was previously demonstrated that such treatment also significantly increases the survival rate over 13 days p.i. (Mazur et al., 2007). However, this was done without looking at the modulation of cytokine/chemokine expression. In both approaches no adverse effects on animal physiology and behavior of tested mice were detected. Taken together, our detailed *in vivo* analysis on antiviral MEK inhibition (Droebner et al., submitted for publication) and the data presented here are, to the best of our knowledge the first demonstration that IV titers can be effectively reduced *in vivo* using a Raf/MEK/ERK pathway inhibitor.

Although there is consensus that IV infection of cells leads to NF-kappaB activity (Bernasconi et al., 2005; Flory et al., 2000; Pahl and Baeuerle, 1995), even though the viral NS1 protein can limit this activation (Wang et al., 2000), the same does not apply to the importance of this activity for virus replication (Ludwig, 2009). There have been several virus supportive mechanisms of NF-kappaB reported, either by induction of pro-apoptotic factors (Wurzer et al., 2003, 2004) facilitating viral RNP nuclear export, or by counteracting cellular antiviral responses by increasing SOCS-3 (Pauli et al., 2008), or directly suppressing ISG promoter regions (Wei et al., 2006). While, on the one hand, it has been shown that the NF-kappaB inhibitors Bay 11-7085, Bay 11-7082 (Kumar et al., 2008; Nimmerjahn et al., 2004) and acetylsalicylic acid (ASA) (Mazur et al., 2007), greatly reduced IV titers in A549 cells and pre-activated NF-kappaB led to enhanced IV replication (Nimmerjahn et al., 2004; Wurzer et al., 2004), on the other hand, studies using an alternative NF-kappaB inhibitor, a cyclopentenone prostanoid (delta^{12}-PGJ_2), were unable to show a decline in A/WSN/33 (H1N1) titres (Bernasconi et al., 2005). Our studies in A549 cells confirmed that NF-kappaB activation caused by avian FPV and human PR8 could be effectively reduced by the irreversible inhibitor of IkappaBalpha phosphorylation (Fig. 1A and B) (Kumar et al., 2008; Mazur et al., 2007; Nimmerjahn et al., 2004; Wurzer et al., 2004). Also inhibition of this pathway using Bay 11-7082 greatly attenuated IV replication in A549 cells (Fig. 1C

and D) as well as in primary AECs (Fig. 4A and B). Furthermore, the *in vivo* use of ASA as a specific NF-kappaB inhibitor reduced IV titers (Mazur et al., 2007) and in our study, daily treatment with Bay 11-7082 demonstrated a significant drop of virus titers in PR8-infected C57BL/6 mice (Fig. 6). Taken together, different *in vitro* cell systems as well as *in vivo* data substantiate that indeed blocking NF-kappaB activation can effectively reduce influenza A virus propagation.

Influenza A virus infection induces cytokine expression both *in vitro* as well as *in vivo* (Chan et al., 2005; Matikainen et al., 2006; Matsukura et al., 1998; Tumpey et al., 2005; Wareing et al., 2004) and it has been postulated that in case of HPAIV infections, the observed increased pathogenesis might not only be due to high viral loads, but also caused by increased and/or deregulated immune responses leading to hypercytokinaemia (Beigel et al., 2005; Chan et al., 2005; de Jong et al., 2006; Guan et al., 2004; Szretter et al., 2007; Tumpey et al., 2005). In this regard, it is important to consider that the expression of many cytokines, including IFN-beta, is modulated by NF-kappaB activation (Hiscott et al., 2001; Ludwig et al., 2006), and recent reports with H5N1 subtype HPAIV in endothelial cells, point to overrepresented viral-induced cytokines being almost all NF-kappaB-dependent (Schmolke et al., 2009; Viemann et al., 2011), and although the Raf/MEK/ERK pathway is generally linked to proliferative mechanisms (Zhang and Liu, 2002), it has also been implicated in cytokine production (Kuderer et al., 2003). Therefore, reducing cytokine expression along with viral replication by interference with these signalling events might offer a beneficial approach in treatment of HPAIV infections.

In our studies, cytokine/chemokine expression profiles varied depending on the virus and model used. In A549 cells, FPV had no effect on IL-8, IL-6 and MCP-1, but led to an increase in RANTES production, whereas PR8 induced IL-8 and IL-6, and suppressed RANTES and MCP-1 expression (Fig. 3A–D). The ability of IV to reduce cellular levels of specific cytokines demonstrates the viral potential to interfere with host defence mechanisms. Markedly, no measurable amounts of IFN-beta upon FPV or PR8 infection could be detected (data not shown). In contrast, the delNS1 virus showed significantly induced IFN-beta secretion (2867 pg/ml – data not shown). This is in agreement with the NS1 protein's ability to down-regulate IFN-beta production (Hale et al., 2008), which might be due at least in part to limiting NF-kappaB activation (Wang et al., 2000). Noteworthy, both inhibitors consistently reduced cytokine/chemokine production.

In primary mouse AECs FPV- and PR8 infection also led to differential regulated cytokine/chemokine levels (Fig. 4C–F), although partially different from A549 cells. It should be noted, that the results for KC and RANTES were near the detection limit and therefore it was difficult to assess any statistical differences. This demonstrates that both IV strains are able to control certain cytokines/chemokines not only in permanent A549 cells, but also in AECs. One of the most obvious differences observed was the virus-induced IFN-beta release in primary AECs which was absent in A549 cells. (Fig. 4G). The importance of NS1 is emphasized by the fact that the FPV- and PR8-induced IFN-beta production was still low compared to that by delNS1 infection (data not shown). Once more, both inhibitors significantly decreased virus-induced cytokine/chemokine production to background levels. In general, the NF-kappaB pathway inhibitor exhibited a stronger effect on virus-induced IFN-beta expression than U0126. This can be expected, since IFN-beta production is known to be regulated by NF-kappaB, IRF3/7 and AP-1 transcription factors (Samuel, 2001). Nevertheless, U0126 also reduced IFN-beta amounts as well as most other virus-induced cytokines/chemokines tested. As U0126 reduced virus titers *in vitro* and *in vivo* better than Bay 11-7082 it can be assumed that MEK inhibition might exert an indirect effect on the cytokine/chemokine profile by efficient reduction of virus titres. Even so, the Raf/MEK/ERK cascade is known to affect IL-8 production (Guillot et al., 2005). Furthermore, we could show that treatment of virus-infected primary lung AECs with both inhibitors showed restrained nuclear RNP export. This demonstrates that the effect observed in A549 cells and other established cell lines (Mazur et al., 2007; Pleschka et al., 2001) also applies for primary lung cells, and consequently it can be assumed that this likely reflects the *in vivo* situation.

As "proof of principle" for our hypothesis that virus propagation and virus-induced cytokines can be reduced concurrently and thereby to provide a rationale for possible therapeutic approaches, *in vivo* studies were performed by infection of mice with mouse-adapted PR8. This model has long been used for *in vivo* studies of IV infections and induces severe pneumonia in mice (Herold et al., 2008). We have previously demonstrated that PR8 infected alveolar macrophages enhanced monocyte transmigration across virus-infected epithelium in a TNF-alpha-dependent manner (Herold et al., 2006). Therefore TNF-alpha was included in our *in vivo* analysis. PR8 infection of C57BL/6 mice resulted in a significant TNF-alpha increase compared to mock-infected mice 2 days p.i. (Fig. 7A). IFN-beta was also significantly elevated in the infected groups (Fig. 7B) as were most other cytokines tested (KC, MCP-1, IL-6, and RANTES) 5 days p.i. (Fig. 7C–F) confirming previous studies (Chan et al., 2005; Dessing et al., 2007; Herold et al., 2006; Hofmann et al., 1997; Lee et al., 2005; Matsukura et al., 1998; Szretter et al., 2007; Wareing et al., 2004; Xu et al., 2006).

Regarding the modulating *in vivo* effects on cytokines/chemokines, Bay 11-7082 considerably decreased IFN-beta, MCP-1, IL-6, and RANTES expression, and although the decrease for TNF-alpha and KC was not significant, a trend was noted. U0126 showed a significant inhibition of PR8-induced TNF-alpha release at day 2 p.i. and of RANTES at day 5 p.i.. For the other virus-induced cytokines (IFN-beta, KC, MCP-1 and IL-6), U0126 still demonstrated a reducing effect.

Recruitment of immune cells into the lung parenchyma and alveolar spaces, although required for host recovery, can sometimes lead to an exaggerated inflammatory immune response, exacerbation of the disease and high mortality observed with some HPAIV (Beigel et al., 2005; Chan et al., 2005; de Jong et al., 2006; Kaiser et al., 2001; Peiris et al., 2004). As such, IL-6 and TNF-alpha are both potent pyrogens with pro-inflammatory activities that seem to act synergistically and have been associated with the aforementioned symptoms and fever in IV infection (Beigel et al., 2005; de Jong et al., 2006; Kaiser et al., 2001). Comparing the results obtained with PR8 in our *in vitro* and *in vivo* systems, we noticed different cytokine induction profiles. This has also been reported by others, indicating that specific results can not be generalized and should always reflect the model system (Guillot et al., 2005; Hofmann et al., 1997; Wareing et al., 2004). In our study, both inhibitors were able to reduce cytokines/chemokines and virus titers although to different intensities. We believe that by this approach we can reduce the detrimental effects of viral tissue destruction and infiltration of inflammatory cells in the alveolar space connected to ARDS and multiorgan failure (Beigel et al., 2005; de Jong et al., 2006).

Although systemic inhibition of signalling pathways brings about concerns as to the possible side effects raised from drug usage (Salomon et al., 2007), local administration could probably be well tolerated (Ludwig, 2009; Ludwig et al., 2003). Drugs targeting these pathways are already in current use, such as NF-kappaB inhibitors like the common aspirin (Karin et al., 2004; Mazur et al., 2007; Yin et al., 1998) and modulators of the Raf/MEK/ERK pathway being under clinical trial for application in cancer therapy (Chang et al., 2003; Pleschka, 2008). Furthermore, in the *in vivo* context it is likely that modulating a specific pathway will not

totally block the overall immune response (as was evident from our animal data), but might attenuate the HPAIV-induced "cytokine burst" while still maintaining important host defence mechanisms active. As the work presented here aims at the "proof of principle" we did not set out to improve the protocol towards optimized therapeutic treatment, or to study combined application of both inhibitors and/or evaluate other substances for inhibition. Further studies will be extremely valuable for fine-tuning the drug effects to ultimately target these pathways within antiviral therapy strategies. In sum, we believe that targeting host pathways by agents which do not bear the risk of emerging viral drug resistance represents a beneficial future therapeutic approach in IV pneumonia.

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