Therapeutic *in vitro* and *in vivo* approach of influenza virus infection by simultaneous reduction of virus titre and cytokine expression through inhibition of virus-induced NF-κB and Raf-MEK-ERK activation

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IV. Abbreviations

aa	amino acids
A549	human alveolar epithelial cell line
AEC	mice primary alveolar epithelial cell
AI	avian influenza
AIV	avian influenza virus
AMs	alveolar macrophages
APCs	antigen-presenting cells
APR	acute phase response
APS	ammonium persulfate
ARDS	acute respiratory distress syndrome
BSA	bovine serum albumin
°C	degree celcius
cm	centimetre
CTL	cytotoxic T lymphocytes
delNS1	A/PR/8/34 with a deleted NS segment
ddH_2O	deionized distilled water
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EBV	Epstein-Barr virus
ECL	enhanced chemoluminiscence
EDTA	ethylenediamine tetraacetic acid
eIF2	eukaryotic translation initiation factor 2
ERK	extracellular signal regulated protein kinase
et al.	et alii (and others)
FasL	Fas ligand
FCS	fetal calf serum
FFU	foci forming unit(s)
FPV	fowl plague virus
g	gram

h	hour(s)
HA	hemagglutinin
HEPES	N-2-hydroxyethylpiperazine
HIV	human immunodeficiency virus
HPAIV	highly pathogenic avian influenza virus
HTLV	human
IAV	influenza A virus
IBV	influenza B virus
IFN	interferon
IκBα	inhibitor of NF-κB alpha
IKK	IκB kinase
IP-10	IFN-γ inducible protein-10
IRF3	IFN regulatory factor 3
IL- 6/8	interleukin 6/8
JNK	c-Jun NH_2-terminal kinases
LPAIV	lowly pathogenic avian influenza virus
M	molar
mAbs	monoclonal antibodies
M1	matrixprotein
M2	ion channel protein
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEK	MAPK/ERK activated kinase
MEKK	MAPK/ERK activated kinase Kinase
mg	milligram
MHC	major histocompatibility complex
MIG	monokine-induced by IFN-γ
min	minute(s)
MIP	macrophage inflammatory protein
ml	milliliter
mM	millimolar
moi	multiplicity of infection

mRNA	messenger RNA
NA	neuraminidase
NEMO	NF-κB essential modulator
NEP/NS2	nuclear export factor
NES	nuclear export signal
NF-κB	nuclear factor kappa B
NIK	NF-κB-inducible kinase
NK	natural killer cells
NLS	nuclear localization signal
NP	nucleocapsid protein
NPC	nuclear pore complex
NS1	non-structural protein 1
ng	nanogram
nt	nucleotide(s)
OD	optical density
PA	subunit of RDRP
PAGE	polyacrylamide gel electrophoresis
PB1	subunit of RDRP
PB2	subunit of RDRP
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit(s)
pg	picogram
p.i.	post infection
PKC	protein kinase C
PKR	(dsRNA activated) protein kinase R
pmol	picomolar
PolyA	polyadenylic acid
RANTES	regulated upon activated normal T cell expressed and secreted
RDRP	RNA-dependent RNA-polymerase
RIG-I	retinoic acid inducible gene-I
RNA	ribonucleic acid
RNPs	ribonucleoproteins

rpm	rotations per minute
RSK	ribosomal S6 kinase
s	second(s)
SCF	Skp-1/Cul/F box
SDS	sodium dodecyl sulfate
TADs	trans-activation domains
TEMED	N,N,N',N'-tetramethylethylenediamine
Th	T helper cells
TLR	Toll-like receptor
TNF-α	tumour necrosis factor alpha
TNFR	TNF-receptor
TRAIL	TNF-related apoptosis inducing ligand
Tris	tris-hydroxymethylaminomethane
TPA	12-O-Tetradecanoylphorbol 13-acetate
Tween 20	polyoxyethylenesorbiten monolaurate
UV	ultraviolet
V	volt
vol	volume
vRNA	viral RNA
v/v	volume percentage
w/v	weight percentage
wt	wild type
µg	microgram
µl	microliter
µM	micromolar

V. Summary

Influenza A virus (IAV) infection can cause severe pneumonia and lead to acute respiratory distress syndrome and ultimately death. Despite damage by viral replication, imbalanced production of anti-viral cytokines ("cytokine storm") resulting in inflammation can also lead to severe lung destruction. Therapeutics as vaccines and anti-viral drugs target components of the virus itself resulting in resistant variants. Therefore new therapeutic measures are urgently needed. IAV has been shown to activate the NF-κB and MAPK (Raf/EK/ERK) signalling pathway. These pathways seem to have both pro- and anti-viral effects, by promoting nuclear export of the viral genome and by inducing expression of anti-viral pro-inflammatory factors.
Therefore it was postulated that the inhibition of these signalling pathways will simultaneously reduce virus replication as well as modulate cytokine production without affecting host defence.
Using specific IKK- (Bay-11-7082) and MEK- (U0126) inhibitors at non-toxic concentrations, I analysed the effect of NF-κB and MAPK pathway inhibition on propagation of a highly pathogenic avian influenza virus (strain A/FPV/Bratislava/79, H7N7) and a human influenza virus (strain A/PR/8/34, H1N1) and the virus-induced cytokine induction in infected human lung epithelia cells (A549) and mice primary alveolar epithelial cells (AECs), *in vitro*. Experiments were also performed in an *in vivo* mouse model for "proof of principle" applying the above inhibitors and the A/PR/8/34 virus for infection.
Results show, (1) by western blot and transcription factor assay that both pathways (NF-κB and MAPK) are activated upon IAV infection in A549 cells; (2) that both inhibitors (IKK-, MEK-inhibitor), used at non-toxic concentrations, lead to decrease in signalling, virus titres (FFU assay) and (3) reduced cytokine expression (multiplex cytokine assay and ELISA), *in vitro* (A549 and AECs) as well as *in vivo* (mice). I also observed differences in the virus-induced (FPV and PR8) cytokine release when comparing different cell types (A549, AECs) as well as in the mouse model.
The results demonstrate that inhibition of NF-κB and Raf/MEK/ERK pathway can be used to simultaneously reduce virus titres and modulate pro-inflammatory cytokine expression *in vitro* as well as *in vivo*. This could be of importance for future therapeutic strategies to treat influenza pneumonia and virus induced "Cytokine Storm".

VI. Zusammenfassung

Eine Infektion mit dem Influenza A virus (IAV) kann schwere Pneumonien verursachen, die zum akuten Atemnotsyndrom und schließlich zum Tod führen können. Neben der Schädigung durch die virale Replikation, kann auch die in einer überschießende Entzündung resultierende, ungeregelte Produktion von anti-viralen Zytokinen („Zytokinsturm“), zu einer ernsthaften Schädigung der Lunge führen. Impfstoffe und anti-virale Substanzen als Therapeutika richten sich in der Regel gegen bestimmte virale Funktionen und Virusbestandteile und führen somit schnell zu Resistenzen. Daher sind neue therapeutische Möglichkeiten dringend notwendig.

Es konnte bereits nachgewiesen werden, dass IAV den NF-κB und den MAPK (Raf/MEK/ERK) Signalweg aktivieren. Diese Signalwege scheinen beide, durch die gleichzeitige Förderung des Kernexports des viralen Genoms und der Expressionsregulation von anti-viralen pro-inflammatorischen Faktoren, pro-und anti-virale Effekte zu besitzen. Daher wurde postuliert, dass durch die Hemmung dieser Signalwege sowohl die Virusvermehrung reduziert werden kann, als auch die Zytokinproduktion moduliert werden kann bei gleichzeitigen Erhalt der Wirstabwehr.

Unter Verwendung spezifischer Inhibitoren beider Signalwege (IKK-Inhibitor: Bay-11-7082, MEK-Inhibitor: U0126) in nicht-toxischen Konzentrationen analysierte ich *in vitro* den Effekt der NF-κB- und MAPK-Signalwegsinhibition in Bezug auf die Vermehrung, eines hochpathogenen aviärern Influenzavirus (HPAIV, Stamm A/FPV/Bratislava/79, H7N7) und eines humanen Influenzavirus (Stamm A/PR/8/34, H1N1), sowie die Zytokininduktion in infizierten humanen Lungenepithelzellen (A549) und alveolären primären Mäuseepithelzellen (AECs). Auch wurden zur Bestätigung der Hypothese in einem Modelorganismus diese Experimente unter Verwendung der oben genannten Inhibitoren und des A/PR/8/34 Virus in einem Mausmodell *in vivo* durchgeführt.

Die Resultate zeigten, dass (1) beide Signalwege (NF-κB und MAPK) durch die IAV Infektion von A549-Zellen aktiviert werden – nachgewiesen durch Westernblot-Analysen und Messung der Transkriptionsfaktor-Aktivierung, (2) beide Inhibitoren (IKK-, MEK-Inhibitor) unter Verwendung in nicht-toxischer Konzentrationen zur Verringerung des Virustiters führen (FFU-Assay) und (3) die Zytokinexpression („multiplex cytokine assay“ und ELISA) *in vitro* (A549 und AECs), sowie *in vivo* (Mausmodel) verringert ist. Dabei konnte gezeigt werden, dass die Zytokininduktion abhängig ist vom (1) Zellmodel (A549 oder AECs) oder Mausmodel und (2) vom Virusstamm (FPV oder PR8)

Die Resultate demonstrieren, dass die Inhibition der NF-κB und Raf/MEK/ERK-Signalwege dazu verwendet werden können gleichzeitig Virustiter zu reduzieren und pro-inflammatorische Zytokinexpression *in vitro*, sowie *in vivo* zu beeinflussen. Dies könnte zukünftig für die Behandlung von Influenzapneumonien im Bezug auf die Hemmung der Virusvermehrung und Modulation der virusinduzierten Zytokinausschüttung von großer Bedeutung sein.

1. Introduction

1.1. Influenza viruses

1.1.1. Different types of influenza viruses

Influenza viruses belong to the family of the *Orthomyxoviridae* and are subdivided into three genera depending on the antigenic differences of their nucleo- and matrix proteins: type A, B and C. They also differ with respect to host range, variability of the surface glycoproteins, genome organization and morphology. The influenza A viruses are responsible for major pandemic outbreaks of influenza and for most of the well-known annual flu epidemics. Influenza C is different from the A and B type, and generally causes only mild cold-type disease in humans. Type A influenza viruses can infect in general birds and mammals (Figure 1.1), whereas type B and C influenza viruses are normally only found in humans. The primary reservoir of all influenza A viruses are wild aquatic birds. Type A influenza viruses are further classified into subtypes based on two surface glycoproteins of the virus; the haemagglutinin (HA: H1-H16) and the neuraminidase (NA: N1-N9) proteins. Unlike influenza A viruses, type B and C influenza viruses are not classified into subtypes [1].

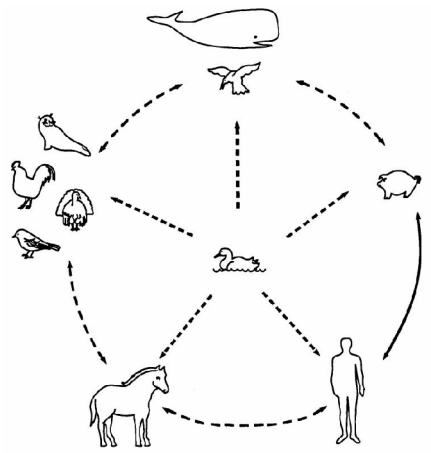

Figure 1.1: The reservoir of influenza A viruses. The hypothesis is that wild aquatic birds are the primordial reservoir of all influenza viruses for avian and mammalian species. Direct transmission of influenza between pigs and humans has been shown (solid line). There is also extensive evidence for transmission between wild ducks and other species. The five different host groups are based on phylogenetic analysis of the nucleoproteins of a large number of different influenza viruses (adapted from: Webster,[2]).

1.1.2. Influenza A virus

1.1.2.1. Morphology and genome structure of influenza A virus

Influenza A viruses (IAVs) are enveloped viruses and consist of eight segmented single-stranded RNAs of negative polarity with between 890 to 2341 nucleotides each that code for at least 10 viral proteins [1, 3] (Figure 1.2 and Table 1.1). Influenza A viruses particles are pleomorphic, in general spherical and have a size of approximately 80 to 120 nm in diameter [1].

The lipid envelope is derived from the host cell membrane during viral budding from cell surface and consists of a lipid bilayer presenting spike-like projections (about 500) on the outside, which represent the structural glycoproteins HA (haemagglutinin) and NA (neuraminidase). The matrix protein M1 underlies the inside, and the M2 (ion channel) protein is also a trans-membrane protein and is found in the lipid bilayer of the viral envelope [1, 3, 4].

The viral HA is a trimer, which comprises three individual HA monomers, whereas the NA is a tetramer. HA is five times more abundant than NA [5]. The HA is synthesized as a single polypeptide chain (HA0), which is subsequently cleaved into two subunits, HA1 and HA2 [5-10]. Cleavage of HA0 is essential for the molecule to be able to mediate membrane fusion between the viral envelope and the host cell membrane and for infectivity [5, 8, 11]. HA1 and HA2 appear as two distinct subunits, with HA1 being the globular domain at the distal end of the spike, which is responsible for binding of the virus to the cellular sialic acid receptor [5]. HA1 is also responsible for the major antigenic epitopes of the molecule to which the host will direct a neutralizing antibody response [6, 8]. The HA2 forms the stem of the viral spike and contains a conserved region of 20-amino acid residues, mostly hydrophobic at the N-terminus [5, 8]. This sequence is generally referred to as the “fusion peptide”, and is responsible for triggering the fusion between the viral envelope and the host cell membrane [5, 8, 9].

The NA is the second major surface antigen of the virus and plays an important role in efficient viral budding [2, 4, 5]. It displays enzymatic activity towards a terminal sialic acid and an adjacent sugar residue [5]. Since sialic acid receptors are present on the cell surface, the neuraminidase activity of NA permits release and prevents reattachment or aggregation, of the newly formed virions from the surface of the infected cells [2, 4, 5].

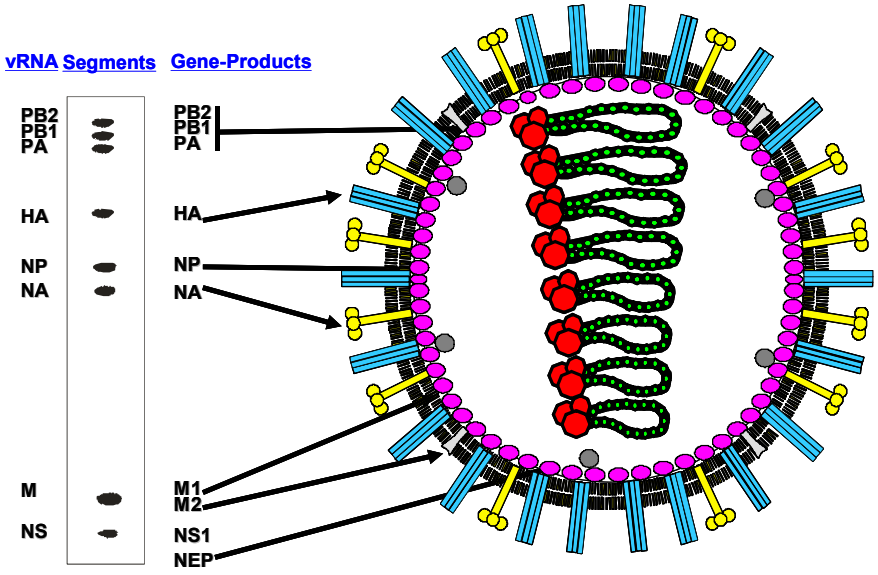

Figure 1.2.A: Cartoon of the influenza virion. The eight viral RNA segments were separated by electrophoresis (left). The corresponding gene products and their localization within the virus particle are depicted on the right. The only non-structural virus protein, the NS1, is only found inside infected cells. (Adapted from Ludwig et al.[12])

Table 1.1.: Influenza A virus proteins and functions (strain A/PR/8/34, H1N1)

(Modified from Ludwig et al. [13])

Segment	vRNA (nt)	Protein	AA	Functions
1	2341	PB2	759	subunit of RDRP; "Cap-snatching"
2	2341	PB1	757	catalytic subunit of RDRP; elongation
		PB1-F2	87	apoptosis?
3	2233	PA	716	subunit of RDRP
4	1778	HA	566	haemagglutinin; surface-glycoprotein; receptor binding; membrane fusion
5	1565	NP	498	nucleoprotein; encapsidation of vRNA and cRNA; part of transcriptase complex; nuclear/cytoplasmic transport of vRNA
6	1413	NA	454	neuraminidase; surface-glycoprotein; receptor disruption, virus releasing
7	1027	M1	252	matrix protein
		M2	97	ion channel activity ; protecting HA-conformation
8	890	NS1	230	post-transcription regulator; inhibition of (i) pre-mRMA splicing, (ii) polyadenylation (iii) PKR-activation
		NEP/NS2	121	nuclear export factor

Oseltamivir (Tamiflu) and zanamivir (Relenza) are neuraminidase inhibitors (NAI) and the only two approved NAI in humans. These drugs are sialic acid analogues, which inhibit the enzymatic activity of NA, thus slowing down the release of progeny virions from infected cells [12, 14, 15].

A third integral membrane protein, the M2, forms a homotetramer and is expressed on the virus surface. M2 functions as a pH-activated proton channel and is essential for viral uncoating in the viral infection cycle [1, 5, 16, 17]. It permits entry of protons from the endosome into the viral particle, thereby weakening and disrupting the interaction between the viral genome and the M1 protein [1, 5]. M2 also seems to be important for viruses with intracellular cleaved HAs (such as H5 and H7 subtypes of avian viruses) by regulating the intra-compartmental pH in the trans Golgi network (TGN) above the threshold at which conformational changes of the HA occur [1, 5].

M2 is the target for the influenza drugs amantadine and rimantadine [17]. Blocking of the M2 channel with amantadine slows the dissociation of M1 from ribonucleoprotein complexes (RNPs) (see below) and the viral membrane, inhibiting subsequent steps in the viral life cycle [1, 5, 17].

Influenza A and B virus genomes consist of eight negative-sense single-stranded RNAs. The viral RNA is assembled with the viral RNA-dependent RNA-polymerase (RDRP), which is itself a complex of three subunits: PB1, PB2 and PA, and the nucleocapsid protein NP to form ribonucleoprotein complexes (RNPs) [1, 5] (Figure 1.2.B). The main function of NP is to encapsidates the virus genome, leading to RNA transcription, replication and packaging [18]. The RNPs are surrounded by a layer of the matrix protein, M1, which is the most abundant structural protein of influenza virus and is thought to act as an adaptor protein between the lipid envelope and the internal RNP particles [19].

All viral segments encode for one protein except for segment 7 and 8 which have overlapping reading frames and will give rise to the proteins M1, M2 and NS1, NS2 (also called nuclear export protein NEP) respectively, by splicing of the primary mRNA transcript [5] (Figure 1.2. and Table 1.1.). Also, some influenza viruses encode for another protein, PB1-F2, in the +1 reading frame of the PB1 gene [20].

M1 protein associates tightly with the vRNP both in the virion as well as during virus assembly in the infected cell [19, 21, 22]. After the protons have entered the virion via the M2, the M1 is released from the RNPs, and the RNPs are then rapidly imported from the virion, which has fused with the endosomal membrane, into the nucleus, by

an active mechanism, through the nuclear pore complexes (NPC) [23]. The M1 protein association to vRNP has been shown to be important also for nuclear RNP export, late in the replication cycle, and this association also seems to prevent the re-import of the newly synthesized vRNPs back into the nucleus [22-24].
NS2 is present in low copies in the virion, whereas the NS1 is the only true non structural protein, but it is highly abundant inside infected cells [5].

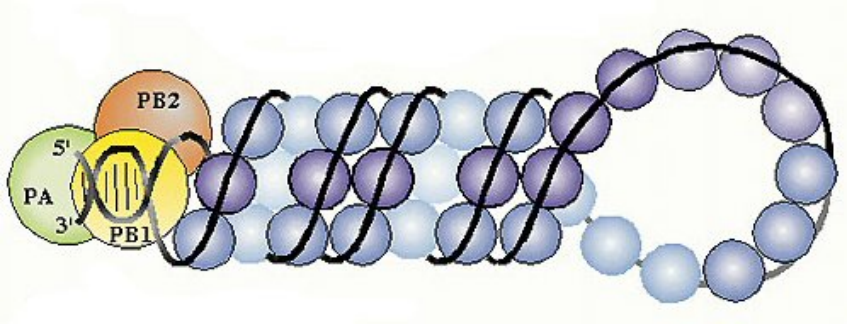

Figure 1.2.B: The influenza virus RNP structure. The NP (blue) is associated with (-) sense single-stranded RNA (black line), and the three subunits of RNA-dependent RNA-polymerase (RDRP): PB1 (yellow), PB2 (orange) and PA (green), which bind at a short duplex region of the vRNA. (Adapted from Portela and Digard, [18])

1.1.2.2. Propagation and genome replication of influenza A virus

Influenza viruses bind to sialic acid residues that are ubiquitously present on glycoproteins or glycolipids on the host cell surface [1, 4] (Figure 1.3). The main targets of IAV in humans are epithelial cells of the upper and lower respiratory tract, but they can also infect macrophages, dendritic cells and other leukocytes [25]. The virus binds to the cell via its receptor-binding pocket at the conserved distal tip of the HA molecule [8]. Different IAV HAs show different receptor binding specificities depending on the nature of the glycosidic linkage between the terminal sialic acid and the penultimate galactose residue on the carbohydrate side chains on the receptor [8]. Human influenza viruses preferentially bind to sialic acids attached to galactose in α2,6 configuration, whereas avian viruses have a preference for sialic acids attached to galactose in an α2,3 linkage [8, 26-29]. In humans influenza viruses infection is mainly a respiratory disease and the sialic acid α2,6 linkage is abundant in the lungs, whereas in birds it is enteric and the sialic acid α2,3 linkage is mainly found in the intestine. This difference is thought to be the basis for the restriction in

transmission of avian influenza viruses directly to humans [8]. On the other hand, pigs have receptors with both type of linkage between sialic acid and galactose, and therefore are susceptible to infection with either human or avian viruses. Co-infection of pigs with different influenza viruses is considered one way by which new viruses with pandemic potential may arise [8, 26].

Virus binding to receptors initiates uptake through so-called receptor-mediated endocytosis. In this process, virus particles are internalised by the host cell plasma membrane. The vesicles thus formed subsequently fuse with intracellular compartments called endosomes, which progress from mild acidic to late endosomes with decreasing internal pH. One of the important steps in IAV infection is the fusion reaction between the viral envelope and the endosomal membrane. For this, the maintenance of a low pH inside the endosomes (pH5-6), by proton pumps within the endosomal membrane, is of utmost importance. Upon pH decrease the HA suffers a major conformational change in its structure thereby leading to movement of the fusion peptide sequences of HA2, previously buried within the stem of the HA trimer, to the distal tip of the HA spike, allowing their insertion into the target membrane (Figure 1.3) [8-10]. This then allows for merging of the two membranes, through which the viral core penetrates into the cell cytosol [30, 31]. M2 proton channel activity is required for the uncoating process. M2 facilitates the flow of ions from the endosome into the virion interior, leading to disruption of protein-protein interactions, thus dissociating M1 from RNPs and viral membrane [1, 5].

The RNP complexes released into the host cell cytosol are transported intact to the nucleus, through the nuclear pore complex (NPC). All four proteins of the RNP complex (NP, PB1, PB2 and PA) contain nuclear localization signals (NLS) and are actively transported into the nucleus, where replication and transcription of viral RNA (vRNA) takes place (Figure 1.3 and 1.6) whereas M1 may enter the nucleus by passive diffusion because of its small size [32-36]. In the nucleus the negative (-) sense vRNAs are transcribed to positive (+) sense messenger RNAs (mRNAs) by the replicase/transcriptase RDRP (PB1, PB2 and PA) carried with the RNPs (Figure 1.3 and 1.6). For initiation of viral mRNA synthesis, in a process referred to as “cap snatching”, the viral transcriptase “steals”, by endonucleolytic cleavage, short cap regions (m7GpppNm) from cellular pre-mRNAs, to act as primers. Thus, this cap snatching process, which is later required for efficient binding of ribosomes to the mRNA, favours production of viral components over synthesis of cellular proteins.

After priming synthesis of mRNAs are terminated 17 to 22nt from the 5' end of the vRNA template at a sequence of 4/5 to 7 uridines (U) [1, 5]. This stretch of uridines act as a polyadenylation signal [37-39] which leads to synthesis of a poly(A) tail by the viral RNA polymerase, by repeated copying of the U sequence, and added to the 3'end of the viral mRNA [38, 39]. The mRNAs are then transported to the cell cytosol for translation to occur [1, 5].

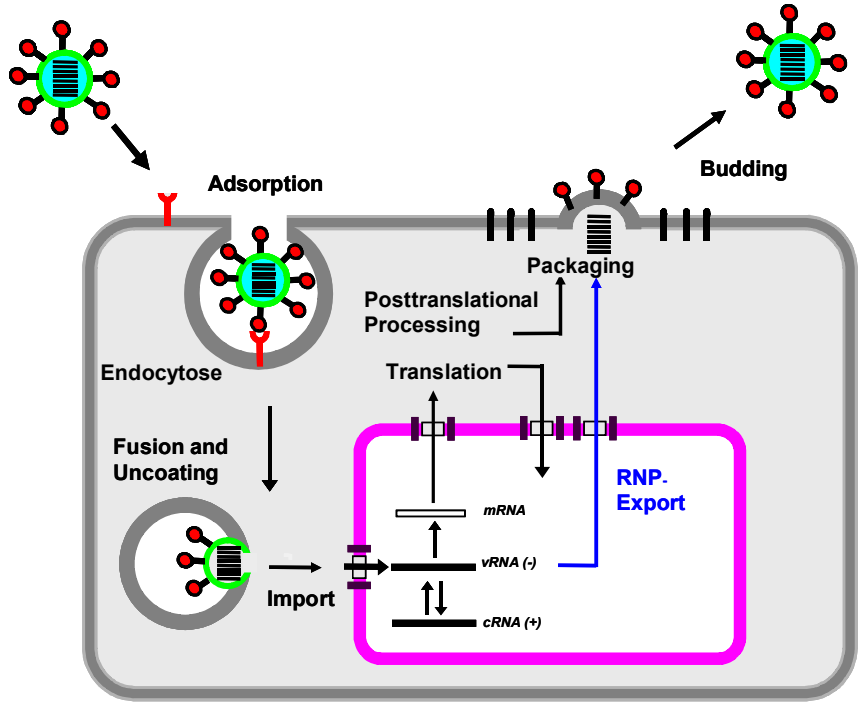

Figure 1.3: The Replication cycle of influenza virus. It begins with binding of the HA-spike to sialic-acid containing receptors on the cell surface. The virion is then taken up into the cell through endocytosis. The RNPs are released into the cell cytoplasm after fusion between the viral and the endosomal membrane, and then transported into the nucleus, where transcription and replication of the viral RNA takes place. Viral mRNA is exported to the cytoplasm and translated into viral proteins. Some replicative proteins (i.e. NP, PB1, PB2 and PA) are transported back to the nucleus and continue the viral genome replication. Viral surface-glycoproteins (i.e. HA and NA) are transported to the cell surface. Late during virus replication cycle, vRNPs are exported out of the nucleus and packed and progeny virions are then released from the membrane by budding. (Adapted from Pleschka)

As mentioned above the polymerase proteins (PB1, PB2 and PA) are required for this process. The PB1 subunit forms the core of the complex and is responsible for polymerase activity, whereas the PB2 subunit is involved in generation of capped

RNA primers for the initiation of transcription by binding the cap structures of host pre-mRNA (cap snatching) prior to their endonucleolytic cleavage by PB1 [1, 2, 5]. The PA subunit is required for both transcription and replication, but its exact role in the virus replication cycle is still unclear [2, 40].

(-) sense viral RNAs (vRNA) also serve as templates for production of exact copies of complementary (+) sense RNA (cRNA), which in turn lead to synthesis of more vRNA molecules [1, 5, 41]. For synthesis of both cRNA (template) and full-length copies of vRNA molecules, initiation is achieved in a primer-independent manner, resulting in triphosphorylated 5' ends. During cRNA synthesis, the polyadenylation signal is ignored [1, 5, 18, 41]. Encapsidation of cRNAs and vRNAs by the NP is a prerequisite for them to be recognized as templates for the viral polymerase. These newly formed vRNAs are later transported into the cytoplasm as vRNPs for assembly into new virus particles [18].

After translation, proteins may remain in the cytoplasm or become associated with the cell membrane. PB1, PB2, PA and NP proteins migrate back into the nucleus, where they associate with newly-synthesized vRNA to form new RNP complexes. NP is thought to control whether mRNA or cRNA are produced, i.e. later in infection when there is a high amount of NP, mRNA synthesis stops whereas cRNA synthesis continues. The amount of free NP is therefore important for switching between expression and assembly during the viral replication cycle [18]. Also, in the nucleus M1 and NS2/NEP bind to the RNPs and are exported through the nuclear pore complexes (NPCs) into the cytosol. Nuclear export of RNPs has been proposed to depend on several viral proteins, such as viral M1 and NEP, and on cellular factors CRM1 and Ran-GTP [22, 32, 42-44]. The M1 retains the RNPs in the cytoplasm for the further maturation [32]. Consequently RNP associates with viral membrane proteins and together are released by budding outwards [5].

During synthesis of viral envelope proteins HA, NA and M2 which starts in the cytosol, the growing polypeptide chains are transported into the endoplasmic reticulum (ER), where the proteins are folded and assembled into trimmers and tetramers. Still in the ER, glycosylation of HA and NA begins, and they are subsequently transported through the Golgi apparatus and the trans-Golgi network (TGN) to the plasma membrane. Many modifications, such as polypeptide folding [45], trimerization [46], N-glycosylation [47], acylation [48] and proteolytic cleavage

[49] are introduced along this pathway, until they reach the cell surface [50]. Normally different proteases are able to cleave the HA0 but this seems to depend on the amino acid (aa) sequence at the HA0 cleavage site. It has been proposed that the HA cleavage site would correlate with the virulence of the virus and that virulent strains (e.g. H5 and H7) would contain a recognition motif of the intracellular protease furin, whereas the avirulent strains would contain only a single arginine residue used by extra-cellular proteases [5, 8, 49]. Another important function of the M2 protein is noticeable at this stage. It protects the HA from a premature fusion-activating conformational change, due to the mildly acidic pH inside the TGN. M2, through its proton channel activity can transiently neutralize the pH within the TGN [2, 16].

1.1.2.3. Viral assembly and Budding

In polarized epithelial cells, HA and NA are transported to the apical side of the plasma membrane, resulting in release of progeny viruses back into the airways and not into the systemic circulation [1, 50]. After budding the viral HA interacts with the host cell receptor, at which point the NA cleaves off this bond and enables virus release from the infected cell and spread to other cells [51]. Sorting of the eight distinct genome segments into each particle is not a purely random process [52-54]. It seems that the packaging signal of vRNA molecules already occurs in the nucleus or during nuclear export through the process of vRNP/cRNP discrimination [55]. In support of this, another study showed that only vRNA molecules and no cRNA molecules are found outside the nucleus in the cytoplasm [56]. Another study suggests that the coding regions of viral RNAs possess signal sequences that promote recruitment of the segments during virion assembly [57]. In support of this theory, recent studies showed that RNPs of influenza A virus are organized in a distinct pattern (seven segments of different lengths surrounding a central segment). The individual segments are suspended from the interior of the viral envelope at the distal end of the budding virion and are orientated perpendicular to the budding tip [52].

As mentioned before the NS1 protein is the only true non-structural protein. It is encoded by segment 8 (which is the shortest RNA segment) of the influenza A virus genome and seems to be extremely important in the pathogenesis of influenza A virus. It performs several functions and the list keeps growing. NS1 has been shown

to inhibit: splicing and polyadenylation of the cellular pre-mRNAs; end-formation of cellular mRNAs; nucleocytoplasmic export of cellular mRNAs; and in contrast to stimulate translation of viral mRNAs [58-65]. Due to the development of reverse genetics techniques, it has been possible to study in detail the function/s of the influenza A virus NS1 protein (NS1 mutants or a deleted NS1 gene – delNS1) [66]. It was interesting to note that the delNS1 virus was unable to replicate in most cells unless they were deficient for an interferon (IFNα/β) system and in wild type (wt) mice the virus was unable to cause disease whereas in an IFNα/β system deficient mouse strain (STAT1$^{-/-}$ mouse), the mice died [66, 67]. The influenza A NS1 has been shown to have ssRNA and dsRNA-binding activity, and as a consequence is able to sequester viral dsRNA and down-regulate the activation of dsRNA-activated protein kinase R (PKR), NF-κB, IFN regulatory factor 3 (IRF-3) and the JNK effectors c-Jun and ATF-2 signalling pathway [63, 68-72]. By blocking activation of NF-κB, IRF3 and AP-1 which together lead to IFNβ gene induction, IFNβ transcription is repressed [70-72]. PKR activation leads to inhibition of eIF-2α and therefore a reduction in protein synthesis. By inhibiting PKR activation the virus counteracts a blockage of protein synthesis, thereby sustaining its own replication [63, 68, 69]. IFNβ is one of the most potent antiviral cytokines [69], and will be further discussed later (cytokine interplay).

Other interesting studies have also shown that NS1 protein can down-regulate apoptosis, indicating anti-apoptotic properties [73].

1.1.3. Antigenic variation of influenza virus infection

Influenza A viruses can increase their antigenic diversity in two ways. It can change by “antigenic drift”, which occurs through small changes (mutations) in the virus HA or NA that happen continually over time due to single amino acid substitutions. This allows the virus to adapt to selective pressure as given by circulating neutralising antibodies [74, 75].

New virus strains arising from antigenic drift generally only result in epidemics and are not as severe, since partial immunity is present in persons with cross-reacting antibodies induced by previous infections.

Pandemics are due to the appearance of new influenza A subtypes against which the population has no previous immunity. This phenomenon is known as “antigenic shift”

[76, 77]. The HA antigen is always involved in antigenic shift as it is responsible for eliciting virus-neutralizing antibodies [1-3]. The neuraminidase (NA) can also be affected. Concerning evidence from past pandemics, there are three possibilities as to how antigenic shift can occur (CDC, Focus on Bird Flu, 2005): (I) Reassortment: where the new virus subtypes are reassortant viruses resulting from double infection, in which eight RNA segments of different viruses swap with each other, producing a new virus. As already mentioned, pigs are thought to serve as a "mixing vessel" for AIV and human influenza virus where genetic reassortment may occur [78-82]. (II) Recirculation of pre-existing subtypes: where a limited number of influenza A subtypes would exist and be recycled in the human population when the antibody status of the population has fallen to levels which would allow for a pandemic infection; a cycle of approximately 70 years (CDC, Focus on Bird Flu, 2005). (III) Gradual adaptation of animal viruses to human transmission: evidence for this hypothesis is the 1918 pandemic. It seems that the pandemic virus was directly descended from an avian ancestor [83-85].

1.1.4. Avian influenza viruses

Even though influenza is thought of as a human disease, the natural reservoir for influenza A viruses (IAV) are aquatic birds and wildfowl. Many different strains actually circulate at any given time, although most don't cause disease in wild birds. As mentioned before, influenza A viruses are divided into subtypes depending on the antigenic nature of the HA and NA proteins. So far 16 different HA and 9 different NA have been described for IAVs and only a limited subset are currently circulating in humans and are cause for annual epidemics and disease; subtypes H1N1, H3N2 and H1N2. As expected they all originated from avian species and adapted to humans following zoonotic events [3, 86, 87].

Influenza viruses that infect birds are called "avian influenza viruses" (AIV).

In wild aquatic birds influenza infection does not cause disease but they can shed large amounts of the virus. In contrast, domestic poultry can develop severe symptoms and die from influenza infections [2].

1.1.4.1. History

Avian influenza (AI) has become an international concern which has grown over the past years due mainly to the highly pathogenic avian influenza subtype (H5N1).

Serious outbreaks have affected almost all areas in Asia and recently also Europe [88, 89]. AI was first recorded in Italy in 1878 as described by Perroncito et al. [90, 91]. As it was the cause of massive poultry epidemics, this disease was then known as the “Fowl Plague”. In 1902 the causative agent was isolated from a chicken, marking the first documented isolation of influenza virus. The first human influenza virus isolation was demonstrated in 1933 [92]. In 1955, it was determined that the virus causing Fowl Plague was a type A influenza virus (http://avianflu.umd.edu/).

1.1.4.2. Current situation (epidemics and pandemics)

AIV has had a devastating impact on the poultry industry throughout the world. In more recent years, east and Southeast Asian countries have been the most affected areas [93, 94]. Destruction of millions of poultry to prevent further spread has resulted in grave economic loses. The first outbreak of human illness due to H5N1 AIV occurred in 1997 in Hong Kong with a mortality of 30% [95]. It was previously accepted that AIV could not directly infect humans, due to the different receptors present on either species and due to the receptor-binding specificities of avian and human viruses, as already explained [89]. However, a new strain of influenza virus can evolve by reassortment (rearrangement and swapping of genetic material when co-infected in the same host) [89, 96]. If this were to happen now, it could be devastating to the human population [79, 96, 97].

So far, the AIV that have been transmitted to humans are of subtypes H5, H7 and H9 [97]. These subtypes of avian influenza A viruses can be further classified as either highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI). So far, all outbreaks of the HPAI have been caused either by the H7 or the H5 virus subtypes [89, 97]. The subtype that has attracted the most attention more recently has been the H5N1, due to the deaths of many human lives [79, 80]. Although the incidence of H5N1 in Eastern Asia greatly increases the risk that it may evolve to a point where transmission between people might be possible, so far, there is no precise evidence that this has happened [98].

In the past century there have been three severe pandemics, all spread worldwide within a year of being detected [97]. The worse flu pandemic across Europe, Asia, and North America occurred in 1918-19 and was termed the “Spanish Flu” [99-102]. This pandemic, (H1N1 strain), is believed to have caused the death of 20 to 50 million people worldwide [85, 99, 100, 103]. Since then, other pandemics have

occurred: the “Asian Flu” (H2N2) pandemic of 1957 claimed over one million lives worldwide after spreading from China [97]; in 1968, the “Hong Kong Flu” an H3N2 virus was responsible for around half a million lives. HPAIV H5N1 was isolated in 1997 for the first time from a human patient in Hong Kong [89, 95, 97]. The virus infected 18 patients resulting in six deaths [89, 97, 104, 105]. Recently HPAIV H7N7 broke out in Netherlands in March 2003 and quickly spread to Belgium and Germany [88, 89]. The virus infected 83/89 people causing conjunctivitis, and caused the death of one veterinarian [105]. After 1997 and 2003, other outbreak of HPAIV subtype H5N1 surfaced again in Vietnam’s and Thailand’s poultry industry in the early 2004 also accompanied with human cases [94]. In 2004 in these two countries the World Health Organization (WHO) confirmed 46 H5N1 infections in people, of which 32 were fatal, but no cases of person to person transmission. Within a few weeks, the disease had spread to ten other countries in Asia, including South Korea, Japan, China and Indonesia [93, 106].

Although spread of AIV from birds to humans is generally rare, it has become quite preoccupying due to the increase in observed frequency. The probability that the virus could gain a form that could pass easily from humans to humans, is a possibility that many scientists consider, and this would have a devastating effect worldwide. From a total of over 385 confirmed cases of avian (H5N1) “flu” in humans, between 2003-2008, 243 have been fatal (WHO, 19 June, 2008). Taken together, the death toll associated with the 1918 influenza virus correlated to the current population could be between 180 million and 360 million human deaths globally [107, 108].

1.1.5. Clinical symptoms of influenza virus infection

Influenza A and B are the major viruses responsible for the annual flu epidemics, which generally occur each winter. In general flu is a self-limiting disease [109], which does not spread further than the respiratory tract, mostly due to the requirement of HA cleavage for efficient virus propagation. The protease needed for this purpose is restricted to the lung and airway epithelium [110, 111].

The common route of “flu” transmission is by aerosol or droplets [111]. Once inhaled, the virus can remain in the mucus of the airways and then be transported by ciliated epithelial cells to the posterior pharynx. Influenza viruses cause a lytic infection of airway epithelial cells [112, 113].

Typically (clinically), influenza viruses cause acute infections characterized by the rapid onset (about 24 – 48h after infection) of chills and fever accompanied by aches and pains throughout the body, malaise, sore throat, nasal congestion and pulmonary complications [113, 114]. Gastrointestinal symptoms have also been reported, particularly vomiting, abdominal pains and diarrhoea [111, 115-117]. Other less common symptoms include secondary bacterial pneumonia, encephalitis and myocarditis. Also, possible risks of complications and death are greatly increased in previously unexposed young children and in the elderly (> 65 years), due to their reduced immune function [109, 111, 116, 117].

However, once a new strain is introduced into the population, the scenario may be more devastating as was the case in previous pandemics and the reported H5N1 avian strains from recent events. In these reports of highly pathogenic influenza virus (HPIV) infections, progressive primary viral pneumonia was generally observed, although secondary bacterial pneumonia was more common in the 1918 cases than in H5N1 infected individuals [109, 118]. Symptoms including extensive pulmonary oedema and acute respiratory distress syndrome (ARDS), characterized by alveolar haemorrhage associated with massive lung infiltration of mononuclear cells are often lethal [109, 119]. In very severe cases, lymphopenia and multiple organ failure with indications of renal and cardiac dysfunction can also occur [115, 118]. There is limited evidence suggesting that these high pathogenic viruses replicate in non-respiratory organs and, therefore, multiple organ failure indicates a deregulated immune response. Furthermore, in the 1918 pandemic there was an unusually high mortality rate in healthy adults ranging from 15 to 34 years, which may reflect immune-mediated pathology [120]. This feature has also been observed in cases of human infections where the avian H5N1 viruses have been highly lethal in individuals of all ages [111]. Another prominent and severe manifestation seen, both in the 1918 pandemic flu and in the more recent avian H5N1 cases, was reactive haemophagocytosis [115, 119, 121], a disorder of the mononuclear phagocytic system characterized by histiocyte proliferation and extensive phagocytosis of erythrocytes, leucocytes, platelets and their precursors by activated macrophages [122, 123]. This pathology profile has been associated with the multiple organ failure observed in very severe cases, both in the 1918 pandemic as well as in H5N1 human cases [109, 115, 121]. Haemophagocytic syndrome is considered to be induced by

increased levels of activating cytokines, with high levels of soluble (s)IL-2r, IL-6, IFNγ and TNF-α associated with a poor clinical prognosis [123, 124].

Furthermore, inflammation due to cytokines (i.e. TNF-α, IFNα/β, IL-1, IL-8 and IL-6) has been associated with the pathogenesis of the disease. And although they are important components in limiting the disease, these cytokines are mainly responsible for the systemic symptoms observed, like fever and myalgias [114, 115, 119, 121, 125-127].

1.2. Mechanisms of intracellular signal transduction and influenza A viruses

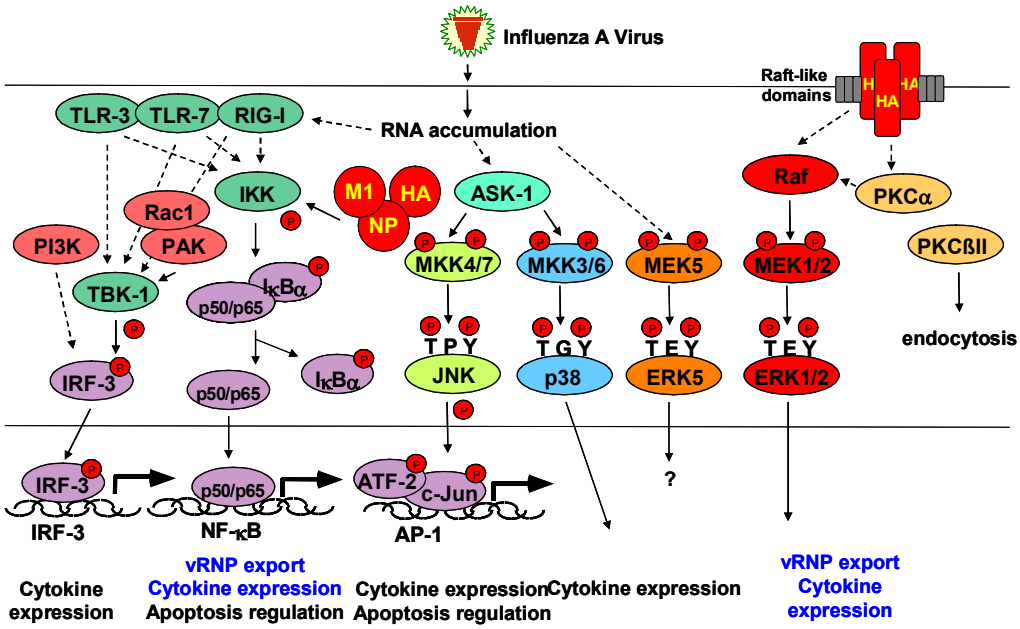

Figure 1.4: Influenza A virus-activated signalling pathways. The schematic representation depicts intracellular signalling pathways that are activated upon IAV infection or by treatment with viral components. (Adapted from Ludwig et al. [128])

Many viruses are known to activate and manipulate cellular signalling pathways [129]. These activated mechanisms lead to expression of different genes that will ultimately lead to viral clearance [130, 131].

Influenza A virus infection have been shown to activate different signalling pathways in the host cell [12, 25, 128]. Some of these cascades are depicted in Fig.1.4. Different pathways seem to be activated at different time points depending on whether it is an early or late phase of virus infection [12, 128]. PKCβII activation

seems to be required for virus entry by endocytosis [128]. IAV leads to activation of all mitogen-activated protein kinase (MAPK) family members, including the extracellular signal-regulated protein kinases (ERKs), the p38 MAPK and the c-Jun NH_2-terminal kinases (JNKs) [132-134]. Activation of the Raf/MEK/ERK pathway has been shown to be required for efficient IAV propagation [132]. p38 MAPK activation has been linked to expression of RANTES and IL-8, which are chemokines responsible for attracting eosinophils and neutrophils to the site of infection [134, 135]. JNK has been shown to be activated upon productive replicating virus and induced by accumulated RNA produced by the viral polymerase [128, 133]. Activated JNK leads to activation of activator-protein 1 (AP-1). AP-1 includes c-Jun and ATF-2 transcription factors which, together with nuclear factor kappa B (NF-κB) and IFN regulatory factor (IRF)-3/-7 are important regulators of the IFNβ expression, one of the most potent antiviral cytokine, and a hallmark in virus infections [69, 128, 136]. As already allured to, NF-κB is activated by IAV, as is the IRF-3/-7 transcription factor [71, 137]. NF-κB seems to be important to mount an antiviral state in cells, since it leads to expression of many pro-inflammatory and antiviral cytokines including IFNα/β. However, in studies using pre-activated NF-κB or impaired NF-κB signalling, it has been demonstrated that this pathway is also important to support virus replication [138, 139].

Most of these activated cascades (mentioned above) have been mainly considered to be events triggered by the cell as a defence mechanism (antiviral response). However, what seems to be evident is that influenza A virus has acquired the capability to evade and manipulate these responses in benefit of its own survival purposes, as shown for the Raf/MEK/ERK and NF-κB pathways [12, 128].

1.2.1. The MAPK pathway (Raf/MEK/ERK signalling cascade)

Mitogen-activated protein kinase (MAPK) cascades are important signalling pathways that transduce many extra- and intracellular signals converting them into several cellular responses that lead to: growth, differentiation, development, inflammation and apoptosis [131, 140, 141]. They also control numerous regulatory processes during development and homeostasis [142-144]. More than a dozen MAPKs have been identified in mammals, and are organized in at least four different families [140, 145]. MAPKs are activated via phosphorylation by distinct upstream kinases on both tyrosine and threonine residues within their catalytic domains. In

mammalian cells, three distinct MAPK families have been well described: the extracellular signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs) and the p38/MAPK [140, 145]. Each cascade consists of at least three enzymes that are activated in series: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAP kinase (MAPK). One of the best characterized MAPK signaling pathways is the Raf/MEK/ERK pathway [140, 141, 145] (Figure 1.5).

In mammals the ERK module, also known as the classical mitogen cascade, consists of serine/threonine kinase Raf, the S/T dual-specificity kinase MEK (Mitogen-activated protein kinase/ERK kinase) [146] and the classical MAPK ERK (Extracellular-signal-regulated kinases). There are two known isoforms for both MEK and ERK (MEK1/2 and ERK1/2), while three isoforms, A-, B- and C-Raf (or Raf-1) have been identified for the Raf kinase. In their protein sequence, ERKs contain a Thr-Glu-Tyr (TEY) motif and become strongly activated, through phosphorylation of the Thr and Tyr residues [131, 141].

The Raf/MEK/ERK cascade is strongly activated by mitogenic stimuli, growth factors and cytokines and transduce signals from cell surface receptors to transcription factors, which regulate gene expression, which in turn can effect cell cycle progression, apoptosis or differentiation [147]. A schematic presentation of the Raf/MEK/ERK cascade is represented in Figure. 1.5.

Binding of cytokines, growth factors or mitogens to their appropriate receptors, leads to the activation of the coupling complex Shc/Grb2/SOS. Upon stimulation by Shc/Grb2/SOS, the inactive Ras exchanges GDP for GTP, leading to its conformational change, thereby becoming active [148, 149]. Phosphorylated tyrosines on receptors or on receptor substrate proteins serve as docking sites for SH2-domain (Src homology) of adapter protein GrB2, which binds itself via its SH3-domain to the proline rich motif of the GDP-GTP exchange factor SOS (son of sevenless) [141, 150]. SOS will interact with Ras and activates the exchange of GDP to GTP [131, 149-151]. Ras is a small GTP-binding protein, and is the common upstream molecule of several signalling pathways including Raf/MEK/ERK, PI3K/Akt and RalGEF/Ral [148, 150, 152-154]. To date four Ras proteins have been identified namely Ha-Ras, N-Ras, Ki-Ras 4A and Ki-Ras 4B. Ras has to be farnysilated or geranylgeranylated at its carboxy-terminus to become active and therefore to be targeted to the cell membrane [150, 154]. GTP loaded Ras leads to recruitment of

the cytosolic serine/threonine kinase Raf to the cell membrane, a membrane shuttle kinase, and to its activation [145, 155, 156].

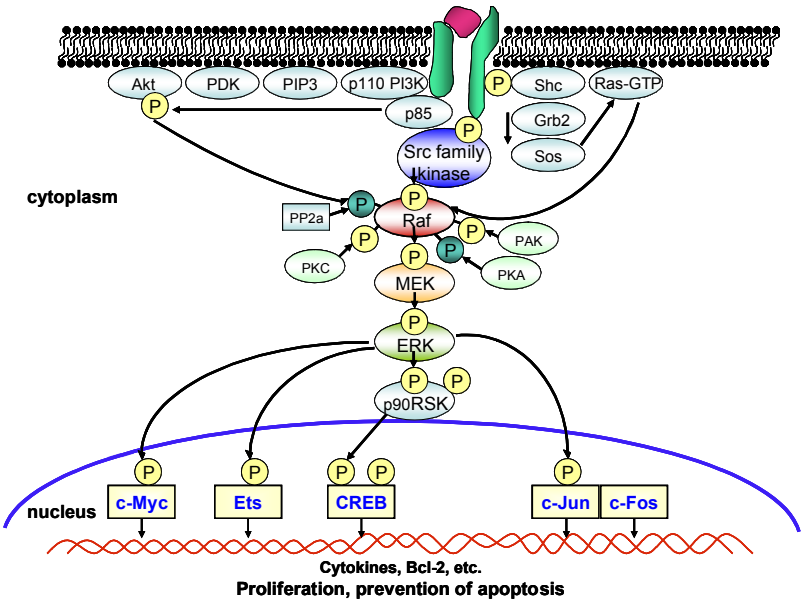

Figure 1.5: Overview of Ras/Raf/MEK/ERK pathway. The picture shows regulation of Raf/MEK/ERK by Ras as wells as other kinases, which serve to phosphorylate S/T and Y resides on Raf. Some phosphorylation (P) events enhance Raf activity (depicted in yellow background), whereas others serve to inhibit Raf activity (shown with dark green background). Moreover, there are phosphatases like PP2a, which remove phosphates on certain regulatory residues. Activation of the PI3K/PDK/AKT pathway is also shown, as this pathway interacts with the Raf/MEK/ERK pathway to regulate its activity. PI3K can be activated by two mechanisms; either the p85 PI3K subunit can bind the activated IL-3Rb chain or Ras. Activated ERK can enter the nucleus and phosphorylate transcription factors. (Adapted from Chang et al. [148])

Raf is a serine threonine (S/T) kinase and its regulation involves a complex series of events involving [131, 157]: (1) recruitment to the plasma membrane mediated by interaction with Ras [154]; (2) dimerization/oligommerization of Raf proteins [158]; (3) phosphorylation/dephosphorylation on different domains [152, 159, 160]; (4) disassociation from the Raf kinase inhibitory protein (RKIP) and (5) association with scaffolding complexes that join the pathway components and ensure efficiency and fidelity of signal transduction [141, 148, 150]. Raf activation is mediated by multiple kinases like Src, PKC (protein kinase C) and Pak3 (p21 (Rac/Cdc42)-activated protein kinase) which lead to phosphorylation and positive regulation of Raf [131,

157, 161-165], whereas other kinases like PKA (protein kinase A) and Akt downregulate Raf activity [166, 167]. Activation of Raf can be further modulated by chaperonin proteins including Bag1, 14-3-3 [168] and heat shock protein 90 (Hsp90) [169]. One of the first characterized substrate of Raf isoenzymes was the dual specificity kinase MEK [170].

MEKs are dual specificity-kinases that are able to phosphorylate both serine/threonine and tyrosine residues on MAPKs [150]. The activity of MEK1 is positively regulated by Raf phosphorylation on S residues in the catalytic domain [146, 150] while activation of ERK1/2, also S/T kinases, are positively regulated by phosphorylation mediated by MEK1 and MEK2. Once activated, MEK transduces the signals through phosphorylation on T-E-Y-motifs in the MAP-kinases ERK1 (p44) and ERK2 (p42) [150] (Figure 1.7.). ERK phosphorylation increases its catalytic activity, mediates oligomerisation and moderates the shuttling of the kinase to the nucleus. ERKs are nuclear shuttle kinases and can directly phosphorylate many transcription factors including Elk-1, ETS-1, c-Jun and c-Myc [171-173]. ERK can also target proteins e.g. serine/threonine kinases like 3pK, RNA-polymerase II, phospholipase A2 [173-175] and phosphorylate and activate the 90kDa ribosomal S6 kinase (p90 RSK), which then leads to the activation of the transcription factor CREB [147, 171-174, 176]. It has also been described that MAPK/ERK activated kinase kinase (MEKK), through an indirect mechanism, can lead to the activation of NF-κB transcription factor, by phosphorylating and activating inhibitor kB kinase (IKK) or/and IκB [141, 150, 177, 178]. Expression of constitutively active components of the ERK pathway cause cell transformation and have been identified in several cancers [150, 153, 175]. In support to this, activated Raf/MEK/ERK cascade has been shown to phosphorylate caspase 9 (an important enzyme in apoptosis induction) on residue T125 which contributes to the inactivation of this protein [179].

Another observation from this pathway is the fact that recent studies in an allergic model, have shown that the Raf/MEK/ERK is also important in controlling interleukin 8 (IL-8) expression [180] (see also Fig. 3.4.1).

1.2.1.2. Role of Raf/MEK/ERK signalling cascade in influenza A virus infection

As mentioned before, both replication and transcription of the influenza virus genome takes place exclusively within the nucleus of the infected cells [181] (Figure 1.6). The viral RNPs will be exported from the nucleus through the cytoplasm and to the cell

membrane to be engulfed into budding progeny virions late in the infectious cycle [5, 132, 181, 182]. Influenza viruses interact with many different cellular functions during their replication, to promote efficient propagation [12, 13, 128, 183]. Activation of the Raf/MEK/ERK (MAPK) signalling cascade is one of the key players for the efficiency of virus propagation, as already mentioned.

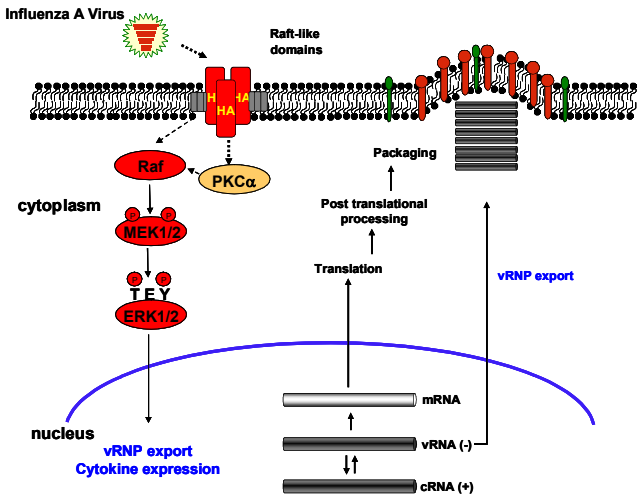

Figure 1.6: Late activation of Raf/MEK/ERK pathway in influenza A virus infection. The cartoon depicts Raf/MEK/ERK activation during late phase of virus replication leading to vRNP export and efficient viral assembly at the cell surface, for budding.

This pathway is activated both early and late during the influenza virus replication cycle and is required for an efficient nuclear RNP export [132, 181, 182]. Analysis of this pathway has been facilitated by the use of highly specific MEK-inhibitors [184, 185]. It was shown for the first time that blocking of this signalling cascade by specific MEK inhibitors, or using dominant negative mutants of ERK or Raf, led to impaired IAV as well as influenza B virus replication [132, 182]. This mechanism seemed to be due to retention of viral RNP in the nucleus and probably due to impaired activity of the viral nuclear export protein (NEP or NS2) [132, 182]. In support, studies using cells expressing active mutants of either Raf or MEK, virus propagation was shown to be increased [182]. This has also been confirmed by in vivo experiments, using transgenic mice expressing an active Raf in lung alveolar epithelial cells [186]. Here virus was shown to preferentially replicate in cells expressing the mutant gene, and mice survival was greatly impaired in this group [186]. What was also interesting was

that inhibition of this pathway with a specific MEK inhibitor, the U0126, did not hamper viral RNA or protein synthesis [132], showed no toxicity effect on cell viability or in an *in vivo* mouse model [132, 182, 187, 188], and did not lead to emergence of resistant virus variants in tests with IAV as well as influenza B virus IBV [182].

1.2.2. The NF-κB pathway

Transcriptional regulators of the NF-κB/IκB family promote the expression of over 150 target genes**,** most of which play an important role in the host immune response [128, 189-192]. These proteins include many pro-inflammatory cytokines and chemokines (IFNβ, TNF-α, IL-1β, IL-6, RANTES and IL-8, etc.) receptors important for immune recognition, proteins involved in antigen presentation, acute phase proteins and adhesion molecules necessary for transmigration across blood vessel walls. Due to this vast role in immune action, NF-κB is known as the central mediator of the immune response [193-197]. NF-κB has also been associated both with apoptosis and anti-apoptotic mechanisms [194, 195, 198].

The NF-κB pathway seems to have been developed early in evolution and has been identified in Drosophila and mollusc [189, 199]. The function and also the components of NF-κB pathway have been evolutionarily conserved in mammals [200].

Nuclear factor-κB (NF-κB) proteins form a group of closely related transcription factors, of which there are five known members in mammals: p65 (RelA or NF-κB3), RelB, and c-Rel (Rel), p50 (NF-kB1) and p52 (NF-kB2) [189, 191, 200, 201].

All five members share an N-terminal NF-κB/Rel homology domain (RHD) that mediates DNA binding, dimerization, nuclear translocation, and interaction with the inhibitory IκB proteins [189, 191, 200-203].

The NF-κB, p65, RelB, and c-Rel contain C-terminal trans-activation domains (TADs) that trigger target gene transcription. p65 is the strongest gene activator and contains two potent trans-activation domains within its C terminus [189, 191, 204]. p50 and p52, are produced as large precursor proteins (p105 and p100, respectively), where p50 is generated by constitutive processing of p105, and the cleavage of p100 to p52 is a regulated event that employs phosphorylation and ubiquitination steps [201, 205]. The p50 or p52 homodimers are also able to repress transcription [204, 206].

The most abundant and fast activated and prototypical form of NF-κB is a heterodimer between p50 and p65 (Figure 1.7), however slow activated dimmers such as p52/RelB can replace the activated p50/p65 heterodimers depending on the promoter context [207]. NF-κB is generally found retained in the cytoplasm in an inactive form when associated with inhibitory NF-κB (IκB) proteins, in most cell types [200, 202, 208]. These inhibitory proteins share a number of protein/protein interaction domains called ankyrin repeats and are part of a large genetic family with eight known mammalian members: IκBα, IκBβ, IκBε, IκBγ, IκBNS, Bcl-3, and the p100 and p105 precursor proteins [189, 200]. While IκBβ protein caps the nuclear localization sequences (NLSs) of both p50 and of p65, the IκBα only contacts the NLSs of p65 [200, 209, 210]. Activated NF-κB also leads to increased synthesis of IκBα which can shut down NF-κB-induced gene expression by mediating nuclear export and dissociation of DNA-binding subunits. Therefore, in this way NF-κB activation can be regulated by a negative feedback mechanism [189, 210, 211].

There are two general major pathways mediating NF-κB activation, the so-called canonical (classical) and non-canonical (alternate) NF-κB pathways [201, 203].

One of the critical requirements of the **canonical NF-κB activation pathway** is the activation of the IκB kinase (IKK) complex. All NF-κB pathways converge in this step which ultimately leads to phosphorylation of IκBα at serines 32 and 36 [212-216].

The IKK complex is composed of three core subunits: the catalytic IKKα and IKKβ subunits and the noncatalytic, regulatory NEMO (NF-κB essential modulator, also known as IKKγ and IKKAP-1) protein [203, 212, 213, 216, 217]. The IKKα and IKKβ share an N-terminal kinasse domain, and a C-terminal containing leucine zipper (required for IKK dimerization), and putative helix-loop-helix motifs (necessary for IKK regulation) [215]. It is believed that activation of both IKKs depends on their ability to dimerize [194, 200, 215, 216]. IKK activation depends on phosphorylation (IKKα or IKKβ) at two conserved serine residues within their activation loops. This phosphorylation may be mediated by trans-autophosphorylation induced by conformational changes or by upstream-acting kinases [200, 206]. Many diverse stimuli can induce NF-κB activation through different signalling pathways that converge on the IKK complex. Some of these include the: NF-κB-inducible kinase (NIK), mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1

(MEKK1), RNA-dependent protein kinase (PKR), protein kinase C, etc. [195, 218]. Also, genetic targeting experiments have shown that both IKKβ and IKKγ (NEMO), but not IKKα, are essential for NF-κB activation by pro-inflammatory stimuli, suggesting that both kinases have different functions [191, 206].

Following phosphorylation of IκBα by IKKβ, a specific ubiquitin ligase, $E3RS^{Ikb}$, belonging to the SCF (Skp-1/Cul/F box) family and an E2 of the UBC4/5 family, will lead to its subsequent polyubiquitination [201, 219]. The ubiquitin marked IκB proteins are then rapidly degraded by the 26S proteasome, thereby releasing NF-κB [193, 212, 219]. Activated NF-κB translocates to the nucleus, where it stimulates transcription of genes containing the consensus sequence 5'-GGGRNNYYCC-3', and transcriptional activity [189, 193, 220] (Figure 1.7).

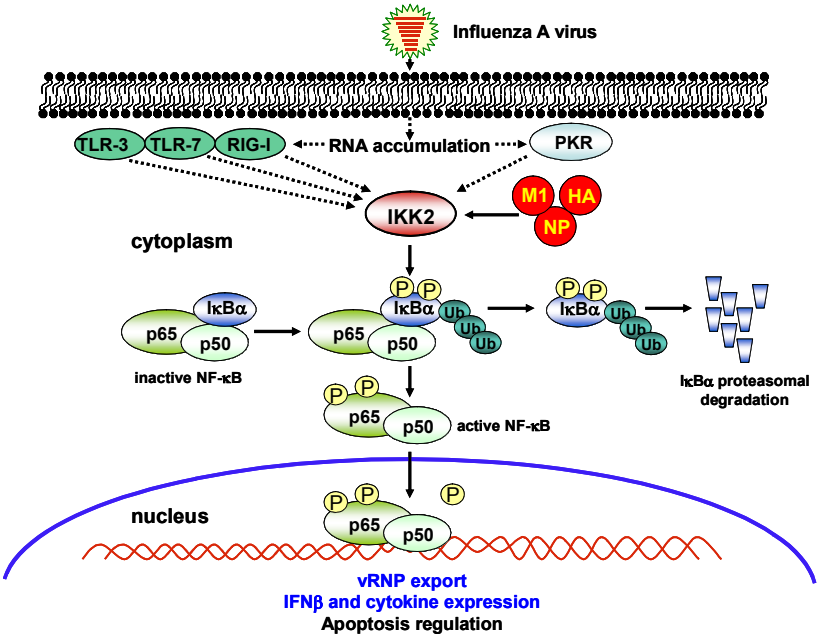

Figure 1.7: Overview of NF-κB pathway. The picture depicts the activation/regulation of NF-κB pathway. Intracellular expression of viral proteins, such as HA, NP and M1 and accumulation of viral RNA species, indirectly via PKR, TLRs or RIG-I, can activate IKK kinase, upstream of NF-κB. Activated IKK phosphorylates of IkBa leading to it's ubiquitination and degradation. NF-κB heterodimer (composed p50 and p65 subunits) is therefore released and can translocate into the nucleus and transactivate responsive genes. (Adapted from Ludwig, [12, 128]).

The **non-canonical pathway of NF-κB** activation is operative mainly in B cells in response to stimulation of a subset of TNF-receptor (TNFR) superfamily. Stimulation of these receptors activates the protein kinase NIK, which in turn activates IKKα. Subsequently IKKα phosphorylates p100 at two C-terminal serine residues, leading to selective degradation of its IκB-like domain by the proteasome. The mature p52 subunit and its binding partner, RelB, translocate into the nucleus to regulate gene expression [201, 203].

1.2.2.1. Role of NF-κB signalling cascade in influenza A virus infection

NF-κB can be activated by an array of different stimuli, such as inflammatory cytokines, mitogens, stress-inducing agents and many bacterial and viral pathogens. This activation is a rapid and immediate event, which occurs within minutes after stimulation [193, 195]. As already mentioned many of its target genes, such as growth factors, cytokines and their receptors, and proto-oncogenes, can influence dramatically the host cell cycle. Probably because of this it constitutes an attractive target to viral pathogens since many viruses have evolved strategies to regulate this pathway, such as: human immunodeficiency virus-1 (HIV-1), human T-lymphotropic virus-1 (HTLV-1), hepatitis B and C viruses (HBV and HCV), Epstein-Barr virus (EBV) and influenza virus [193, 195]. In a viral context NF-κB modulation can promote several functions: sustain viral replication, host cell survival and mediate the immune response to the invading pathogen. Also, since NF-κB has been shown to modulate both pro- and anti-apoptotic mechanisms [221] some viruses have been shown to exploit the anti-apoptotic properties of NF-κB to evade the host defence mechanisms, which limits replication by killing infected cells, or in contrast to induce apoptosis as a mechanism to increase virus spread [193].

It has been shown that influenza A virus infection results in activation of the NF-κB pathway, either by over-expression of viral proteins such as HA, M1, and NP [222, 223] as well as double-stranded (ds) and single-stranded (ss) RNA. The sensor mechanisms for this can be Toll-like receptors, such as TLR-3 -7/8, and RNA helicase proteins, such as RIG-I and mda-5 [224-229]. Furthermore, studies on RIG-1 and mda-5 in influenza virus infections show a significant increase in IFNβ promoter activity [230] (Figure 1.7). Nevertheless, it is important to mention here that, in part, the levels of NF-κB activation seem to be limited by the viral NS1 protein [71].

As mentioned before NF-κB controls many antiviral and pro-inflammatory cytokines, and along with IRF-3/7 and AP-1, forms the important regulators of IFNβ, one of the most potent antiviral cytokines of the innate immune response. Therefore, upon activation one would expect an anti-viral effect of this pathway. Supporting this notion, in A549 cells (human alveolar epithelial cell line) infected with IAV, the kinetics of IFNβ gene expression correlated with NF-κB activation [231]. Also IAV induction of NF-κB was related to increase in expression of other pro-inflammatory cytokines and production of high levels of IL-8 [232, 233]. In other experiments this was again demonstrated, using trans-dominant negative mutants of IKKβ or IκBα, IFNβ promoter activity significantly decreased upon IAV infection [71].
However, in part contradicting this theory, were studies showing that in cells with a pre-activated NF-κB, influenza viruses showed higher replication levels [139]. And confirming these findings impaired NF-κB signalling led to a reduction in virus titres [71]. This function was shown to be in part due to NF-κB-dependent viral expression of pro-apoptotic factors, such as TNF-related apoptosis inducing ligand (TRAIL) or FasL, which enhanced virus propagation in an autocrine and paracrine fashion [128, 138]. Also studies using specific inhibitors of this pathway showed impaired virus propagation, both *in vitro* as well as *in vivo* [234]. Therefore this pathway, in the context of an IAV infection, presents both an antiviral as well as a pro-viral effect, where the pro-viral seems to prevail over the antiviral effect. One can speculate whether these two functions are consistent for all influenza A viruses, or whether either effect shows different prevalence's, depending on which virus and at what time point in the infection cycle NF-κB is activated by one specific strain. Nevertheless, most reports seem to point to this pathway being important for both, host as well as viral regulating mechanisms. One study, however, has shown that NF-κB was not essential for virus replication [232].

1.3. Immune response and cytokine interplay

Upon influenza A virus infection, the epithelial cells of the respiratory tract and leukocytes which are the primary targets of the virus, will induce both innate and adaptive immune responses [113, 235, 236]. Respiratory epithelial cells can produce large amounts of virus, which can further infect alveolar macrophages (AMs) [231, 236] (Figure 1.8). Upon infection cells will undergo either apoptosis or necrosis [113,

237] and trigger immune responses and the production of cytokines and chemokines [25, 236, 238]. These inflammatory mediators have been shown to be up-regulated both in *in vitro* [238-242] as well as in *in vivo* experimental influenza infection [120, 243, 244].

Mutant viruses expressing the HA and NA of the 1918 influenza virus or all 8 segments have been shown to induce significantly higher levels of IFNγ, TNF-α, monocytes chemotactic protein-1 (MCP-1), RANTES, IL-6, IL-8 and up-regulated related genes, such as NF-κB, *in vivo* supporting the case that elevated chemokine and cytokine levels were a hallmark of the human disease elicited by this virus [120, 243, 245]. Also *in vitro* studies have demonstrated superior induction of pro-inflammatory cytokines in macrophages, in particular TNF-α and IFN-β by H5N1 viruses compared to other human H3N2 and H1N1 viruses [126]. In another study, by the same group, different H5N1 viruses (A/Hong Kong/483/97, A/Vietnam/1194/04 and A/Vietnam/3046/04) induced higher IFN inducible protein-10 (IP-10), IFN-β and RANTES compared to H1N1 in primary human alveolar and bronchial epithelial cells [240]. Generally, disease severity was strongly associated with cytokine levels [114, 115, 127]. Chemokine release, including MCP-1 and RANTES, was found increased in adult/neonatal macrophages infected by avian H5N1 and H9N2 compared to human H1N1-infected cells [244].

In a study using human alveolar epithelial A549 cells, type I IFNs were shown to be important for the activation of antiviral response genes, such as the MxA [231].

How influenza viruses effect hypercytokinaemia is still poorly understood, but both epithelial cells as well as monocytes/AMs and dendritic cells can activate different transcription factors upon viral infection that lead to production of pro-inflammatory and chemotactic cytokines, such as IFNα/β, IL-6, IL-8, MCP-1, RANTES, TNF-α and others [25, 128, 227, 233, 237, 246-248].

In general the most likely *in vivo* situation is that upon cytokine/chemokine production by infected epithelial cells and/or AMs, monocytes/macrophages, neutrophils and T cells are induced to migrate from the blood stream through the endothelial barrier into the site of infection [249] (Figure 1.8). The recruitment of monocytes/macrophages into the lung parenchyma and alveolar spaces, is a hallmark of the initial adaptive immune response [237, 249, 250], and although they are required for host recovery [251-253], an increase of their presence can lead to exaggerated inflammatory and

immune responses, as mentioned above, which may contribute to the exacerbation of the disease and the high mortality observed with some highly pathogenic influenza viruses, such as H5N1 and the 1918 pandemic virus H1N1 [112, 114, 115, 119, 127, 249, 254].

Whereas the innate immune response is mainly responsible for controlling virus replication in the early stages of infection, the adaptive immune response is responsible for limiting progression of the disease. Adaptive immune responses are generally essential to eliminate the virus completely [235, 253, 255, 256]. The innate immune response is also responsible for triggering adaptive immune response mechanisms by secretion of cytokines which will further lead to the recruitment of other immune cells (macrophages, neutrophils and natural killer – NK cells) to the site of infection [235, 255, 257]. In turn these cells will lead to further production of cytokines, chemokines and other anti-viral proteins [257]. NK cells which are large granular lymphocytes, detect and bind to virus-infected cells [255]. Upon binding NK cells release the contents of their granules (containing perforins and granzymes) into the infected cell, thereby inducing apoptosis [115, 258].

A brief overview of some of the cytokines/chemokines and their main targets and functions are explained later.

As mentioned above, the innate immune response slows virus replication and prevents spread during the first few days of infection but survival might only be achieved by the “adaptive immune response” [255]. Moreover, one of the important features of the adaptive immune response is the “immunological memory” that is achieved for a specific pathogen. If an individual has been previously exposed to the same antigen, the adaptive immune response is much faster, due to this “immunological memory” [259].

Adaptive immune responses rely on the activation of antigen-specific B and T lymphocytes and the production of antibodies. Adaptive immune responses begin when T cells recognise viral peptides presented in the context of major histocompatibility complex (MHC) on antigen presenting cells (APCs), such as macrophages or dendritic cells [250]. Immunological memory of B cells is lifelong but where influenza is concerned not very efficient since it is strain specific within an HA subtype, whereas T cell memory can vary from months to years or longer, but can distinguish between types (A or B) and not between the influenza A subtypes [253, 260, 261].

Antibodies produced by B cells bind and neutralize virus on the mucosal surface to prevent cell entry and further replication of viruses [261].

There are two main types of T lymphocytes; T helper (Th) cells – mainly $CD4^+$, and cytotoxic T lymphocytes (CTLs) – mainly $CD8^+$. Memory Th cells can be subdivided into Th1 or Th2 types, and these secrete specific subset of cytokines. Th1 cells stimulate cell-mediated CTL immune response, whereas Th2 responses lead to antibody production. Th1 can best deal directly with intracellular invaders [259, 262].

CTLs recognize cells expressing surface major histocompatibility complex class I (MHC I)-viral peptide complexes which results in production of pro-inflammatory cytokines such as IFNγ or, like NK cells, granule-mediated killing of the target cell [261]. These granules, containing perforins and granzymes, are transported to the surface of activated CTLs where contact with the target cell occurs. Granzymes are then transported across the cell membrane into the cytoplasm of the target cell and lead to induced apoptosis of the same [258, 261, 263].

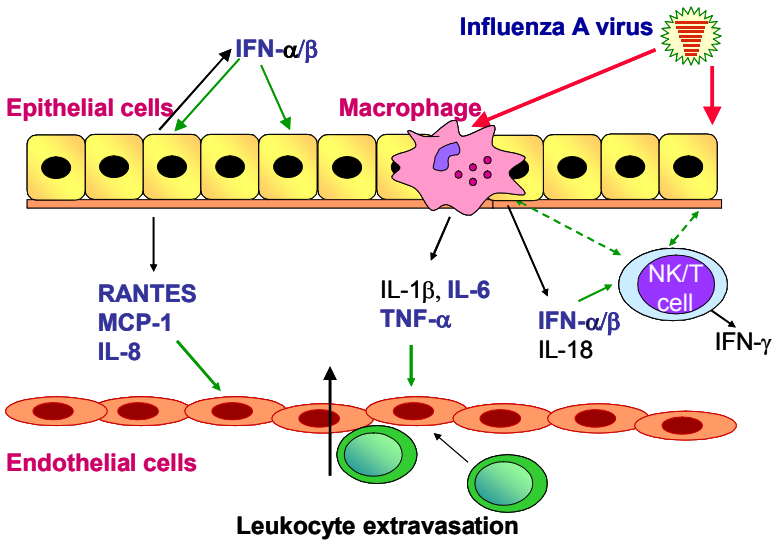

Figure 1.8: Cytokine interplay. A schematic picture showing some of the key cytokines/chemokines and their interplay during influenza a virus infection. The cytokines highlighted in bold blue are the key cytokines studied in this work. Red arrows indicate the cells the virus infects, black arrows indicate the secreted cytokines and green arrows indicate to which cells cytokines will exert an affect. (Adapted from Julkunen [25]).

H5N1 viruses have also been shown to affect specific immunity. In the presence of H5-bearing cells, perforin expression was suppressed and the cytoxicity of CD8+ T

cells reduced, and this led to lymphoproliferation, overproduction of IFNγ and macrophage activation [264, 265].

Cytokines

One of the most potent anti-viral cytokines produced by the innate immune response is the interferon. Interferon (IFN) was discovered as an antiviral agent during studies with influenza virus interference.

- IFNs can stimulate cells both in an autocrine as well as in a paracrine manner. In general, cells can synthesize IFN in response to an external stimulus such as viral infection and cells can respond to IFN by establishing an antiviral state [69, 266, 267]. IFNs are commonly grouped into two types [69, 262]. Type I IFNs are also known as viral IFNs and include IFN-α, IFN-β. Type II IFN is also known as immune IFN (IFN-γ). Interferons (IFN-α/β) are one of the most important cytokines in viral infections and have several functions: (1) they can induce an anti-viral state to neighbouring cells by promoting production of anti-viral proteins; (2) they lead to recruitment of monocytes/macrophages and T cells (including natural killer (NK) cells); (3) they stimulate increase production of MHC (major histocompatibility complex) class I and II, which will enhance antigen presentation; and (4) increase maturation of antigen-presenting cells (APCs), thereby leading to adaptive immune responses [266, 268, 269].
- IFN-γ, type II interferon, is secreted by activated T cells (Th1 cells) and NK cells. IFN-γ can stimulate macrophages, increase antigen processing and expression of MHC [250, 266, 267].
- IL-1α and IL-1β are produced mainly by mononuclear and epithelial cells upon inflammation, injury and infection. They trigger fever, induce a wide variety of acute phase response (APR) genes and activate lymphocytes.
- IL-18 is a pro-inflammatory cytokine which in synergy with IL-12, enhances NK cell activity and promotes inflammatory Th1 cell responses [250].
- IL-6 is generally produced by fibroblasts, endothelial cells, macrophages and leukocytes and is a primary inducer of fever, hormones, acute phase proteins and T and B cell expansion upon injury and infection. IL-6 can also act as an anti-inflammatory agent in some instances.

- IL-12 is produced by antigen presenting cells (APCs) and has immunoregulatory effects on NK cells and T cells. IL-12 is important in cell-mediated immunity by pushing the balance between Th1 cells and Th2 cells towards Th1-type predominance. IL-12, in synergy with TNF-α, can also elicit the production of large amounts of IFN-γ by NK cells.
- TNF-α is a pro-inflammatory cytokine originally identified as a tumor cell killer, and mainly produced by activated macrophages, NK cells and T cells (mainly Th1 cells). TNF-α plays a role in endothelial activation and lymphocyte movement and is one of the crucial mediators in acute and chronic inflammatory conditions [250].

Chemokines

These are small secretory molecules that are produced by a variety of cells constitutively or in response to microbial/viral infection [250, 270]. Chemokines bind to specific cell surface receptors on leukocytes, which will lead to a rapid change in the cell shape and behaviour enabling them to migrate from the blood stream through the vascular endothelium into the site of inflammation [270-272].

CC group of chemokines target many different cells, such as: monocytes, T cells, dendritic cells, eosinophils and NK cells. These include monocyte chemotactic protein (MCP)-1/CCL2, macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, regulated upon activated normal T cell expressed and secreted (RANTES)/CCL5 and eotaxin/CCL11 [250, 273].

The CXC group of human chemokines mostly mediates neutrophil chemotaxis and include: IL-8/CXCL8, monokine-induced by IFN-γ (MIG)/CXCL9 (nonELR), IFN-γ inducible protein-10 (IP-10)/CXCL10 and stromal cell-derived factor-1 (SDF-1)/CXCL12.

Lastly, the sole CX3C type chemokine, namely fractalkine/CX3CL1, binds CX3CR1 and attracts T cells and monocytes but not neutrophils [273, 274].

Cytokine	Abbreviation	Cell Source	Target Cells	Primary Effects
Type I Interferons (IFN)	IFN-α IFN-β	All	All	Antiviral, NK cell cytotoxicity, induction of class I MHC
Type II Interferons (IFN)	IFN-γ	NK cells	Monocytic cells, most others	Activation, induction of class II MHC, CD4 T-cell differentiation
Tumor necrosis factor (TNF)	TNF-α	Monocytic cells, NK cells, others	PMN, endothelial cells, hypothalamus, others	Activation of adhesion, inflammation, fever, cell death, antiviral, induction of class I MHC
Interleukin 1	IL-1 α and β	Monocytic cells, others	Endothelial cells, hypothalamus, others	Inflammation, fever
Interleukin 6	IL-6	Monocytic cells, others	B cells, liver	Growth, acute phase reactants, fever
Interleukin 8	IL-8	Neutrophils and T cells		Chemoattractant and activates neutrophils
Interleukin 12	IL-12	Monocytic cells, dendritic cells	NK and T cells	IFN-γ production, CD4 T-cell differentiation
Interleukin 18	IL-18	Monocytic cells, few others	NK and T cells	Enhances IFN-γ production
monocyte chemotactic protein -1	MCP-1 (CCL-2)	Respiratory epithelium and monocytic cells	Monocytes, T cells and dendritic cells	Chemoattractant
Regulated upon activation normal T cells expressed and secreted	RANTES (CCL-5)	Respiratory epithelium and T cells	Monocytes, T cells and dendritic cells	Chemoattractant and activates T cells

Table 1.2: List of some important cytokines and chemokines in influenza virus infection. Adapted from La gruta, Immunology and Cell Biology, 2007 and Biron and Sen, Innate responses to viral infections Chp 9, Fields Virology, 5th edition.

1.4 Aims

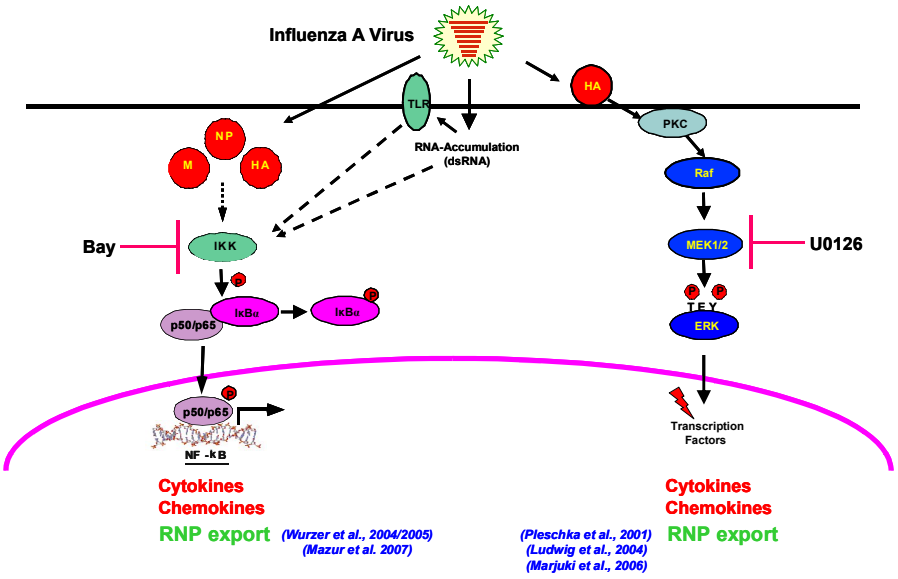

Figure 1.9: The two cellular pathways targeted in this study (NF-κB) and (Raf/MEK/ERK). The cartoon shows the activation of the two pathways by IAV particles, and the specific targets of the inhibitors used in this study; the IKK inhibitor (Bay 11-7082) for the NF-κB pathway and a MEK inhibitor (U0126) for the Raf/MEK/ERK pathway. Both pathways are required for (1) efficient virus propagation, by means of vRNP export from the nucleus, as well as (2) antiviral functions, by means of cytokine/chemokine expression, upon virus infection. (Adapted from Pleschka).

Influenza A viruses are segmented ssRNA viruses which mainly target the epithelial cells of the respiratory tract in humans and generally cause acute, self-limiting infections [1, 25, 109]. Possible risks of complications and death are, however, greatly increased in young children, the elderly [109, 111, 116, 117] or when the population comes into contact with new highly virulent virus strains [76, 109, 126]. Increase virulence and high viral loads as well as severe lung inflammation due to cytokines (i.e. TNF-α, IFNα/β, IL-1, IL-8, MCP-1 and IL-6), has been linked with the pathogenesis of these HPIV strains. The associated hypercytokinaemia is thought to be responsible for the main systemic symptoms observed and the severity of the disease [114, 115, 119, 121, 125-127].

Therapeutics strategies in the form of antiviral drugs such as amantadine and rimantadine, which target the viral M2 ion channel protein as well as neuraminidase inhibitors, zanamivir and oseltamivir, can reduce the duration of symptoms of clinical

influenza, but the appearance of drug-resistant variants have already been reported [12, 106, 275-278]. Vaccination is also an option, but in the possible appearance of a pandemic situation, the appropriate vaccines would not be produced in time [279, 280] to avoid disaster. Therefore the need for new therapeutic strategies is urgent.

Upon IAV infection cells activate many signalling pathways such as; the Raf/MEK/ERK, the NF-κB, the p38 MAPK, the JNK, the IRF-3 and PI3K pathways. [12, 13, 183, 281].

Activation of the NF-κB transcription factor has been shown to be important for the expression of many pro-inflammatory and antiviral cytokines (anti-viral function) [232, 233] as well as important in supporting virus replication (pro-viral function) [138, 139, 234]. The Raf/MEK/ERK (MAPK) signalling cascade, on the other hand, has generally been linked to efficient influenza virus propagation [132, 182, 282]. This pathway has, however, in other studies also been shown to be involved in cytokine regulation [135, 283].

Specific inhibitors that block activation of the NF-κB pathway, such as Bay 11-7082 (Bay) by selectively inhibiting TNF-α inducible phosphorylation of IκBα (www.calbiochem.com), or U0126 a MEK inhibitor [284] which inhibits downstream activation of the Raf/MEK/ERK pathway, have already been previously used in studies with influenza virus [132, 139, 181, 182].

The aim of this study was to modulate both virus replication as well as virus-induced cytokine production simultaneously, by targeting these two pathways (NF-κB and Raf/MEK/ERK) using the specific inhibitors mentioned above (Figure 1.9). This idea envisioned targeting these specific cellular pathways shown to be important for virus replication and for virus-induced cytokine expression with the purpose of limiting the main factors linked to the severe disease outcome of infection with certain HPIV strains.

The aim also included comparison/correlation studies in cell culture experiments using either permanent cell lines (human alveolar epithelial cells – A549 cells) or primary cells (mice alveolar epithelial cells – AECs), as well as *in vivo* studies using C57BL/6 mice.

2. Materials and Methods

2.1. Materials

2.1.1. Instruments

Abbocath-T (26Gx19mm)	Hospira
Bio Imaging Analyzer (BAS 2000)	Fuji Film
Cell culture incubator	Heraeus; Nuaire
Cell culture microscope	Hund
Confocal laser scanning microscopy (TCS SP5)	Leica
Culture Hood (HB2448)	Heraeus
Developing machine	Optimax, Protec
Disposable Razor Med Comfort	AMPri GmbH
Electrophoresis apparatus system	Institute for Medical Virology
Electrophoresis power supply (EPS500/400)	Pharmacia
ELISA reader (Type LP 400)	Diagnostic Pasteur
FACScan	Becton Dickinson, USA
Fine scale (Mettler PM460)	Mettler Waagen GmbH
Heat block	Jumotron
Luminex™ Reader	Biorad
Magnetic stirrer	IKA Labortechnik
Megafuge 1.0 R	Heraeus
Microwave oven	Quelle
Mini centrifuge	Biofuge 13, Heraeus
Neubauer chamber	Optik Labor
Omincan 50	Braun Medical
pH meter (Type 632)	Metrohm
Shaker (Type 3013)	MSGV GmbH
Scale (P1200)	Mettler
Scanner Canonscan 9900F	Canon
SDS-PAGE gel system	Institute for Medical Virology
Sonicator (Type HD70)	Bondelin Sonoplus
Spectrophotometer (DU-70)	Beckman

Sterile needles	BD Microlance 3 BD
Syringe (microliter, serial 700)	Hamilton
Syringe 20ml single use	Braun, Melsungen AG
Tissue Ruptor	Qiagen
Vortex (Vibrofix VF1)	IKA Labortechnik
Water bath (SW-20C)	Julabo
Western-Blot chamber	Institute for Medical Virology

2.1.2. Reagents and general materials

Acrylamide	Bio-Rad
Ammonium persulfate (APS)	Serva
Annexin V-FLUOS	Roche
Aprotinin	Roth
β-mercaptoethanol (MetOH)	Roth
Benzamidin	Sigma
Blotting papers (GB004)	Scheicher & Schuell
Bradford reagent	Biorad
Bromophenol blue	Merck
BSA (Solution, 35%)	MP Biomedicals
BSA (Powder)	Roth
Chloroform	Roth
Coomassie brilliant blue R 250	Merck
Cryotubes	Nunc
DAB Peroxidase substrate (3,3'-Diaminobenzidine)	Sigma
DAPI (stock 1mg/ml)	Roth
DEAE Dextran (MW: 500,000)	Pharmacia Biotech
1,4-Diazabicyclo [2,2,2]octane (DABCO)	Merck
Dimethylsulfoxid (DMSO)	Sigma
1,4-Dithiothreit ($C_4H_{10}O_2S_2$) (DTT)	Roth
Cuvette	Biorad
Eppendorf tube	Eppendorf
Ethanol (absolute)	Roth

Ethylenediamine tetraacetic acid (EDTA)	Fluka
Falcon centrifuge tube	Falcon
Glycerol	Sigma
Glycine	Roth
Hydrochloride (HCl)	Roth
Isopropanol	Roth
Leupeptin	Sigma
Magnesium chloride ($MgCl_2$)	Merck
Methylcellulose (methocel MC)	Sigma
Microtiter plate (96 wells)	Greiner
Mowiol 40 - 88	Aldrich
Methanol	Roth
MTT (dimethylthiazole-diphenyl tetrazolium bromide)	Sigma
N-2-hydroxyethylpiperazine (HEPES)	Sigma
Paraformaldehyde (PFA)	Merck
Pefablock	Roth
Potassium chloride (KCl)	Roth
Precision Plus Protein Standards (All Blue)	BioRad
Potassium dihydrogen phosphate (KH_2PO_4)	Roth
Propidium Iodide	Sigma
PVDF-Membrane Immobilon-P transfer membrane	Millipore
Rainbow marker	Amersham
Roti-Free, ready-to-use Stripping Buffer	Roth
Scientific Imaging film BioMax MR	Kodak
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	Merck
Sodium-β-glycerophosphate	Sigma
Sodium hydroxide (NaOH)	Merck
Sodium hydrogen carbonate ($NaHCO_3$)	Fluka
Sodium orthovanadate	Sigma
Sodium pyrophosphate	Sigma
TEMED (N,N,N',N'-Tetramethyl-ethylene diamine)	Serva

Tris HCl	Roth
Triton X-100 (t-Octylphenoxypolyethoxyethanol)	Sigma
Tween 20	Roth
Trypan blue	Gibco
Whatman 3MM Papier	Schleicher & Schüll
WST-1 Cell proliferation Reagent	Roche

2.1.3. Monoclonal and polyclonal antibodies

Antibody	Company	Dilution
Anti-P-ERK (E-4) mouse monoclonal IgG	Santa Cruz Biotechnology	1:250
Anti-ERK2 (C-14) mouse monoclonal IgG	Santa Cruz Biotechnology	1:500
HRP conjugated goat anti-mouse IgG	Santa Cruz Biotechnology	1:1000
Anti-flu A NP (FPV) mouse (clone 1331)	Biozol–Biodesign Internat.	1:6000
Anti-influenza A PR/8/34 chicken polyclonal IgY	Biomol – US Biological	1:50
Texas Red conjugated rabbit anti-mouse	Dianova	1:200
biotinylated rat anti-mouse CD16/32	BD Pharmingen	1:?
biotinylated rat anti-mouse CD45 mAbs	BD Pharmingen	

2.1.4. Materials for cell culture

Dulbecco's Modified Eagle's medium (DMEM)	Invitrogen/Gibco
Fetal calf serum (FCS)	PAN
penicillin-streptomycin solution (100x)	PAA
Trypsin-EDTA (10x)	PAA
Tissue culture dish	Becton Dickinson
Tissue culture flask	NUNC
Bovine Albumin	ICN

2.1.5. Materials for mice experiments

Agarose low melting	Sigma Aldrich
Dispase	BD Biosciences
DNase	Serva
HBSS	PAA
Isofluorane	Abbott/Baxter
MagneSphere Paramagnetic Particles	Promega
Syringe (microliter, serial 700)	Hamilton
Mesh filter membrane 100μM and 40μM	Beckton Dickinson
Filter paper 20μM	Milipore

2.1.6. Kits

AEC staining kit	Sigma
Anti-mouse IFNβ ELISA kit	BioSource/Invitrogen
Anti-human IFNβ ELISA kit	BioSource/Invitrogen
ECL (enhanced chemiluminescence) solution kit	Amersham/GE
Multiplex human cytokine kit	BioRad
Multiplex mouse cytokine kit	BioSource/Invitrogen
NF-κB TransAM ELISA kit	AtiveMotiv
Opteia set mouse TNF-α kit	BD Biosciences

2.1.7. Virus strains and cell lines

A/FPV/Bratislava/79 (H7N7)	Strain-collection in Giessen
A/PR/8/34 (H1N1)	Strain-collection in Giessen
A549 (Human Alveoar Epithelial cells)	Strain-collection in Giessen
MDCK-S (Madin-Darby canine kidney cells)	Strain-collection in Giessen
Vero (African Green Monkey)	Strain-collection in Giessen

2.1.8. Inhibitors and solvent

Bay 11-7082 (IKK inhibitor)	Calbiochem
U0126 (MEK inhibitor)	Promega
Cremophor EL	Fluka

2.1.9. Media

Dulbecco´s Modified Eagle Medium (DMEM 10L)

1x DMEM powder high glucose
37 g/10 L bicarbonate
100 ml/10 L sodium pyruvate
100 ml/10 L 100x penicillin/streptomycin
sterile filtered

Freeze medium

90% Complete DMEM
10% DMSO

DMEM/10% FCS/antibiotics (complete DMEM 0,5L)

445 ml DMEM
50 ml FCS (heat inactivated)
5 ml 100x penicillin/streptomycin

DMEM/BA (0,5L)

492 ml DMEM
5 ml penicillin/streptomycin (100x)
3 ml Bovine Albumin (BA) (35%)

2x DMEM/BA for Plaque-Assay (0,5L)

100 ml MEM (10x)
10 ml penicillin/Streptomycin (100x)
20 ml $NaHCO_3$ (7.5%)
6 ml Bovine Albumin (BA) (35%)
354 ml ddH_2O, autoclaved

2.1.10. Buffers and solutions

100x Ca^{2+}/Mg^{2+} solution (100ml)

1 g $MgCl_2$

1.32 g $CaCl_2$

ddH_2O added up to 100 ml

autoclaved, then filtered with the 0.2µm filter column

Phosphate-Buffered Saline (10x PBS) (1L)

0,137 M NaCl

0,27 mM KCl

8,1 mM Na_2HPO_4

1,47 mM KH_2PO_4

adjust total volume to 1 L with ddH_2O, autoclaved

1x PBS^{++} buffer (0,5L)

495 ml 1x PBS (autoclaved)

5 ml Ca^{2+}/Mg^{2+} solution (100x)

PBS/Ca^{2+}/Mg^{2+}/BA/antibiotic (200ml)

20 ml 10x PBS (see above)

174,8 ml ddH_2O (sterile)

2 ml penicillin/streptomycin (100x)

1,2 ml BSA (35%)

2 ml Ca^{2+}/Mg^{2+} (100x)

SDS-PAGE loading buffer (2x) (Laemmli Buffer)

100 mM Tris-HCl, pH 6.8

200 mM DTT (dithiothreitol)

4% SDS

20% glycerol

0.2% bromophenol blue

SDS-PAGE running buffer (5x) (1L)

1 g SDS

6 g Tris HCl

28,8 g glycin

adjust total volume to 1 L with ddH_2O

SDS-PAGE transfer buffer (1L)

5,8 g Tris HCl

2.9 g glycin

0.17 g SDS

200 ml methanol

adjust total volume to 1 L with ddH_2O

TLB buffer

20 mM Tris HCl, pH 7.4

137 mM NaCl

10% (v/v) glycerol

1% (v/v) Triton X-100

2 mM EDTA

50 mM sodium-β-glycerophosphate

20 mM sodiumpyrophosphate

(Addition of inhibitors for lysis buffer, see below)

Lysis buffer (10ml)

10 ml TLB buffer (see TLB buffer)

10µl Pefablock (200 mM)

10µl Aprotinin (5 mg/ml)

10µl Leupeptin (5 mg/ml)

100µl Sodium orthovanadate (100 mM)

50µl Benzamidine (1 M)

Tris-Buffered Saline (10x TBS) (1L)

24.2 g Tris HCl

80 g NaCl

dissolve by adding 900 ml ddH_2O

adjust pH to 7.6 and total volume to 1 L with

ddH_2O

TBS/Tween (0.05%) (1x TBS-T) (1L)

100 ml 10x TBS

900 ml ddH_2O

0.5 ml Tween 20

Blocking buffer and antibody diluting solution

5% (w/v) nonfat dry milk

in 1x TBS-T (see above)

2.1.11. Gels and other media

SDS-PAGE stacking gel

2,9 ml ddH_2O

1,25 ml Tris HCl, pH 6.8 (0.5 M)

50 µl SDS (10%)

750 µl polyacrylamide (PAA) (30%)

50 µl APS (10%)

4 µl TEMED

SDS-PAGE resolving gel (10%)

4 ml ddH_2O

2,5 ml Tris HCl, pH 8.8 (1.5 M)

100 µl SDS (10%)

3,3 ml polyacrylamide (PAA) (30%)

50 µl APS (10%)

6 µl TEMED

Cell fixing buffer (100 ml)

95 ml PBS++

4 ml Formaldehyde (PFA)

1 ml Triton X-100 (t-Octylphenoxypolyethoxyethanol)

Methyl cellulose stock, 3% stock for Focus assay (200ml)

6 g methylcellulose

200 ml ddH_2O

heat ddH_2O up to 90°C

add methylcellulose by using a sieve, mix thoroughly and autoclave

ripen it at 4°C for three days

aliquot in 50 ml and freeze at -20°C

Methyl cellulose media, 1.5% stock (100 ml) for Focus assay

50 ml methyl cellulose stock (3%)

1 ml Penicillin/Strepromycin (100x)

1 ml BA (30%)

10 ml MEM (10x)

4 ml $NaHCO_3$ (7.5%)

1 ml DEAE Dextran (MW: 500,000) (1%)

33 ml ddH_2O

mix well and store at 4°C

Mowiol DABCO

2.4 g Mowiol

6 g Glycerol

6 ml ddH_2O

mixed thoroughly over night. Next day add 12 ml 0.2 M Tris-HCl (pH 8.5) and incubated at 50°C for 30 min. Centrifuge the viscous mixture at 12000 g (Megafuge 1.0R, 6000 rpm) at room temperature for 15 min, and mix the supernatant with 2.5% DABCO.

U0126 and Bay 11-7082 stock

U0126 M [380,5g/L] and Bay 11-7082 [207.2g/L]

Both inhibitors were prepared to a stock concentration of 100mM dissolved in DMSO. Aliquots were then made and stored at -20^{o}C.

Cremophor EL [(Polyoxyethylenglyceroltriricinoleat 35 (DAC), Polyoxyl 35 Castor Oil (USP/NF) is a trademark of BASF, Germany] is a non-ionic solubilizer and emulsifier. It can convert hydrophobic drugs into aqueous solutions. Addition of Cremophor EL to a drug, allows for fine degree of dispersion and therefore the drug is more readily absorbed and its efficiency is increased.

For the dissolving U0126, we first used DMSO and then further added Cremophor EL in a ratio of 1:4. The above solution was then further diluted in filtered PBS (total volume 250μl/mouse) for *in vivo* application.

2.2. Methods

2.2.1. Working with cell cultures

2.2.1.1. Maintenance of cell culture

Mardin-Darby canine kidney cells (MDCK) and human Alveolar epithelial carcinoma cells (A549) were maintained in Dulbecco modified Eagles medium (DMEM) containing phenol red as a pH-indicator, supplemented with 10% heat inactivated fetal calf serum (FCS) and streptomycin/penicillin. The cells were incubated at 37°C with 5% CO_2 and 95% humidity. They were routinely cultured to 100% confluence and than passaged according to the needs.

2.2.1.2. Storage of cell cultures

For freezing; cells were washed with 1x PBS and 5 ml of 1x trypsin-EDTA was then added. They were then incubated at 37°C with 5% CO_2 and 95% humidity and left until cell detachment, after which 5ml complete media was added. Cell suspensions were then centrifuged at 350 g (Megafuge 1.0R, 1000 rpm), 4°C for 5 min. The cell pellet was gently resuspended with 1 ml freeze medium (90% complete DMEM and 10% DMSO) and transferred into cryotubes. These were set into a styropore box and

left to freeze gradually in the -80°C freezer. The DMSO prevents ice crystal formation and allows the cells to remain intact. After 24 hours, cells were transferred into liquid nitrogen where they can be kept for a longer period of time.

For thawing; cells were removed from liquid nitrogen and immediately transferred into a 37°C water bath for 5 min. Cells were then resuspended carefully and transferred into a cell culture flask filled with complete DMEM (19 ml). After 24h cells will have reached 100% confluency and should be passaged for further propagation.

2.2.1.3. Infection of cells

Influenza virus, avian A/FPV/Bratislava/79 (H7N7) and human A/PR/8/34 (H1N1), were used for infection of A549 cells. The virus inoculum was prepared by adding the according amount of virus stock to a certain volume of PBS/BA/P/S/$Ca^{2+}Mg^{2+}$ depending on the desired multiplicity of infection (moi) used for experiment. A549 cells were previously (one day before) seeded on 3.5 cm dishes and grown to confluence. The cells were then washed with 1x PBS^{++} and 100 µl of virus inoculum was laid on top by creating bubbles in the middle of dish, to ensure a consistent virus distribution. The cells were further incubated at room temperature for 1 hour, after which the inoculum was removed by aspiration and 2 ml of DMEM/BA media was added with the desired treatment (DMSO, Bay 11-7082 or U0126) or untreated. Cells were then further incubated at 37°C with 5% CO_2 for the desired time points, and further treated.

The calculation of moi was done as follows:

$$\frac{1000\ \mu l}{\text{Virus titer [PFU]}} = \frac{X\ \mu l\ \text{virus}}{\text{moi x cell amount in the culture}}$$

2.2.2. Preparation of cell lysates for Western blot analysis

At a certain time point after infection and/or treatment, cells (from 3.5cm dishes) were washed with cold 1x PBS^{++}. An extra 1 ml of cold 1x PBS^{++} was added to the cells with which they were scraped off and transferred into eppendorf cups. This was then centrifuged at 25000 g (Biofuge 13, 13000 rpm) for 1 min and the cell pellet carefully resuspended in 75 µl lysis buffer by pipetting up and down. The lysis was performed by incubating cells for 25-30 min on ice and vortexing at every 5 min intervals. The

lysed cells were then centrifuged at 25000 g (Biofuge 13, 13000 rpm), 4°C for 15 min. The supernatant was finally transferred into new eppendorf cup and stored at -70°C until further requirement.

2.2.3. Cell viability (cytotoxicity) analysis

In order to determine whether the concentration of inhibitors used for experiments would affect cell viability, MTT-assay or WST-1-ssay was performed. Both these assays measure the activity of mitochondrial dehydrogenase in the living cells. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) or (4-(3-(-iodophenyl)-2-(4-nitrophenyl)-2H-5tetrazolio)-1,3-benzene disulfoonate) (WST-1) is taken up by cells and can be reduced either enzymatically (by mitochondrial dehydrogenase/reductase enzymes) or through direct interaction with NADH, which is reduced to NADPH. This reaction only takes place when enzymes are active in living cells and therefore conversion is directly related to number of viable cells and can be analyzed photometrically in an enzyme-linked immunosorbent assay (ELISA) reader.

This reaction produces blue formazan crystals (MTT) or yellow formazan (WST-1) in living cells.

2.2.3.1. MTT-assay

A549 cells were seeded in 96well cell culture plates (150µl/well) and grown in complete DMEM media overnight at 37°C with 5% CO_2 so that they were confluent on the day of the experiment. After addition of the inhibitors (mixed in DMEM/BA), cells were incubated further for 4, 6, 8, 10, 24 and 48 hours. Cell media was then replaced with 150µl of complete DMEM media and incubated for 1 hour to allow for cell proliferation. 7µl of 5mg/ml MTT stock solution was diluted in 193µl complete DMEM media (175µg/ml final concentration) and added into each well after aspirating the old media. Cells were incubated for a further 90 min and subsequently fixed with 4% paraformaldehyde (PFA, in 1x PBS) at room temperature for 30 min. The fixing solution was aspirated and the plates were dried under the hood for 10-15 min. The tetrazolium crystal was dissolved by adding 200µl of isopropanol to each well and the plates left shaking for 10 min on a 96-well plate shaker. The plates were analyzed photometrically at 550 nm excitation in an enzyme-linked immunosorbent assay (ELISA) reader.

2.2.3.2. WST-1-assay

This assay can be performed in living cells, without any need of fixing the cells.

The media was always prepared fresh (for 24 well plates – 300µl/well), by diluting 1:50 of WST-1 stock in DMEM without phenolred.

The supernatants were collected from the treated cells/samples and kept at RT for the duration of the WST-1 experiment (it was later added back to the cells).

The cells were washed once with PBS, after which 300µl of the 1:50 prepared solution (see above) was added per well. The cells were then incubated for 1h at 37°C and 5% CO_2 (for A549 cells) or 7.5% CO_2 (for mice primary cell culture). After the elapsed time 100µl of supernatant was removed and placed in duplicate into a 96-well ELISA plate. The absorbance was measured at 450nm – reference set to 620nm – in an enzyme-linked immunosorbent assay (ELISA) reader.

Absorbance results from time point post-treatment (viral infection and/or +/- inhibitors) and for each individual well were then correlated to initial results before treatment (time point zero) and given as percentage values.

2.2.3.3. Trypan Blue dye exclusion

For the exclusion of dead cells, 10ml of cell suspension (in media) was added to 90ml of Trypan blue. It was mixed gently up and down with a pipette and 10ml of this dilution was placed in a Neubauer chamber and counted. Trypan blue enters and stains dead cells are whereas the unstained cells are live.

The cell number (dead/alive) was counted taking into account the dilution factor (10) from above and the Neubauer chamber multiplication factor 1000.

2.2.4. Raising virus stocks

T-75 flasks were seeded with MDCK-S or Vero cells one day before and allowed to grow to +/- 90% confluency. A preparation of the virus stock was made to infect bottles at a moi=0.01. The bottles were washed with 5ml PBS^{++} and then infected with 5ml of virus stock in PBS/BA/P/S/$Ca^{2+}Mg^{2+}$ for 1hour at RT. After this incubation, the inoculum was removed and 10ml of DMEM/BA media was added to the bottles and incubated at 37°C and 5% CO_2 for approximately 48h or until liquid plaques were seen. In the case of A/PR/8/34 virus, Trypsin at 2µg/ml was additionally added to the media for cleavage of the HA, which is necessary for efficient replication.

Supernatant was then removed, centrifuged at 350 g (Megafuge 1.0R, 1100 rpm), 4°C for 5-10 min, and the clear supernatants collected from the tubes. The analysis of titres and HA were then calculated, as described below.

2.2.5. Analysis of infectious virus titres by immunohistochemistry

Virus dilution was prepared in a 96-well microtiter plate with U-form bottom. First 180µl of PBS/BA/P/S/$Ca^{2+}Mg^{2+}$ was pipetted into each well and 20 µl of the virus stock was added into the well of the first row. The virus dilution (200 µl) was mixed by pipetting up and down and 20 µl of it was transferred into the well of the second row. The same steps were repeated up to the last row to get 10^{-1} to 10^{-8} dilution series.

MDCK cells were seeded in 96-well plates and grown over night at 37°C with 5% CO_2 so that they were 90% confluent on the day of infection. The cells were washed once with 1x PBS^{++}, then infected with 50µl of virus dilution and incubated at room temperature for 1 hour. Virus inoculum was aspirated and 150µl methylcellulose media was added into each well. In the case of A/PR/8/34 virus titration, Trypsin at 2µg/ml was additionally added to the methylcellulose media for the reason stated above (section 2.2.4.). The plate was placed at 37°C with 5% CO_2 for 30 or 48 hours, for FPV or PR8, respectively. After time of incubation, methylcellulose media was removed by aspiration. Cells were washed twice with 1x PBS^{++} and fixed, as well as permeabilized, with 100µl/well of 4% PFA/1% TritonX-100 in 1x PBS^{++} overnight at 4°C or alternatively for 1 hour at RT. Afterwards cells were washed three times with 1x PBS/0.05% Tween20 and incubated with 50 µl of primary antibody (anti NP-mAb, 1:6000 diluted in PBS^{++}/3% BSA) for 45 min at room temperature. After aspirating the primary antibody dilution, cells were again washed three times with PBS/0.05% Tween20, followed by secondary antibody incubation (Horse Radish Peroxidase (HRP)-conjugated anti mouse, 1:1000 diluted in PBS^{++}/3% BSA) for 45 min at room temperature. Cells were washed as before and 100µl DAB-substrate (first, one silver pill (Sigma FAST™ DAB) dissolved in 15 ml 1x PBS followed by dissolving one golden pill (Sigma FAST™ UREA H_2O_2)) or 50µl of AEC staining kit solution was used and was added into each well and placed at room temperature for 10-15 min. The **AEC** (3-Amino-9-ethylcarbazole) Staining Kit is used for staining peroxidase labelled compounds in immunohistochemistry or immunoblotting techniques. AEC produces an insoluble end product which has a red colour. Brown or red stained foci

were observed under microscope, wells were washed with normal water to remove the rest of salts and air dried at room temperature. After drying, the plates were scanned using the Canonscan 9900F at 1600 dpi and virus foci counted. The viral titre was determined as follows:

FFU/ml = number of foci x volume factor x dilution factor

Volume factor: FFU (Foci forming unit) is related to 1 ml. If a dish was infected with 50 µl viral dilution solution, the factor is 20.

One foci was considered when more than 3-5 adjacent cells were stained in one particular area, as opposed to single cell staining which would probably mean, that the cell had not produced an infectious virus.

2.2.6. Haemagglutination (HA) Assay

2.2.6.1. Preparation of red blood cells (RBCs) from chicken blood

Red blood cells should be taken from Specific Pathogen Free (SPF) chickens. If SPF chickens are not available, blood may be taken from normal chickens that are shown to be free from antibodies to avian influenza. First, about 20-30 ml fresh chicken blood was transferred to a 50 ml sterile Falcon centrifugation tube containing 10 ml of 3.7% sodium citric acid. The RBCs were washed by filling the tube to 50 ml with PBS and centrifuged at 700 g (Megafuge 1.0R, 1100 rpm) at 4^{o}C for 10 min. The supernatant above the RBC-fraction containing serum, white blood cells and fat was carefully removed by aspiration, then RBCs pellet was washed again with PBS and centrifuged as mentioned above. This washing step was repeated twice. Finally the pellet of RBCs was diluted to 0.5% (v/v) with PBS for haemagglutination assay.

2.2.6.2. HA assay

50 µl PBS was distributed into each well of a plastic U- or V-bottomed 96-well plate. 50 µl of virus suspension (from cell culture supernatant) was placed in the first well and two-fold dilutions performed from well to well (left to right) in a row (12 wells), so that the final dilution on well no.12 was 1:4096. Subsequently 50 µl of 0.5% chicken RBCs was added to each well and the plate gently tapped to allow for even mixing.

The RBCs were then allowed to settle for about 30-60 min at 4°C. The Haemagglutinating Units (HAU) are measured as: 2^x, where X is the number of the last well without blood precipitated on the bottom (with a mesh of erythrocytes).

2.2.6.3. HI assay

25 µl PBS was distributed into each well of a plastic U- or V-bottomed 96-well plate. In the first well, 50 µl of antibody dilution (Chicken anti-influenza A, Puerto Rico 8/34 (H1N1) Pab – IgY) (1:50) (dilution previously tested) was added and then a 2-fold dilution was performed (25µl from first well to second, etc…) until well no.12 and discarded 25µl from this last well. To each well 25µl of virus dilution was added (previously tested for HA – 3 wells below the last positive well dilution) (i.e. if HA=2^6; then dilute the virus to 2^4 = 1/16 = 100µl + 1500µl PBS/BA/P/S/$Ca^{2+}Mg^{2+}$) and incubated for 30min-1h at RT.

After the incubation 50 µl of chicken erythrocytes were distributed to each well; only pipetted on top and not shaken. The plate was then left at 4°C for 45min-1h.

Haemagglutination inhibition was visualized, in contrast to HA, by the wells that had RBC precipitated at the bottom.

2.2.7. Confocal Laser Scanning Microscopy and Immunofluorescence Assay (IFA)

Confluent cells were trypsinized by 1x trypsin-EDTA, reseeded in the 3.5 cm dish containing sterile glass cover-slips (12 mm) and incubated at 37°C with 5% CO_2. On the next day, the cells were confluent. After infection and treatment of cells, the growth medium was removed from the culture dish, and the cells were washed once with 1x PBS^{++}, then the cells were fixed with 1 ml 4%PFA in 1x PBS^{++} over night at 4°C. After fixation, cells were washed twice with 1x PBS^{++} and subsequently incubated with 1ml 1% Triton X-100 for 45 min. Cells were then washed 3 x with PBS and incubated with 20 µl of the primary Anti-flu A NP (FPV) mouse (clone 1331) (1:200 dilution in PBS^{++}/3% BSA) for each cover-slip for 1 hour at room temperature. Afterwards cells were washed three times as before, and further incubated with 20 µl of the secondary TexasRed-labeled goat anti-mouse IgG (1:200 diluted in PBS^{++}/3% BSA) for 1 hour at room temperature. The cells were then washed again three times and incubated with 20µl DAPI stock (1:200 diluted in PBS^{++}/3% BSA) for 5 minutes.

After a further three washes (as above) and an extra wash with ddH_2O, the glass cover slip was fixed on a glass slide with Mowiol, and allowed to harden overnight. Fluorescence was visualized, and pictures taken the following day with a TCS NT confocal laser scanning microscope.

2.2.8. Western blotting (Semi-dry)

2.2.8.1. Measurement of relative protein concentration (Bio-Rad protein assay)

The Bio-Rad Protein Assay is based on the observation that the absorbency maximum for Coomasie Brilliant Blue G250 shifts from 450 nm to 595 nm when binding to protein [285]. 5 µl of cell lysate (as described in section 2.2.2.) was added into diluted Bio-Rad Dye Reagent (1:5 dilution of Dye Reagent concentrate in ddH_2O). This was then mixed well and after a period of 10 min, the protein content was determined by measuring the absorption at wavelength 595 versus reagent blank (containing the lysis buffer only). This was done to apply an equal amount of protein from all samples onto the SDS-PAGE gel. The calculation was done as follows: the OD value (OD_{ref}) of the sample with lowest concentration (Reference sample C_{ref}) was divided by the OD values (OD_x) from the other samples. This would give a factor value (F_x) that would be multiplied by a constant volume (i.e. 70µl). The respective volume obtained (V_x – representative of the volume needed from each individual sample) would then be subtracted from the respective 70µl and this would give the amount of lysis buffer volume (L_x) that would be needed to add to the V_x to obtain the same amount of protein concentration from the lowest sample concentration.

2.2.8.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The eletrophoresis apparatus was assembled according to manufacturer's (Bio-Rad) instruction, and the resolving gel was poured in between the two glass plates. A space of about 1 cm plus the length of the teeth of the comb was left uncovered. Isopropanol or 100% ethanol was added to the surface of the gel to apply achieve an even gel surface. After the resolving gel was polymerized, isopropanol was removed and the stacking gel was poured on top of the resolving gel. The comb was then inserted on the top and the gel and allowed to polymerise. 35 µl of 2x Laemmli buffer, containing 10% β-mercaptoethanol to reduce disulphide bonds, was added to 70 µl of

sample (after determining the protein concentration) and was incubated for 5 min at 95°C and cooled on ice for 1 min, then shortly centrifuged and then 25µl of the adjusted protein concentration (see section 2.2.8.1.) was loaded into the wells of the gel. Rainbow protein marker or Precision Plus protein standard (2 µl marker + 8 µl Laemmli buffer) was loaded as control. Electrophoresys buffer was added and the gels were run at about 20 V/cm gel length. The negatively charged SDS-proteins complexes will migrate in the direction of the anode at the bottom of the gel. Small proteins move rapidly through the gel to the bottom, whereas large ones move slower and stay on the top. Proteins that differ in mass by about 2% can be distinguished with this method. The electrophoretic mobility of many proteins in SDS-polyacrylamide gels is proportional to the logarithm of their mass.

2.2.8.3. Transfer to membrane in a "Semi-dry" electroblotter

After the cell extracts were subjected to SDS-PAGE, the proteins were transferred onto a PVDF-membrane by electroblotting. The membrane had been previously incubated in 100% methanol for 1-2 min, washed for 5 min in ddH_2O, and further equilibrated for 5 min in transfer buffer. A sandwich of two blotting papers, the PVDF-Membrane, the polyacrylamide gel and again 2 blotting papers (without any bubbles), was laid in a "Semi-dry" Electroblotter. The current was set to 0.8 mA/cm^2 for 90 min, for protein transfer. The negatively charged proteins will migrate from the gel on top in the direction of the anode at the bottom and transfer onto the PVDF-membrane placed underneath the gel.

2.2.8.4. Immunodetection of proteins

After transferring the proteins, the PVDF-membrane was washed for 5 min in 1x T-TBS buffer, and then blocked in blocking buffer for 1 hour at room temperature or overnight at 4°C. The membrane was then washed for 5 min in 1x T-TBS buffer and incubated with the primary antibody (e.g.: P-ERK, 1:200 in blocking buffer) for 1 hour at room temperature or overnight at 4°C. After washing three times (5 min each) in 1x T-TBS buffer, the membrane was incubated with the secondary antibody solution (e.g.: HRP-conjugated anti-mouse monoclonal antibody, 1:1000 diluted in blocking buffer) for 1 hour at room temperature.

2.2.8.5. Enhanced Chemiluminescence (ECL) reaction

The membrane was washed three times, as before (5 min each) in 1x T-TBS buffer and once in 1x TBS, then incubated for 1 min in ECL (enhanced chemiluminescence ECL) solution which was prepared according to the manufacturer's instructions. After 1 min, the membrane was laid between a glass plate and a clear plastic membrane, into a photo cassette. A light sensitive film was then placed on top of the membrane and exposed for 1-5 min or longer. The film was then developed in a developing machine.

In order to detect the ERK2 protein (as a loading control), the previously attached antibody was stripped from the membrane by placing it in with 20ml stripping solution (Roti-Free, ready-to-use Stripping Buffer, Roth) and incubating for 60 min at 37°C. After washing with 1x T-TBS buffer for 5 min, the membrane was incubated in blocking buffer for 1 hour at room temperature. After a 5 min wash in 1x T-TBS buffer the membrane was incubated with anti ERK2 monoclonal antibody (1:500 diluted in blocking buffer) solution for 1 hour at room temperature or overnight at 4°C. After three times (5 min/each) washing with T-TBS, the membrane was incubated with the secondary antibody solution (e.g.: HRP-conjugated anti-mouse monoclonal antibody, 1:1000 diluted in blocking buffer) for 1 hour at room temperature. Subsequently the membrane was washed as before three times in 1x T-TBS buffer and one more time in 1x TBS, and incubated for 1 min in ECL solution and further analysed as before.

2.2.8.6. Quantification of protein bands

Protein bands exposed on the film were scanned at 800 dpi and the picture was saved in grey scale as a TIFF file. The intensity of protein bands was densitometrically determined by means of PC-BAS software. Both P-ERK and ERK2 bands were analysed. For normalisation, the lowest band value measured for ERK2 (loading control) was set to one and divided by all other band values of ERK2. This gave a ratio (factor) value for each band to which each corresponding P-ERK band value was multiplied. This calculation was calculated to normalize the amount of P-ERK by the amount of ERK-protein loaded for each sample.

2.2.9. NF-κB analysis

NF-κB activity was measured by commercially available kit (TransAM from ActiveMotif). This kit uses an ELISA based high-throughput screening system in

which 96-well plates are covered with immobilized oligonucleotides containing an NF-κB (5'-GGACTTTCC-3') consensus binding sequence. Only the active form of NF-κB will bind to this site. Also, the primary antibodies used then to detect NF-κB will recognise an epitope on p65 that will only be accessible when NF-κB is active and bound to the target DNA. A secondary HRP-conjugated antibody is then used for colorimetric analysis via spectrophotometry. The sample can be either analysed from whole-cell or nuclear extracts. The detection limit for this assay is < 0.5μg of cell extract or < 0.4ng of recombinant p65 protein/well.
For our study lysates were prepared from infected and treated A549 cells, as mentioned before (2.2.1.3.), and collected for further analysis. Assay was performed according to manufacturer's protocol and analysed by ELISA reader at 450nm wavelength.

2.2.10. Cytokine analysis

Suspension protein arrays (multiplex bead immunoassay) are designed in a capture solid sandwich immunoassay format and permit high throughput of multiple markers in individual samples. The system employs colour-coded beads as the solid support, each of which is conjugated (covalently bound) with capture antibodies. These antibodies will react with the specific analyte (cytokine) of interest from the unknown sample or standard. After a series of washing steps to remove unbound protein, detector antibodies (biotinylated) are allowed to react with the beads, followed by addition of streptavidin-phycoerythrin (streptavidin-PE) – (Biorad) or an R-Phycoerytherin (RPE) – (Biosource), which binds to the biotinylated detection antibodies. The spectral properties of each bead are then monitored with the Luminex 100^{TM} instrument. The constituent of each well is drawn up into the flow-based instrument which measures each specific reaction based on bead colour and fluorescence. Results are calculated by interpolation from the standard curves.

Supernatants from infected and control samples were collected at the respective times and analyzed for cytokine expression. Human IL-8, IL-6, MCP-1 and RANTES were analysed by a multiplex cytokine array kit (Biorad), mouse KC (mouse analogue of human IL-8), IL-6, MCP-1 and RANTES were analysed by a multiplex cytokine array kit (Biosource, Invitrogen). Both were performed according to manufacturer's

instructions. For human and mouse IFNβ analysis, the supernatants were measured with commercially available ELISA kits (Biosource, Invitrogen), according to manufacturer's instructions.

2.2.11. Mice

C57BL/6 mice (weight of 18-21g) were purchased from Charles River Laboratories (Sulzfeld, Germay). Mice were bred under specific pathogen-free (SPF) conditions. All experiments were approved by the local government committee of Giessen.

2.2.12. Mice primary cell isolation

Briefly, mice primary alveolar epithelial cells (AECs) were isolated as previously described (Corti et al., Am J Respir Cell Mol Biol, 1996) but with some modifications. C57BL/6 mice were euthanised by an overdose of isoflurane and exsanguinated by cutting the inferior vena cava. Lungs were then perfused with 20 ml of sterile HBSS via the right ventricle until they were clean of blood. A shortened 21-gauge cannula was then firmly fixed to the exposed trachea through a small incision performed on the trachea. 1.5 ml of sterile dispase (enzyme used for digestion) was then applied through the needle into the lungs followed by 500μl of sterile 1% low melting agarose in PBS (37^{o}C). After 2 min of incubation, the lungs were removed into a 15ml tube containing 2ml of dispase and allowed to incubate for a further 40 min at RT. The lungs were then placed in a culture dish containing DMEM/2.5% HEPES buffer/0.01% DNase (also enzyme used for digestion), and the tissue was carefully dissected from the airways and large vessels. The cell suspension was successively filtered (first through a 100μM then through 40μM mesh filter membranes and finally through a 20μM filter paper) to obtain a single cell suspension and resuspended in 10ml of complete DMEM media. The cells were then incubated with biotinylated rat anti-mouse CD16/32 and rat anti-mouse CD45 mAbs (specific leukocyte antibodies) for 30 min at 37^{o}C. After this period, cells were washed and incubated with streptavidin-linked MagneSphere Paramagnetic Particles for 30 min at RT with gentle rocking, followed by magnetic separation of contaminating leukocytes for 15 min. The purity of freshly isolated mice primary alveolar epithelial cells (AECs) in the supernatant was always >90% (assessed by trypan blue dye exclusion – see section 2.2.3.2.). Cells were then seeded on 24-well cell culture plates at a density of 4 x 10^5

cells/well and grown to 90% confluence for 2 days with complete DMEM media. On day 2 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.

2.2.13. *in vivo* mice experiments

Mice were either infected intra-tracheally with 500PFU of influenza virus A/Puerto-Rico/8/34 (H1N1; PR8) diluted in sterile PBS in a total volume of 70μl or with 70μl sterile PBS (mock controls). Mice were treated intra-peritoneally with inhibitors (Bay or U0126) or solvent controls (DMSO, DMSO/Cremophor EL – see below) every 24h until day of sacrifice, starting at 24h before infection. Mice were sacrificed on day 2 or 5 by an overdose of isofluorane. After opening the abdominal cavity the peritoneum was cut open and blood was taken from the vena cava inferior (+/- 400μl). For the bronchoalveolar lavage (BAL) fluid, 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 1ml (4 x 250μl PBS) collected and analysed for virus titres and cytokines. All samples were kept at -80^{o}C, until further analysis.

2.2.14. Statistical analysis

Each point corresponds to the mean +/- S.D. 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: significant * < 0.5; very significant ** <0.01; and very very significant *** < 0.001.

3. Results

Human alveolar epithelial cell line (A549 cells)

Since activation of both the NF-κB and the Raf/MEK/ERK pathway seem to be important for virus survival as well as immune regulation, I wanted to analyse the effect of inhibiting these pathways in human alveolar epithelial cells, in order to reduce both virus replication and cytokine induction, simultaneously, in an attempt for an anti-viral therapy.

For this purpose I chose a specific IKK inhibitor, Bay 11-7082, and a highly specific MEK inhibitor, U0126. Bay 11-7082 (Bay) blocks activation of the NF-κB pathway by selectively inhibiting TNF-α inducible phosphorylation of IκBα (www.calbiochem.com), whereas U0126 is a selective inhibitor of MEK [284] and therefore inhibits downstream activation of the Raf/MEK/ERK pathway.

3.1. Viability of A549 cells upon treatment with specific inhibitors

To ensure that treatment with these inhibitors would not affect cell viability, an MTT assay was performed. This assay measures cellular proliferation by a colorimetric technique which correlates to mitochondrial enzyme activity (reductases). (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT) is taken up by cells and can be reduced either enzymatically (by mitochondrial dehydrogenase/reductase enzymes) or through direct interaction with NADH, which is reduced to NADPH. This reaction only takes place when enzymes are active in living cells and therefore conversion is directly related to number of viable cells and can be analyzed photometrically in an enzyme-linked immunosorbent assay (ELISA) reader.

In order to investigate pulmonary epithelial cells which are the primary targets of IAV infection in humans [110, 111], the human alveolar epithelial cell line (A549) was treated for different time points with the respective inhibitors at the concentrations of 25μM (Bay) or 50μM (U0126) and a control (untreated) or solvent (DMSO) in the according amount.

Results show that the percentage of viable cells (Figure 3.1), was not affected by treatment with the inhibitors tested at concentrations of 25μM (Bay) or 50μM (U0126), demonstrating that these concentrations were not toxic for the cells. Cell viability was maintained for up to 48h post treatment.

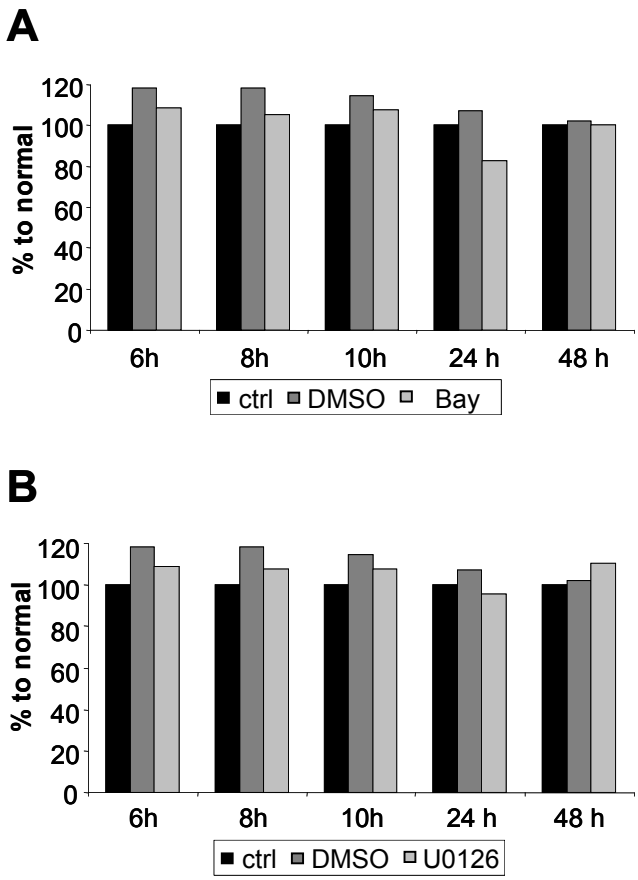

Figure 3.1: Viability test on A549 cells. A549 cells were treated with the respective inhibitor concentrations (**A**) Bay at 25µM and (**B**) U0126 at 50µM, incubated for 6, 8, 10, 24 and 48h at 37°C/5%CO_2 and tested for viability by MTT assay. All groups were assayed with a sample number of at least 16.

3.2. Virus infection induces the NF-κB signal cascade in A549 cells and Bay 11-7082 can inhibit this activation as well as decrease virus titres

Infection of cells with IAV has been reported to lead to activation of NF-κB pathway [222, 223]. Also IAV induction of NF-κB has been correlated to increase in expression of pro-inflammatory factors [232, 233]. Contradicting this alluded anti-viral effect of NF-κB activation in IAV infection, other studies have shown that the activation of this pathway is extremely important for efficient viral propagation [138, 139, 234] representing thus a pro-viral function. Since the aim of this study was to modulate both virus replication as well as virus-induced cytokine production simultaneously, this pathway seemed a perfect target.

3.2.1. Virus-induced NF-κB activation can be inhibited by Bay 11-7082

In order to investigate whether A/FPV/Bratislava/79, H7N7, (FPV) and A/PR/8/34, H1N1, (PR8) could also induce the NF-κB signalling cascade in A549 cells, cells were infected with either FPV or PR8 at a moi=1, and lysates were obtained. Activity was monitored at 6 and 8h post infection (p.i.) via an ELISA oligonucleotide based method.

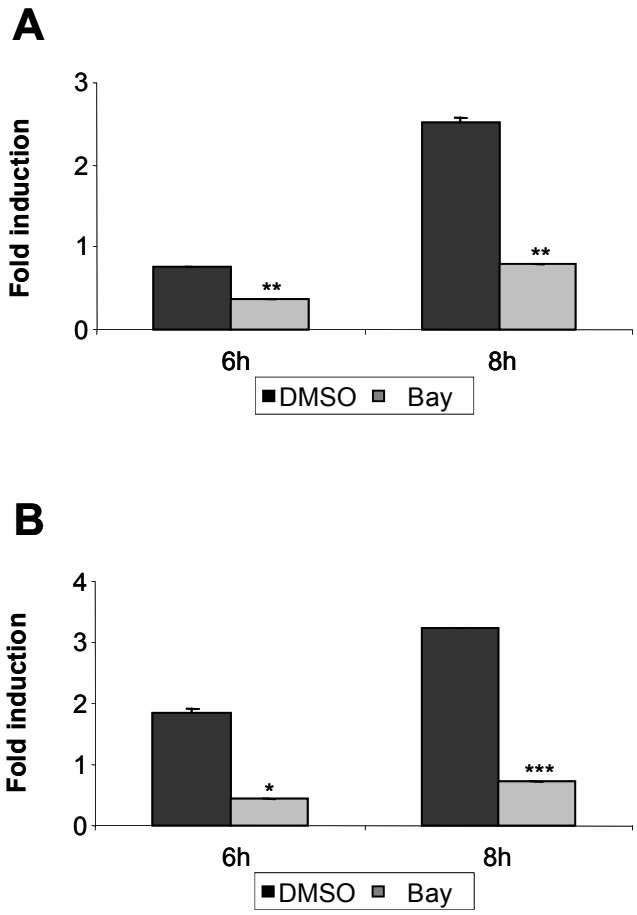

Figure 3.2.1: Virus-induced NF-□B activity can be reduced by Bay. A549 cells were infected with FPV (**A**) or PR8 (**B**) (moi=1), and incubated for different time points at 37°C/5%CO_2 with DMSO or with Bay 11-7082 (25μM). Cell lysates were then used to analyse NF-κB activity by an ELISA based oligonucleotide method. The results are representative of three independent experiments. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to infected cells without inhibitor treatment.

Both viruses led to NF-κB activation, and activity was shown to be decreased upon incubation with the specific IKK inhibitor, Bay 11-7082 (25μM), for both FPV by 70% (Figure 3.2.1(**A**)) and PR8 by 80% (Figure 3.2.1(**B**)).

3.2.2. Virus titres can be inhibited by Bay 11-7082

As I also wanted to affect virus propagation in this setting by blocking this pathway, analysis was performed on the supernatants from infected A549 cells, at different time points (4, 6, 8 and 10h p.i.) treated or untreated with Bay inhibitor, for virus titres.

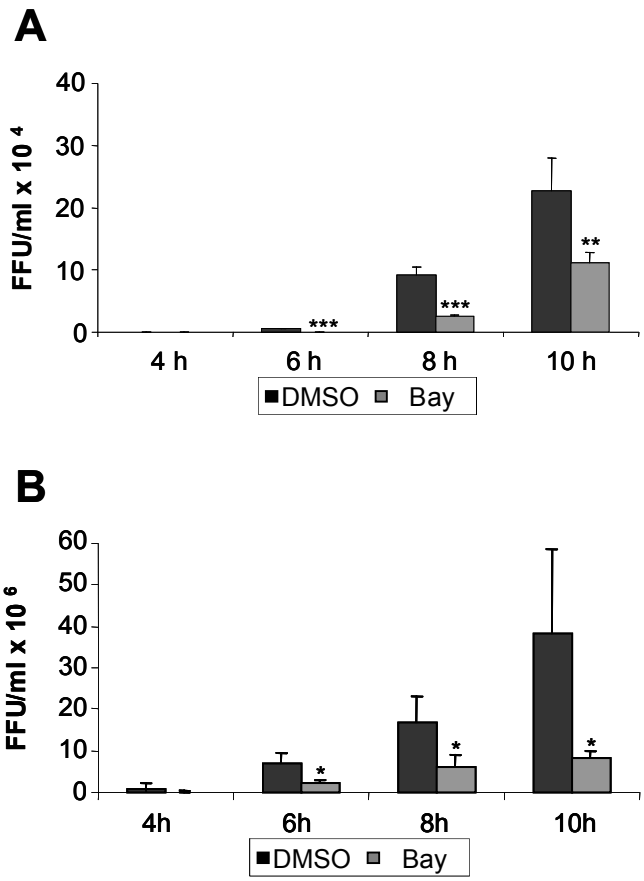

Figure 3.2.2: Virus titres can be reduced by Bay. A549 cells were infected with FPV (**A**) or PR8 (**B**)(moi=1), and incubated for different time points at 37°C/5%CO_2 with DMSO or with Bay 11-7082 (25µM). Virus titres were analysed from supernatants of the according sample by FFU assay. The results are representative of three independent experiments. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to cells infected without inhibitor treatment.

As depicted in Figure 3.2.2, a significant decrease was observed in virus titres upon treatment, using the specific IKK inhibitor (Bay at 25µM). This was true for both the avian (FPV) by 70-50% and human viruses (PR8) by 65-80% analysed in these experiments.

3.3. Virus infection induces the Raf/MEK/ERK signal cascade in A549 cells and U0126 can inhibit this activation as well as decrease virus titres

Influenza virus infection of cultured cells has been shown to lead to the activation of the classical Raf/MEK/ERK (MAPK) signalling cascade. As also previously demonstrated activation of this pathway has mainly been linked to efficient influenza virus propagation [132, 182, 186].

3.3.1. Virus-induced Raf/MEK/ERK can be reduced by U0126

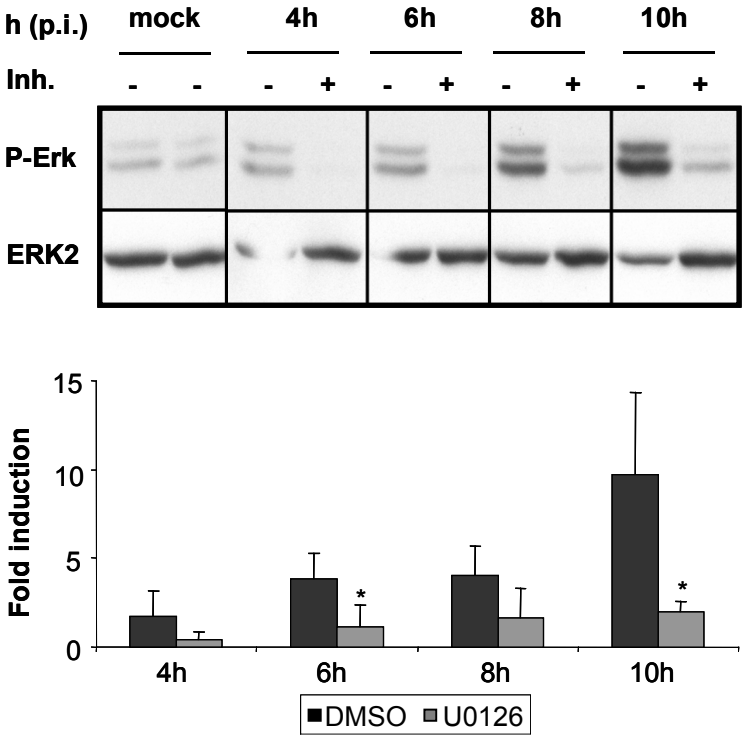

Figure 3.3.1.A: FPV-induced ERK activity can be reduce by U0126. A549 cells were infected with FPV (moi=1) and incubated for different time points at 37°C/5%CO_2 with DMSO or with U0126 (50µM). At the respective time points (4, 6, 8, and 10h) cell lysates were prepared and later analysed by Western blot analysis, using a specific anti-P-ERK monoclonal mouse antibody for the detection of activated ERK. Respective bands of three independent experiments were quantified and relative ERK activation was calculated and normalized to the loading control (ERK2). p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to infected cells without inhibitor treatment.

In order to determine whether FPV and PR8 also have the capacity to induce activity of the Raf/MEK/ERK signalling cascade in A549, cells were infected with either virus

at a moi=1, and activity was monitored at 4, 6, 8 and 10h p.i. via western blot analysis.

As demonstrated, both viruses were able to lead to ERK activation, with a gradual increase over time. This activity was shown to be dramatically decreased upon incubation with the specific MEK inhibitor, U0126, for both FPV by 60-80% and PR8 by 85-95% viruses (Figure 3.3.1.A and 3.3.1.B).

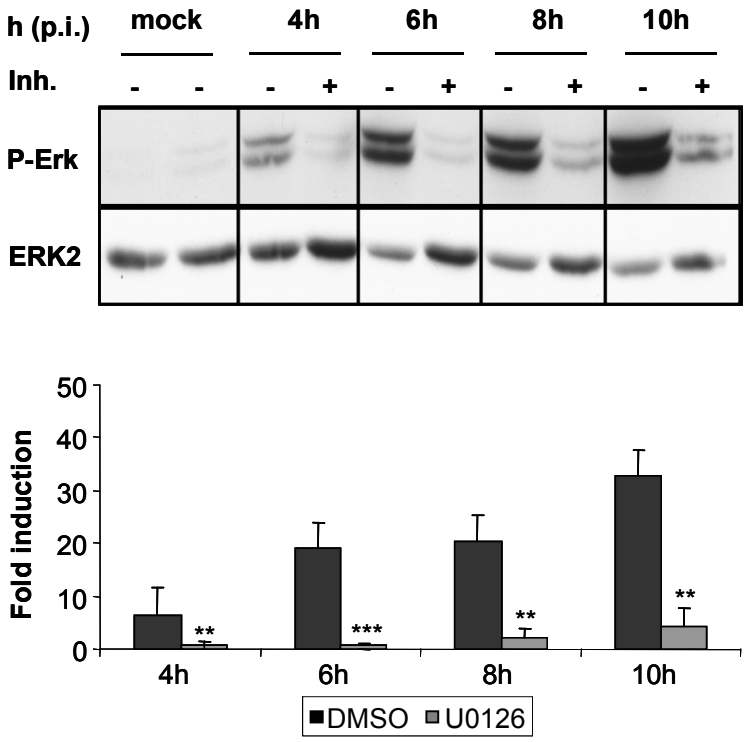

Figure 3.3.1.B: PR8-induced ERK activity can be reduce by U0126. A549 cells were infected with PR8 (moi=1). At the respective time points (4, 6, 8, and 10h) cell lysates were prepared and later analysed by Western blot analysis, using a specific anti-P-ERK monoclonal mouse antibody for the detection of activated ERK. Respective bands of three independent experiments were quantified and relative ERK activation was calculated and normalized to the loading control (ERK2). ERK activation from mock-infected cells was accordingly set to 1. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to infected cells without inhibitor treatment.

3.3.2. Virus titres can be inhibited by U0126

Now, to identify if the inhibition induced by U0126 on ERK activity, could lead to reduced virus replication in A549 cells, supernatants were analysed for virus titres

(by FFU assay) from FPV or PR8 (moi=1) infected A549 cells, at different time points (4, 6, 8 and 10h p.i.). In agreement with previous results [132], I was able to demonstrate that inhibition of ERK activity, using the specific MEK inhibitor U0126 (at 50μM), effectively reduced FPV by 75-60% and PR8 by 65% virus titres in human alveolar epithelial (A549) cells (Figure 3.3.2).

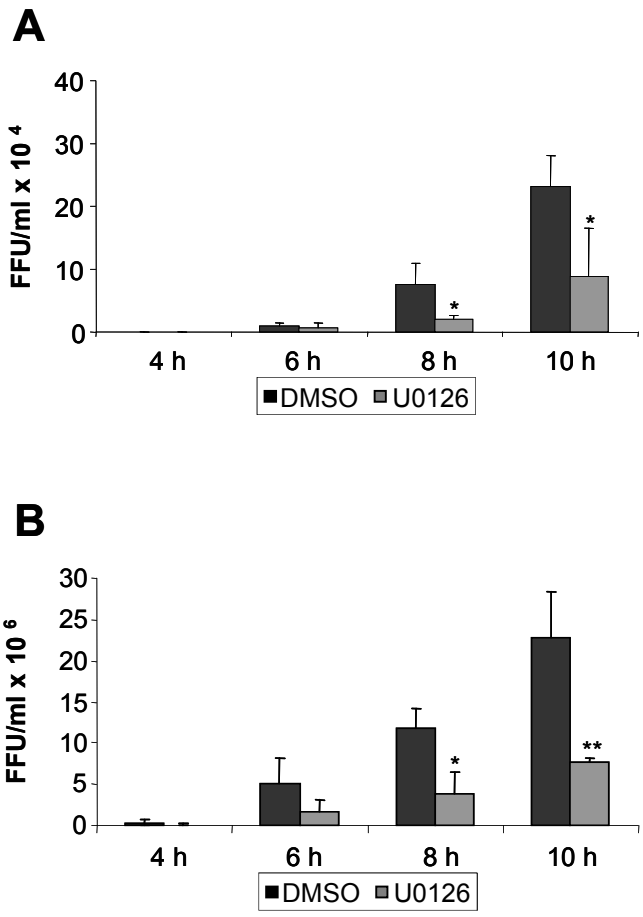

Figure 3.3.2: Virus titres are decreased by U0126 treatment. A549 cells were infected with avian FPV (**A**) or human PR8 (**B**) (moi = 1), and treated with solvent (DMSO) or U0126 (50μM) for different time points (4, 6, 8 and 10h). The supernatants were collected at the different time points and later analysed for virus titres by FFU assay. All groups were assayed in triplicate. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to infected cells without inhibitor treatment.

3.4. Bay 11-7082 and U0126 can decrease influenza A virus-induced cytokine production in A549 cells

Since I could show that in the A549 cell system, both pathways could be activated by either virus (FPV and PR8), and that I could efficiently reduce both activation of the respective pathways as well as virus titres with specific inhibitors (Bay – IKK inhibitor and U0126 – MEK inhibitor), I now wanted to investigate the effect of these inhibitors in virus induced cytokine secretion in A549 cells.

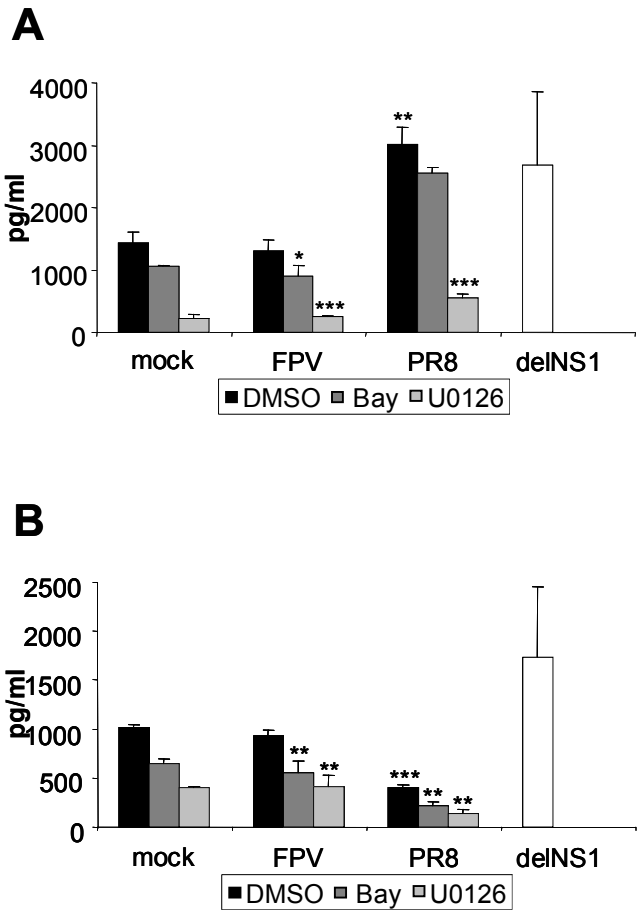

Figure 3.4.1: Virus-induced cytokine release is decrease upon inhibitor treatment. A549 cells were infected with either FPV or PR8 (moi=1), for 1h RT, then incubated for 10h at 37°C. Supernatants were analysed for cytokines/chemokines IL-8 (**A**) and MCP-1 (**B**), expression by Multiplex assay. All groups were assayed in triplicate. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to mock vs. infected or infected (–) inh. vs infected (+) inh. treatment.

To this end A549 cells were infected, as previously described, with FPV, PR8 and delNS1 (see below) (moi=1), and supernatants collected at 10h p.i.. The samples were always kept at -70^{o}C until further analysis. Cytokine analysis was performed using specific immunoarray kits (see methods).

In these cytokine/chemokine studies the delNS1 virus (a PR8 virus with a deleted NS1 segment) was used as a positive control, since the NS1 protein of influenza virus, as previously explained, has been linked to down-regulation of host immune responses, mainly to the attenuation of IFNβ production [66, 67].

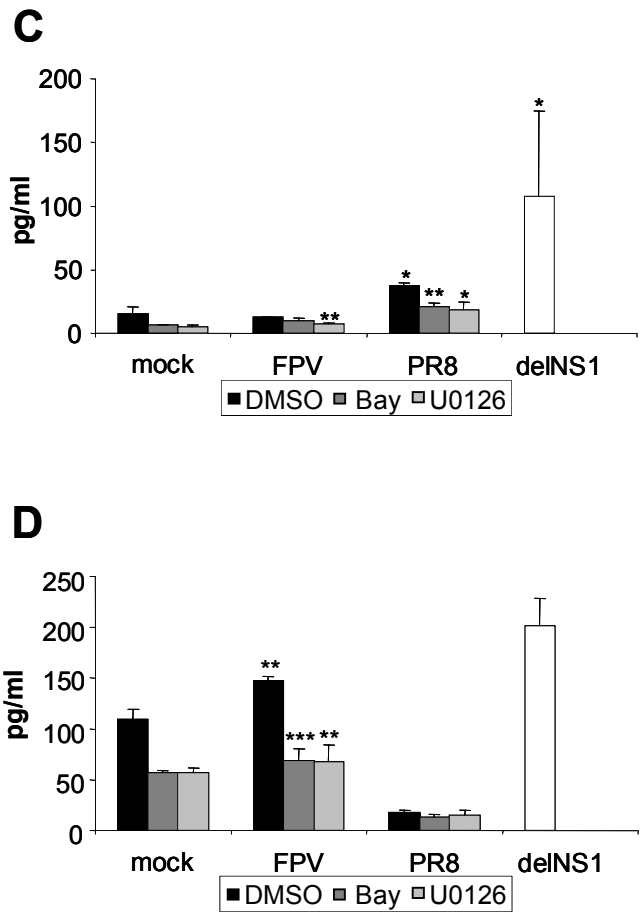

Figure 3.4.1: Virus-induced cytokine release is decrease upon inhibitor treatment. A549 cells were infected with either FPV or PR8 (moi=1), for 1h RT, then incubated for 10h at 37°C. Supernatants were analysed for cytokine/chemokine IL-6 (**C**) and RANTES (**D**) expression by Multiplex assay. All groups were assayed in triplicate. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to mock vs. infected or infected (–) inh vs. infected (+) inh treatment.

Expression profiles of cytokines/chemokines varied depending on the virus used (Figure 3.4.1). FPV had no effect on IL-8 (**A**), MCP-1(**B**) and IL-6 (**C**), but lead to an increase in RANTES (**D**) production, whereas PR8 was able to induce IL-8 and IL-6, and reduce MCP-1 and RANTES expression. What was consistent for all results though was that both inhibitors were able to reduce cytokine/chemokine production. In the case of IL-8, the U0126 was a better inhibitor than Bay 11-7082.

The delNS1 virus was also found to increase IL-6 production, but had no effect on the other cytokines tested (IL-8, MCP-1 and RANTES) at 10h p.i.

IFNβ secretion was measured by use of a specific human ELISA kit. I was unable to detect IFNβ, both in FPV as well as PR8 virus infection and also at different time points tested (10 and 24h p.i.) (Figure 3.4.2). But, as expected, the delNS1 virus was able to efficiently induce secretion of this cytokine.

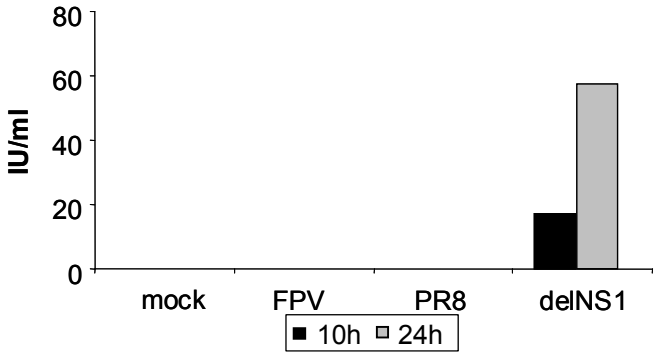

Figure 3.4.2: IFNβ induction in A549 cells. A549 cells were infected with either FPV, PR8 or delNS1 (a NS1 deletion mutant of PR8 – used as a positive control) (moi=1), then incubated for 10 and 24h at 37°C/5%CO2. Supernatants were collected and IFNβ analysed by commercial available human IFNβ ELISA kit. All groups were assayed in triplicate.

3.5. FPV and PR8-induced nuclear RNP export is efficiently blocked by Bay 11-7082 and U0126 in A549 cells.

As both NF-κB and ERK pathway activation have been associated with efficient viral nuclear RNP export [132, 181, 234], I decided to confirm these findings in the A549 system. A549 cells were infected with either FPV or PR8 viruses and treated with either inhibitor (Bay or U0126) for different time points (6, 8 and 10h p.i.). At each selected time point cells were fixed until further analysis. Evaluation of the

intracellular RNP localization was analysed at the different time points by means of immunofluorescence with a confocal microscope.

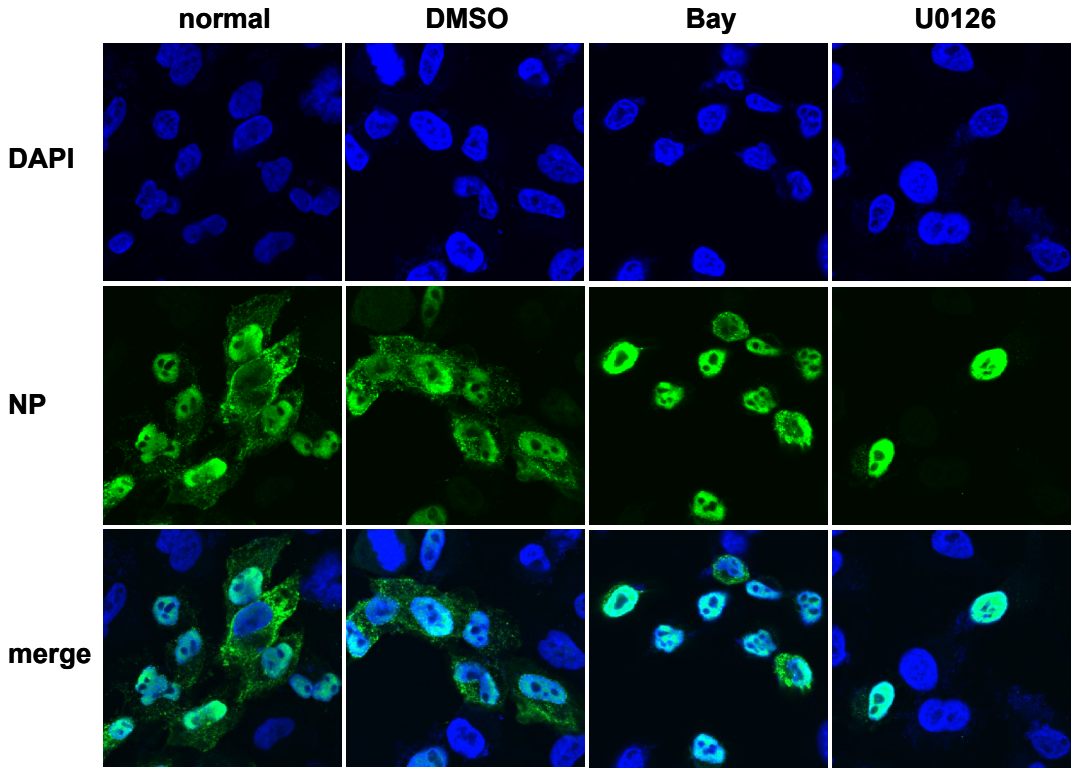

Figure 3.5.1: vRNP nuclear export in A549 cells. A549 cells were infected with FPV (moi=1), and incubated for 10h p.i. at 37°C/5%CO_2 with normal media or treated with DMSO, Bay 11-7082 (25µM) or U0126 (50µM). Cells were fixed and analysed by immunofluorescence with a confocal microscope. Representative pictures of three independent experiments are shown.

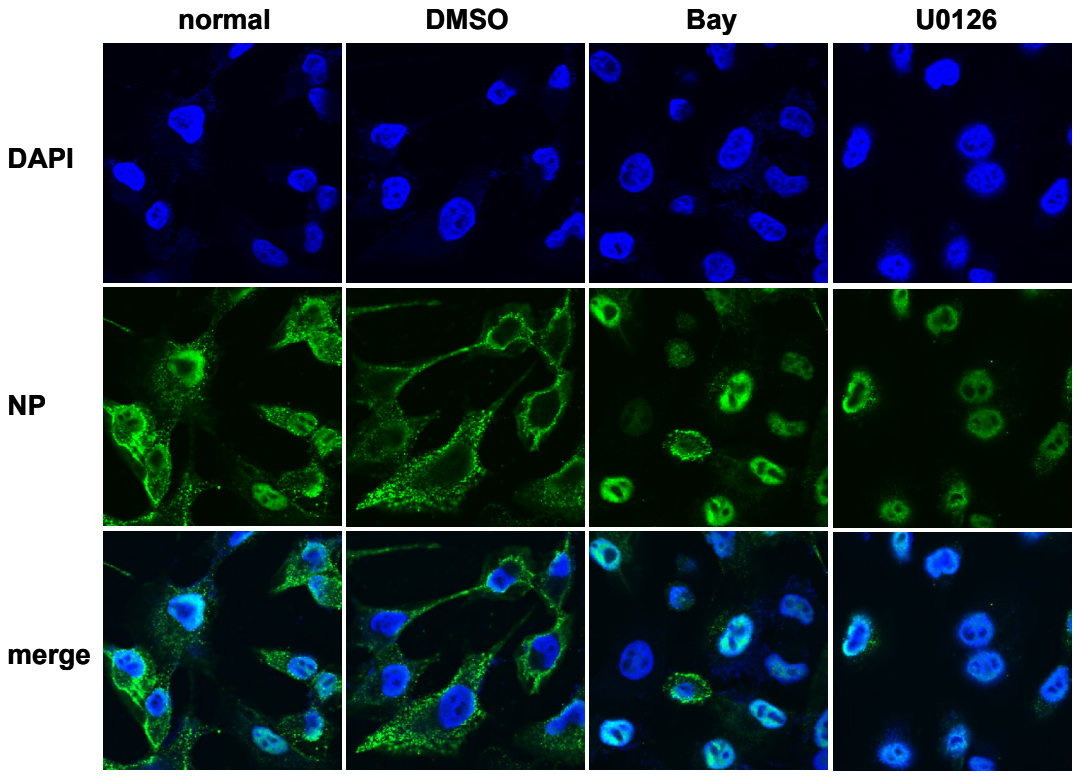

Figure 3.5.2: vRNP nuclear export in A549 cells. A549 cells were infected with PR8 (moi=1), and incubated for 10h p.i. at 37°C/5%CO_2 with normal media or treated with DMSO, Bay 11-7082 (25µM) or U0126 (50µM). Cells were fixed and analysed by immunofluorescence with a confocal microscope. Representative pictures of three independent experiments are shown.

Results demonstrate that in A549 cells both avian (FPV) as well as human (PR8) influenza virus show nuclear RNP export, however, upon inhibitor treatment (with Bay or U0126), the RNP export was significantly affected as seen at 10h p.i. (Figure 3.5.1 and 3.5.2). The results confirm that the activation of both NF-κB as well as Raf/MEK/ERK pathways is necessary for efficient viral nuclear RNP export.

Primary mice alveolar epithelial cells (AECs)

The findings so far had demonstrated that both inhibitors (Bay and U0126) tested for the two pathways, NF-κB and ERK, were efficient at decreasing virus titres as well as simultaneously reducing virus-induced cytokine release in A549 cells, thus, I decided to test this theory in a system closer to the *in vivo* situation. For this I chose to analyse mice primary alveolar epithelial cells (AECs) upon influenza virus infection and treatment with the previously selected inhibitors.

Isolation of type II AECs are characterized by pro-SPC accumulation in the cells visualized by antibody staining. By day 3 or 4 (day of infection), the main type of AECs present are of type I, characterized by loss of pro-SPC staining and an abundance/increase of presence of T1α staining (specific marker antibody for type I alveolar epithelial cells).

At this point it is important to mention that all the work concerning primary cell isolation was kindly performed by Lidija Cakarova (PhD student) from Prof. Juergen Lohmeyer's laboratory in Medical Clinic II, in Giessen.

Mice were bred under specifc pathogen-free (SPF) conditions. All mice experiments were approved by the local government committee of Giessen.

3.6. Viability of mice primary alveolar epithelial cells upon treatment with specific inhibitors

To determine the optimal inhibitor concentration for experiments in AECs, a concentration curve was drawn for the inhibitors Bay 11-7-82 and U0126. Due to the obvious difficulty and ethical reasons in obtaining primary cells, an alternative assay for cell viability was chosen instead of the traditional MTT assay. WST-1 assay permits cell viability analysis during the course of the experiment (see methods), and given the amount of AEC cells available, was the most reasonable choice for these tests.

Previous initial results had proven that the inhibitor concentrations (Bay – 25μM and U0126 – 50μM) used in A549 cells were toxic for these AECs. Therefore, AECs were treated for different time points with the respective inhibitors at different decreasing concentrations of Bay (15; 10; 7.5; and 5μM) or U0126 (40; 30; 20 and 10μM) and a control (PBS) or solvent (DMSO). Cell viability was tested for up to 30h post treatment.

Results (Figure 3.6) demonstrate that none of the concentrations tested here, from both inhibitors (Bay or U0126), had any compromising effect on AEC viability, demonstrating that these concentrations were not toxic for the cells. I therefore used in the following experiments the highest non-toxic concentration for both inhibitors, namely Bay – 15μM and U0126 – 40μM.

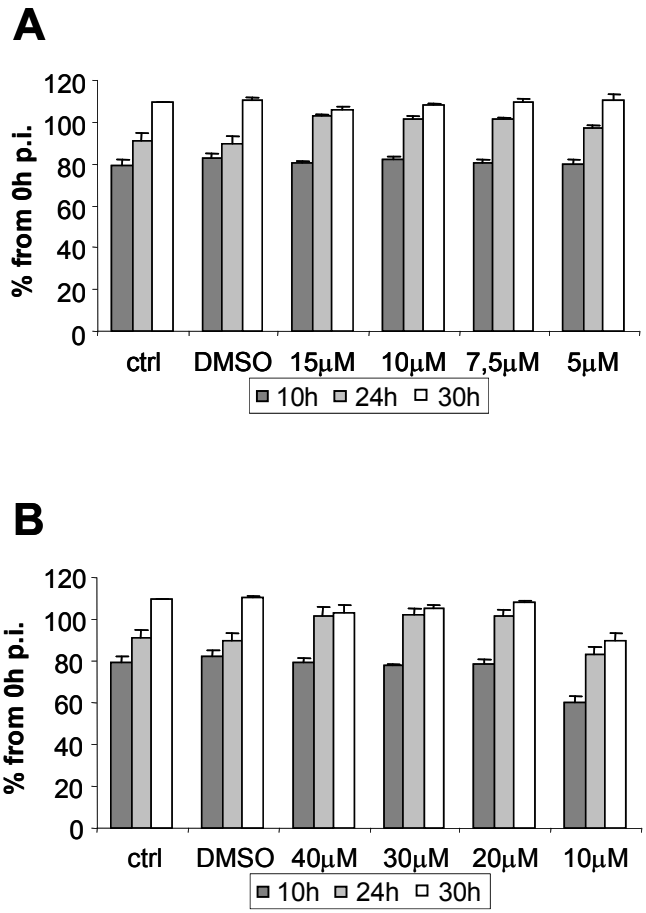

Figure 3.6: Viability test on mice primary alveolar epithelial cells. Primary mice AECs were treated with Bay (**A**) or U0126 (**B**) inhibitor at indicated concentrations, further incubated for 10, 24 and 30h at 37°C/7.5%CO2 and tested at the indicated time points for viability by WST-1 method. All groups were assayed in triplicate.

3.7. Viability of mice primary alveolar epithelial cells upon treatment with specific inhibitors during the course of infection

As mentioned above, one can analyse cell viability during the experimental procedure, when using the WST-1 method. I therefore decided to evaluate the viability of AECs during the course of a viral infection. A zero hour (before infection)

WST-1 test performed, and then continued, as explained earlier, by infecting for 1 hour at RT. Following this one hour incubation, inoculum was aspirated and media with solvent or inhibitors was added to wells, for different time points (10, 24 and 32h p.i.). At each time point the media was removed and kept separately at RT while performing the WST-1 assay. After the one hour incubation of the cells with the WST-1 media, the original media was returned to the wells and the experiment continued until the 32 h time point.

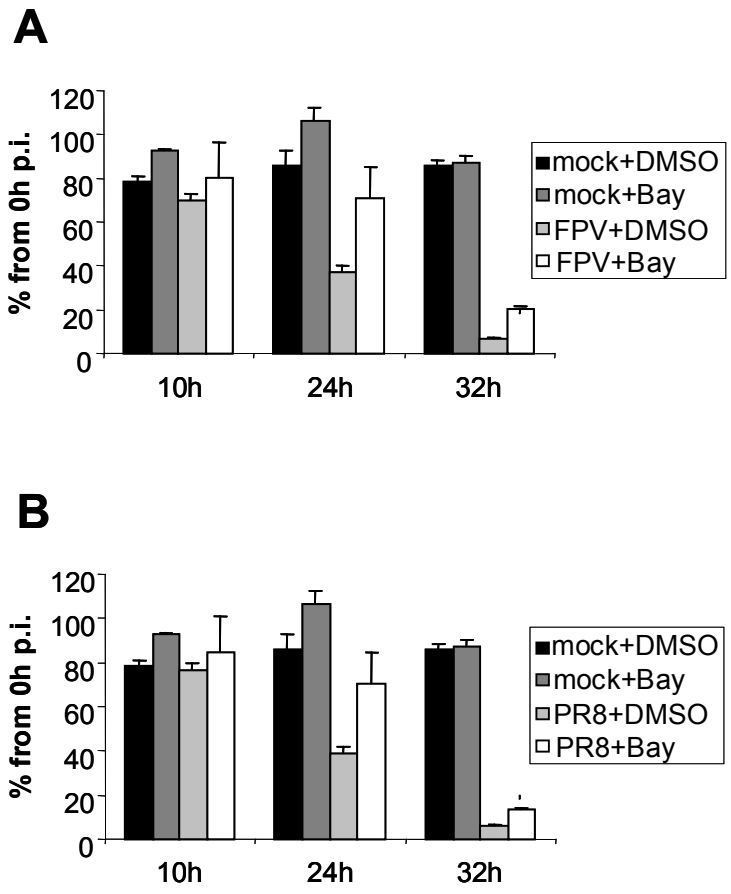

Figure 3.7: Viability test on mice primary alveolar epithelial cells during infection. Primary mice AECs were infected with either FPV (moi=0.1) or PR8 (moi=0.01) and incubated for 10, 24 and 32h at 37°C/7.5%CO2. At each time point cells were analysed for viability according to WST-1 protocol (refer to Methods). Results show for FPV (**A**) and PR8 (**B**) with Bay at 15μM. All groups were assayed in triplicate.

Figure 3.7 confirms previous findings, showing no toxic effect of the inhibitors during the course of the infection, as seen by the mock treated AECs. It was interesting to

observe that upon infection and treatment, the Bay inhibitor could actually increase cell survival of infected and Bay treated cells compared to infected and solvent (DMSO) treated cells. This was observed with both FPV (**A**) and PR8 (**B**) viruses at 24 and 32h p.i., although more apparent and significant at 24h p.i. This effect was not observed in the cells treated with the U0126 inhibitor.

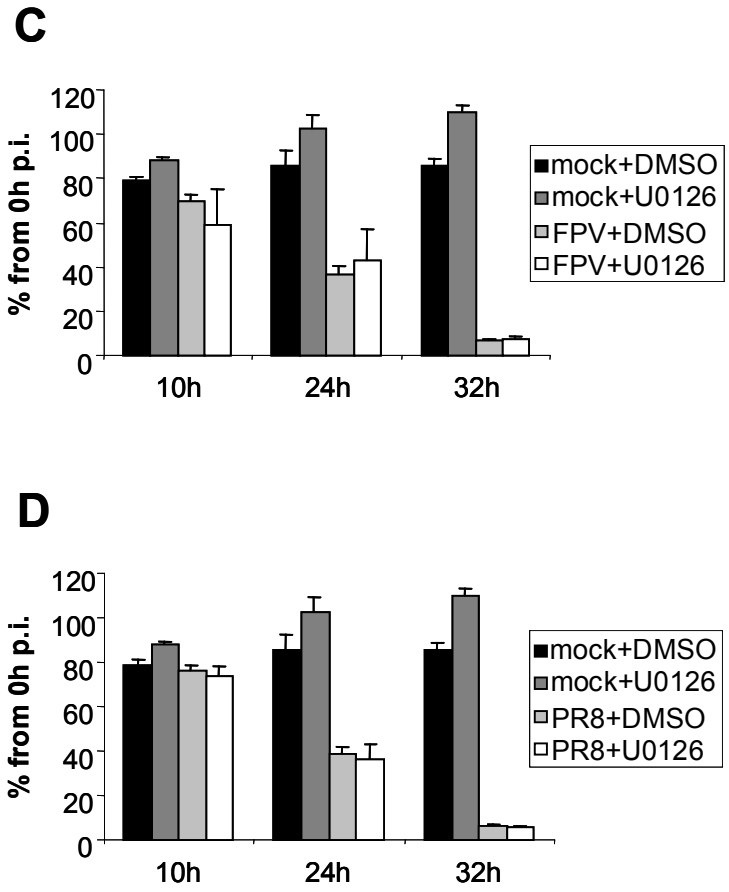

Figure 3.7: Viability test on mice primary alveolar epithelial cells during infection. Primary mice AECs were infected with either FPV (moi=0.1) or PR8 (moi=0.01) and incubated for 10, 24 and 32h at 37°C/7.5%CO2. At each time point cells were analysed for viability according to WST-1 protocol (refer to Methods). Results show for (**C**) FPV and (**D**) PR8 with U0126 at 40μM. All groups were assayed in triplicate.

3.8. Both Bay 11-7082 nd U0126 can decrease virus titres in mice primary AECs

In earlier experiments I could demonstrate that both inhibitors (Bay and U0126) were capable of inhibiting virus replication in A549 cells. The aim now was to test this effect on primary cells, and specifically in mice primary alveolar epithelial cells.

To this end, AECs were infected with FPV (moi = 0.1) or PR8 (moi = 0.01) and treated with DMSO, Bay (15μM) or U0126 (40μM) for different time points (10, 24 and 32h p.i.). Supernatants were collected at each time point, and analysed for virus titres (by FFU assay).

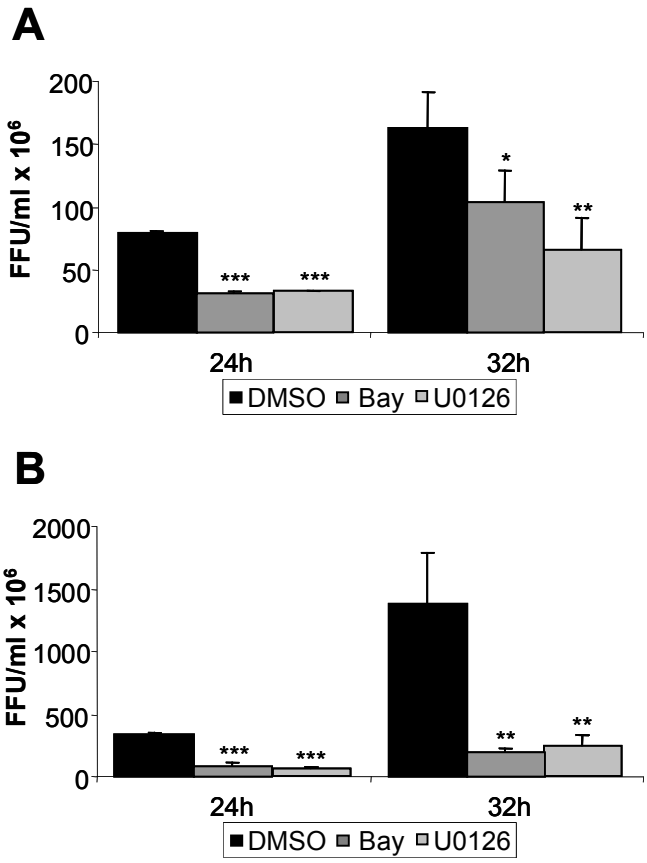

Figure 3.8: Virus titres in mice primary alveolar epithelial cells upon treatment. Primary mice AEC's were infected with either FPV (moi=0.1) (**A**) or PR8 (moi=0.01) (**B**), and treated with DMSO (solvent), Bay (15μM) or U0126 (40μM) and incubated for 24 and 32h at 37°C/7.5%CO2. Supernatants were collected and analysed for virus titre by FFU assay. All groups were assayed in triplicate. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to cells infected (–) inh. vs. infected (+) inh. treatment.

Confirming earlier A549 results, both the IKK inhibitor (Bay 11-7082) and the MEK inhibitor (U0126), were able to efficiently and significantly decrease virus titres in AECs (Figure 3.8). Virus titres were reduced with Bay by 60-40% in FPV and 75-85% in PR8, while U0126 reduced FPV titres by 60% and PR8 by 85%. This

demonstrated that activity of both NF-κB as well as Raf/MEK/ERK pathway is also important for viral propagation in mice primary alveolar epithelial cells.

3.9. Bay 11-7082 and U0126 can decrease influenza A virus-induced cytokine production in mice primary AECs

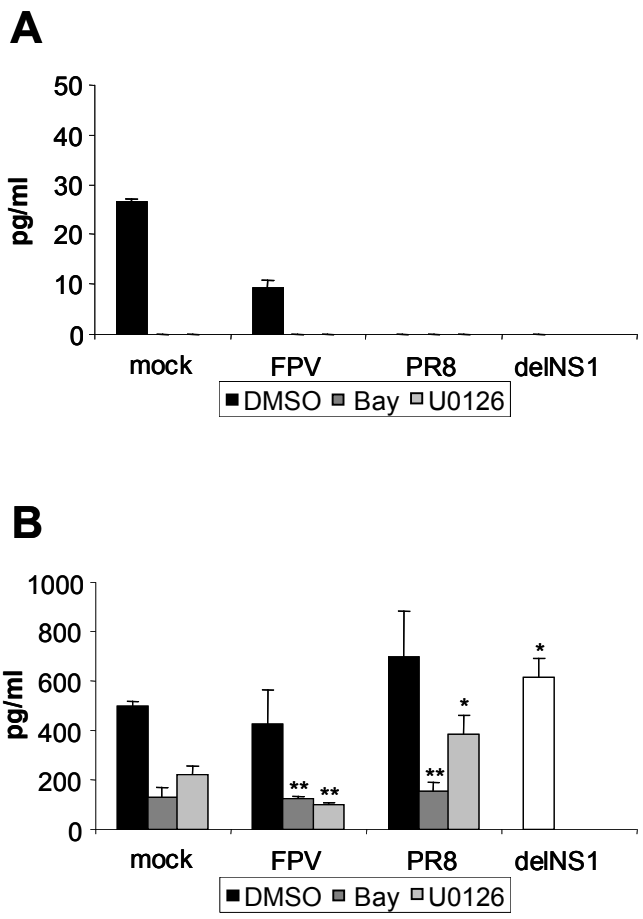

Figure 3.9.1 (A and B): Cytokine induction in primary alveolar epithelial cells. Primary mice AECs were infected with either FPV (moi=0.1), PR8 (moi=0.01) or delNS1 (moi=0.1) and treated with either solvent (DMSO), Bay (15μM) or U0126 (40μM) for 10h at 37°C/7,5%CO2. Supernatants were collected and analysed for KC (mouse analogue for human IL-8) (**A**) or MCP-1 (**B**) by specific multiplex assay kits. All groups were assayed in triplicate. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to mock vs. infected or infected (–) inh. vs. infected (+) inh. treatment.

I was now able to demonstrate that inhibiting NF-κB and Raf/MEK/ERK pathways by use of specific inhibitors led to reduction of virus titres in A549 as well as in mice

primary alveolar epithelial cells (AECs). I could also show that in A549 cells, inhibition of these pathways could reduce virus-induced cytokine production. I now wanted to prove whether this effect would still hold true for AECs.

For this purpose AECs were infected as before with either FPV (moi = 0.1) or PR8 (moi = 0.01) and treated with solvent (DMSO) or the specific inhibitors (Bay or U0126) and analysed cytokine/chemokine secretion from supernatants at 10h p.i..

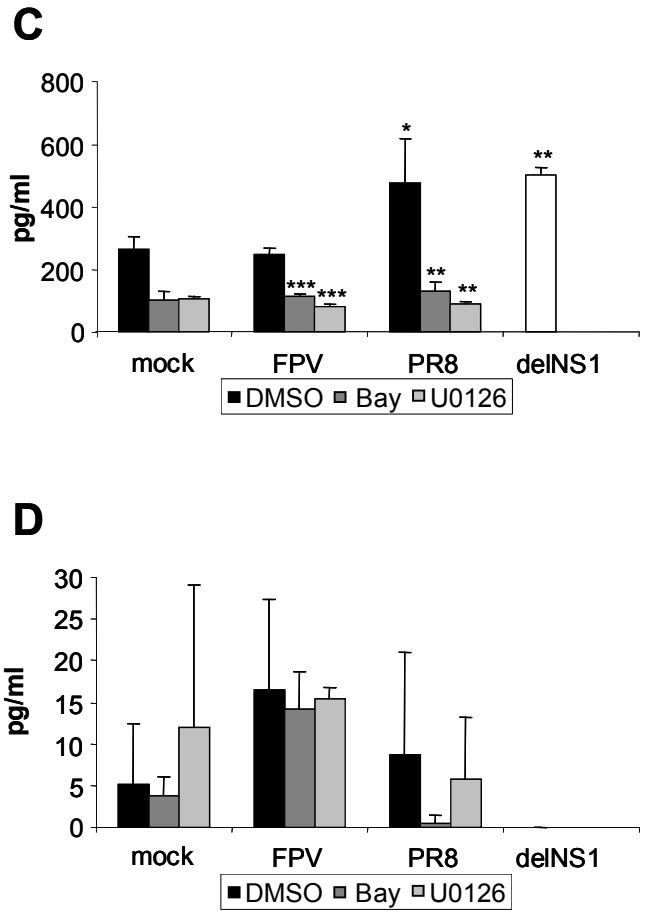

Figure 3.9.1 (C and D): Cytokine induction in primary alveolar epithelial cells. Primary mice AECs were infected with either FPV (m.o.i.=0.1), PR8 (m.o.i.=0.01) or delNS1 (m.o.i.=0.1) and treated with either solvent (DMSO), Bay (15μM) or U0126 (40μM) for 10h at 37°C/7,5%CO2. Supernatants were collected and analysed for IL-6 (**C**) or RANTES (**D**) by specific multiplex assay kits. All groups were assayed in triplicate. p values (* < 0.5; ** <0.01; *** < 0.001) are given as compared to mock vs. infected or infected (–) inh. vs. infected (+) inh. treatment.

As depicted in Figures 3.9.1, FPV was unable to induce KC (the human IL-8 analogue in mice) (**A**), MCP-1 (**B**) or IL-6 (**C**) secretion whereas it slightly increased

RANTES (**D**) production at 10h p.i. However PR8 led to a reduction in KC whereas MCP-1 was only slightly increased. PR8 was, however, able to significantly induce IL-6, but had no effect on RANTES induction as compared to mock at 10hp.i.. Both inhibitors were, in any case, able to efficiently reduce cytokine production for both viruses. The delNS1 was also able to induce increase in MCP-1 and IL-6 in mice primary alveolar epithelial cells (AEC), but had no effect on KC and RANTES.

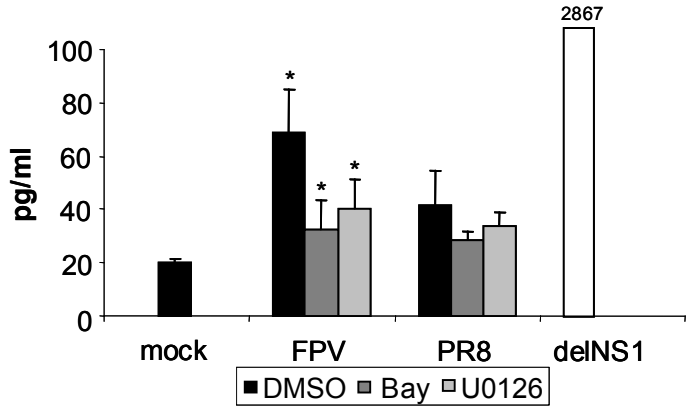

Figure 3.9.2: IFNβ induction in primary alveolar epithelial cells. Primary mice AECs were infected with either FPV (moi=0.1), PR8 (moi=0.01) or delNS1 (moi=0,1) and treated with either solvent (DMSO), Bay (15μM) or U0126 (40μM) for 10h at 37°C/7,5%CO2. Supernatants were collected and analysed for IFNβ by ELISA. All groups were assayed in triplicate. p values (* < 0.5; ** <0.01; *** < 0.001) are given as compared to mock vs. infected or infected (–) inh. vs. infected (+) inh. treatment.

IFNβ evaluation was accomplished by analyzing supernatants using a specific mouse ELISA kit. As shown in Figure 3.9.2 FPV was able to induce significant IFNβ production whereas PR8 lead to a slight increase in this cytokine. As seen before in A549 cells delNS1 virus was capable of inducing very high IFNβ secretion (2867pg/ml), again demonstrating the importance of the NS1 protein in viral defence.

3.10. FPV and PR8 induced nuclear RNP export is efficiently blocked by Bay 11-7082 and U0126 in AECs.

As both NF-κB and ERK pathway inhibitors, Bay and U0126 respectively, had been previously shown to lead to nuclear RNP retention in A549 cells, and to exclude that this effect was exceptional to established permanent cell lines, as also previously

observed by others [132, 181, 234], I decided to confirm these findings in mice primary alveolar epithelial cells. AECs were therefore infected with either FPV or PR8 viruses (moi = 0.02) and treated with either DMSO (solvent), Bay (15μM) or U0126 (40μM) for 10h p.i.. At the selected time point cells were fixed and further analysed for the intracellular RNP localization by means of immunofluorescence with a confocal microscope.

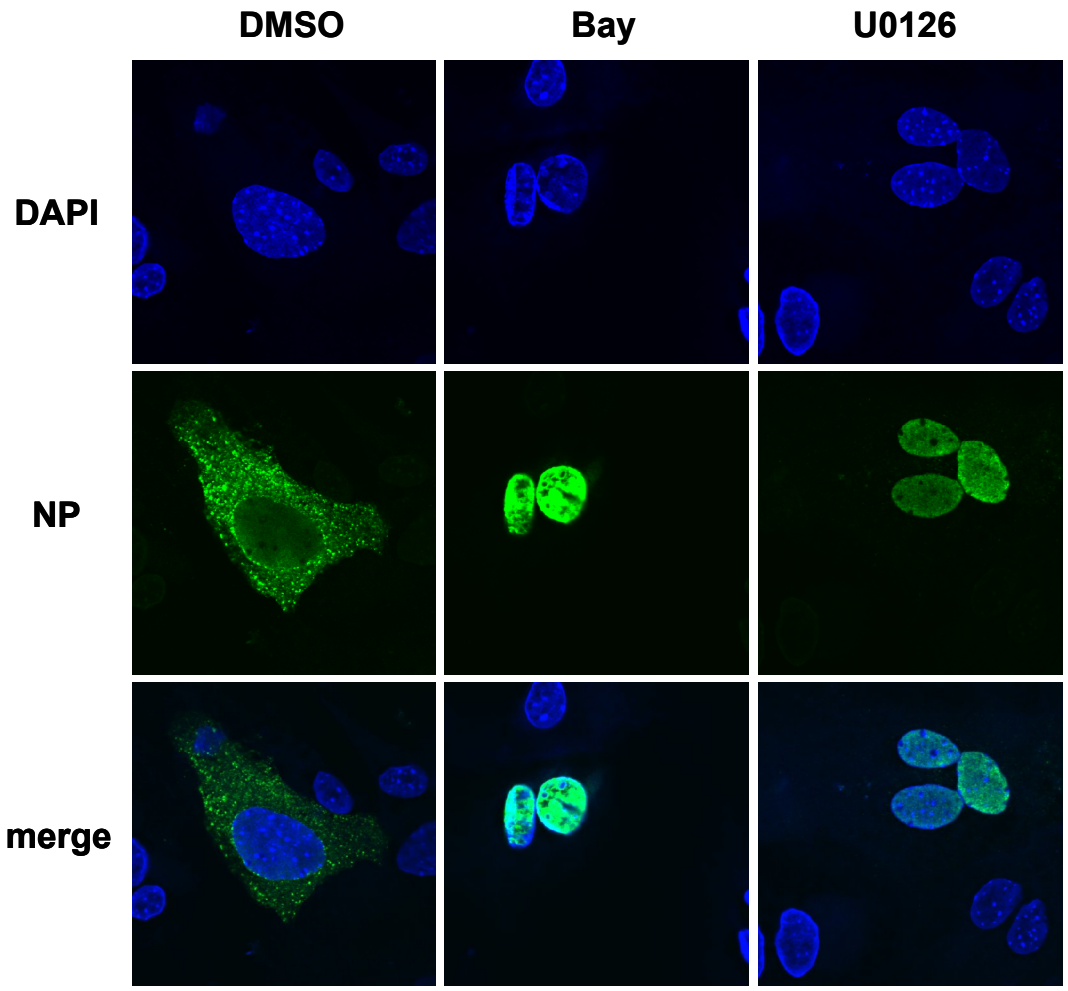

Figure 3.10.1: vRNP nuclear export in mice primary alveolar epithelial cells. AECs were infected with FPV (moi=0.02), and incubated for 10h p.i. at 37°C/7.5%CO_2 with normal media or treated with DMSO, Bay 11-7082 (15μM) or U0126 (40μM). Cells were fixed and later analysed by immunofluorescence with a confocal microscope. Representative pictures of three independent experiments are shown.

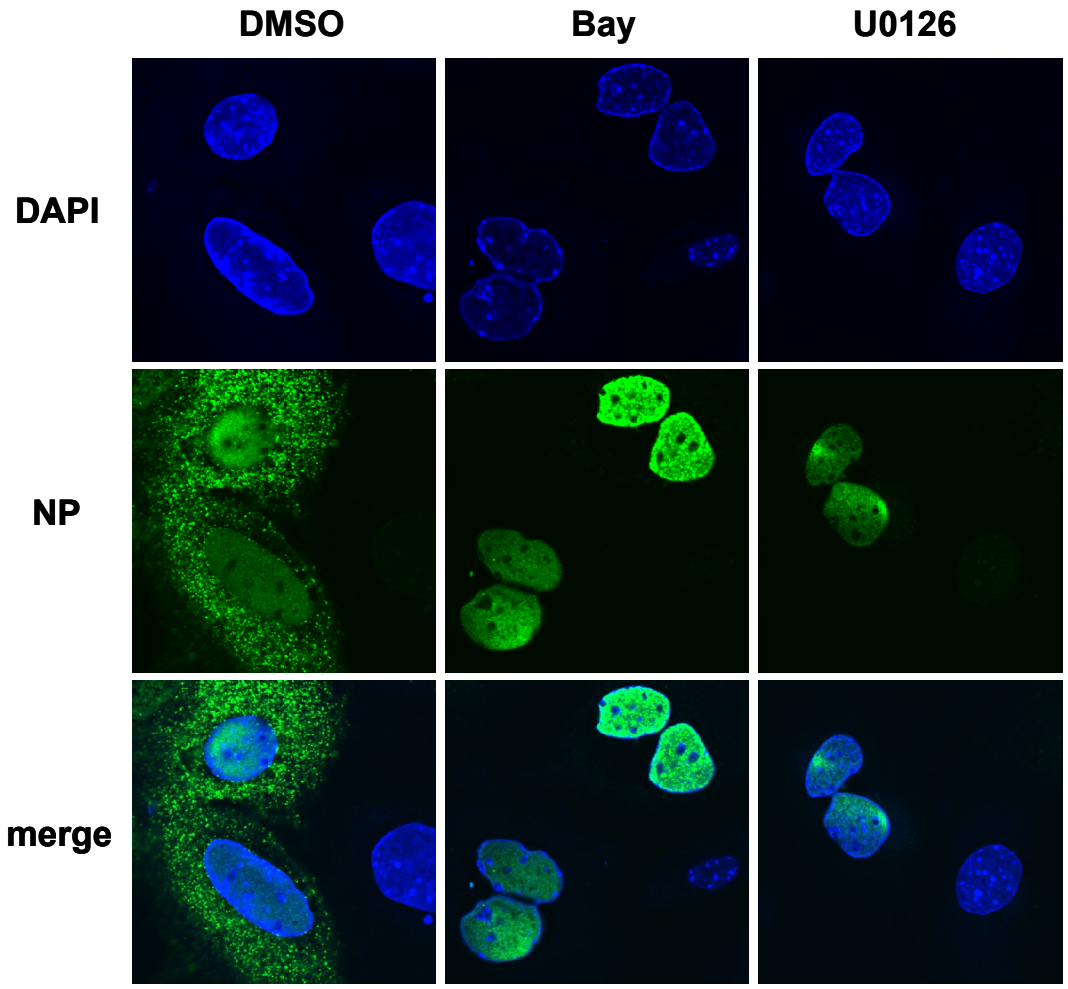

Figure 3.10.2: vRNP nuclear export in mice primary alveolar epithelial cells. AECs were infected with PR8 (moi=0.02), and incubated for 10h p.i. at 37°C/7.5%CO_2 with normal media or treated with DMSO, Bay 11-7082 (15µM) or U0126 (40µM). Cells were fixed and later analysed by immunofluorescence with a confocal microscope. Representative pictures of three independent experiments are shown.

Results demonstrate that in AECs, influenza virus induced-nuclear RNP export at 10h p.i. was impaired upon inhibitor treatment (with Bay or U0126) (Figure 3.10.1 and 3.10.2). Taken together the results demonstrate that the effect of blocking RNP nuclear export by inhibiting NF-κB or Raf/MEK/ERK pathways, till now only shown in permanent cell lines, could be reproduced in mice primary alveolar epithelial cells.

Cocktail treatment

Since both inhibitor systems had proven to inhibit virus-induced cytokine induction as well as virus propagation, simultaneously, both in human alveolar epithelial cell lines (A549) as well as in mice primary alveolar epithelial cells (AECs), I wished to determine whether the combination of both inhibitors used in lower concentration in a "cocktail" could further enhance this effect. The aim was to reduce single inhibitor treatment by a combined therapy, therefore lower inhibitor concentration were tested in combination.

3.11. Cell viability in A549 cells with combination treatment (Bay and U0126).

To test this hypothesis, evaluation of combination treatment concentrations that would not compromise cell viability had to be analysed. So as not to sacrifice mice unnecessarily, A549 cells were first analysed. Again an MTT assay was performed on A549 cells, as before, treated with different combinations of lower inhibitor concentrations for different time points.

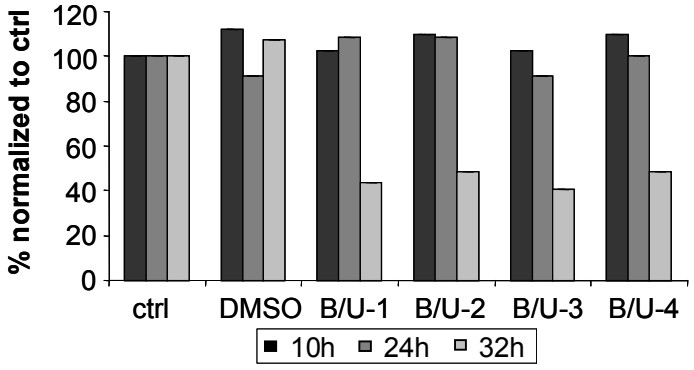

Figure 3.11: Viability of A549 cells with combination treatment (Bay + U0126). A549 cells were treated with the respective inhibitor cocktail concentration (Bay/U0126, B/U-1:15μM/30μM; B/U-2:10μM/30μM; B/U-3:10μM/25μM; B/U-4:7.5μM/25μM) , incubated for 10, 24 and 48h at 37°C/5%CO2 and tested for cell viability by MTT assay. Mean and standard deviation is representative of sample number of 16.

Results demonstrate that all the concentrations tested up to 24h p.i. had no toxic effect on the cells, and only had adverse effects at 48hp.i. on cell viability (Figure 3.11). Therefore I decided to perform further tests with two of the highest concentration.

3.12. Virus titres treated with combination treatment (Bay and U0126).

To evaluate whether a combination of the inhibitors could further decrease virus propagation, I infected A549 cells with either FPV or PR8 (moi =1) and treated them with DMSO or the two previously selected inhibitor "cocktails" (Bay-15μM/U0126-30μM and Bay-10μM/U0126-30μM) (see section 3.11.).

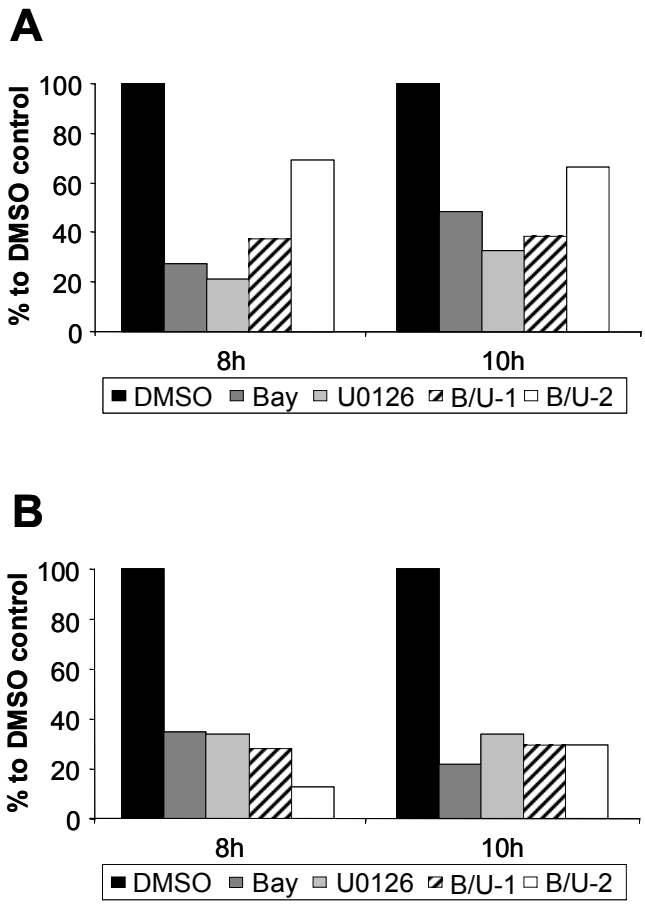

Figure 3.12: Virus titres with combined inhibitors on A549 cells. A549 cells were infected with FPV (**A**) or PR8 (**B**) (moi=1) and treated with the respective inhibitor cocktail concentration (Bay-25μM; U0126-50μM; Bay/U0126, B/U-1:15μM/30μM; B/U-2:10μM/30μM) and incubated for 8 and 10h at 37°C/5%CO2 and virus titres were analysed from supernatants by FFU assay. All groups were assayed in triplicate.

As seen in Figure: 3.12, there was no significant enhanced decrease using the lower combination treatment (Bay-15μM/U0126-30μM and Bay-10μM/U0126-30μM) in the

observed virus titre when comparing to results with single inhibitor treatment (Bay – 25μM or U0126 – 50μM).

3.13. Cell viability in AECs with combination treatment (Bay and U0126).

Not wanting to abandon this hypothesis entirely in regard to combination treatment, although the observed results in A549 cells were not promising, I decided to evaluate this effect in primary alveolar epithelial cells, since previous results from cytokine analysis had demonstrated that the effects seen in A549 cells not always corresponded to AEC results.

For this reason viability tests with several combination treatments were performed on AECs and cell toxicity analysed with the WST-1 method.

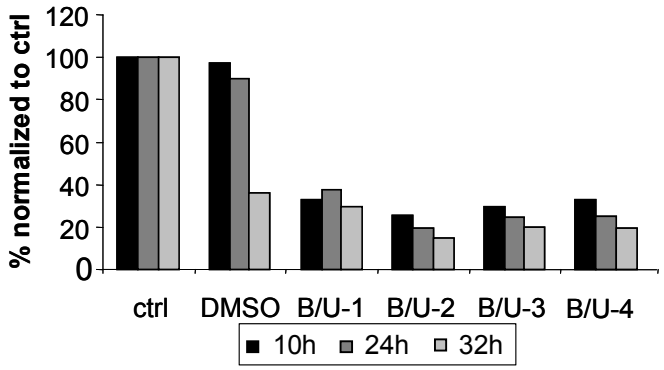

Figure 3.13: Mice primary alveolar epithelial cells viability test (WST assay) with combination treatment. Primary mice AECs were treated with the respective inhibitor cocktail concentrations (Bay/U0126, B/U-1:15μM/30μM; B/U-2:10μM/30μM; B/U-3:10μM/25μM; B/U-4:7.5μM/25μM) and incubated for 10, 24 and 32h at 37°C/7.5%CO2. Supernatants were then collected and cells were then washed once and 300μl of WST-1 media was added for 1h and incubated at 37°C/7.5%CO2, and further analysed according to WST-1 protocol. All groups were assayed in triplicate.

As shown in Figure: 3.13 (above), all the inhibitor "cocktails" tested (even the lowest concentrations) had very toxic effects on cells as early as 10h after treatment. For this reason, and the fact that in A549 cells the results with the combined treatment were also not alluring, I decided to abandon this investigation.

***in vivo* mice experiments**

All the results so far suggested that the hypothesis to decrease both virus titres and virus-induced cytokine secretion was valid in my experimental cell culture setup. This idea is thought to be meaningful in "real life" situations specially when considering the reports on HPAIV, such as the H5N1. These reports associate pathology and symptoms with increase inflammatory responses as well as high viral loads [110, 111, 115, 126]. Since the opportunity to analyse these effects in an *in vivo* mouse model arose I decided to take this approach to study a closer to "real-life" situation.

Previous studies using mice *in vivo* experiments have shown that the A/PR/8/34 virus induces cytokine/chemokine expression and is lethal in mice [242, 286, 287]. For this reason and the one stated above, the next step was to study the effect of the specific IKK and MEK inhibitors (Bay and U0126, respectively) in an *in vivo* mouse model, using this A/PR/8/34 virus strain as a "proof of principle".

Again, in this situation, it is important to mention, that most of the mice handling was performed by either Katrin Hoegner or Susanne Herold, from the laboratory of Prof. Joergen Lohmeyer, in Medical Clinic II, Giessen.

Mice were bred under specific pathogen-free (SPF) conditions. All mice experiments were approved by the local government committee of Giessen.

3.14. C57BL/6 mice

To determine whether the results observed for A549 as well as primary alveolar epithelial cells (AECs) could be reproduced in an *in vivo* model, *in vivo* experiments were conducted in C57BL/6 mice.

Previous reports from Susanne Herold (unpublished data) had given some idea on viral as well as cytokine kinetics after intratracheal inoculation of C57BL/6 mice with the PR8 virus. For this reason the same protocol was maintained, so as not to waste unnecessary mice to build up a new kinetics curve.

Also, previous studies performed by Oliver Planz's group (Tuebingen, Germany) on an appropriate solvent for U0126, gave us a head start as to a means of minimizing DMSO solvent application in *in vivo* experiments. Cremophor EL (CremEL) is a non-ionic solubilizer and emulsifier and can convert hydrophobic drugs into aqueous solutions allowing the drug to be more readily absorbed. By using this oil, we could minimize DMSO solvent administration.

Report on U0126 intraperitoneal application in mice had been shown earlier by Duan et al. [288] in a mouse asthma model. I decided to use the same concentration of inhibitor in this model therefore a dose of 30mg/kg mouse was selected and applied, dissolved in DMSO/CremEL (see methods, section 2.2.13) via intraperitoneal injection to mice every 24h, starting 24h before infection.

For the IKK inhibitor, Bay 11-7082, I decided to administer a concentration based on the same molarity ratio used in our previous A549 cell culture experiments. Therefore a concentration of 8.2g/kg mouse was used. The amount of DMSO solvent necessary for the Bay inhibitor application (7.89μl/mouse) was in the range of the DMSO present in the U0126 DMSO/CremEL solvent (6μl/mouse), and therefore, was considered minimal. Bay inhibitor was also administered to mice every 24h starting 24h before infection.

3.14.1 Bay 11-7082 and U0126 can decrease virus titres in in C57BL/6 mice

Earlier tests in both A549 as well as AECs demonstrate that both inhibitors (Bay and U0126) were capable of inhibiting virus replication *in vitro*. The aim now was to test this effect in an *in vivo* mouse model.

Titres from infected solvent treated (DMSO or CremEL/DMSO) mice versus infected and inhibitor treated (Bay or U0126) mice, both at day 2 and day 5 p.i., from bronchoalveolar lavage fluids (BAL) showed a significant decrease in virus titres observed at both time points (as analysed by FFU assay) when administering the individual inhibitors (Figure: 3.14). PR8 was effectively reduced by 60% at both time points (day 2 and 5) with Bay and by 95% (day 2) and 50% (day 5) with the U0126. This demonstrated that activity of both NF-κB as well as Raf/MEK/ERK pathway is also important for viral propagation in mice. During the course of the treatment there no differences were observed in the behaviour and appearance of mice from the individual groups.

One can speculate that it could be possible that, by using another route of administration as well as further optimized concentrations of the inhibitors, better results would be achieved in further decreasing virus titres.

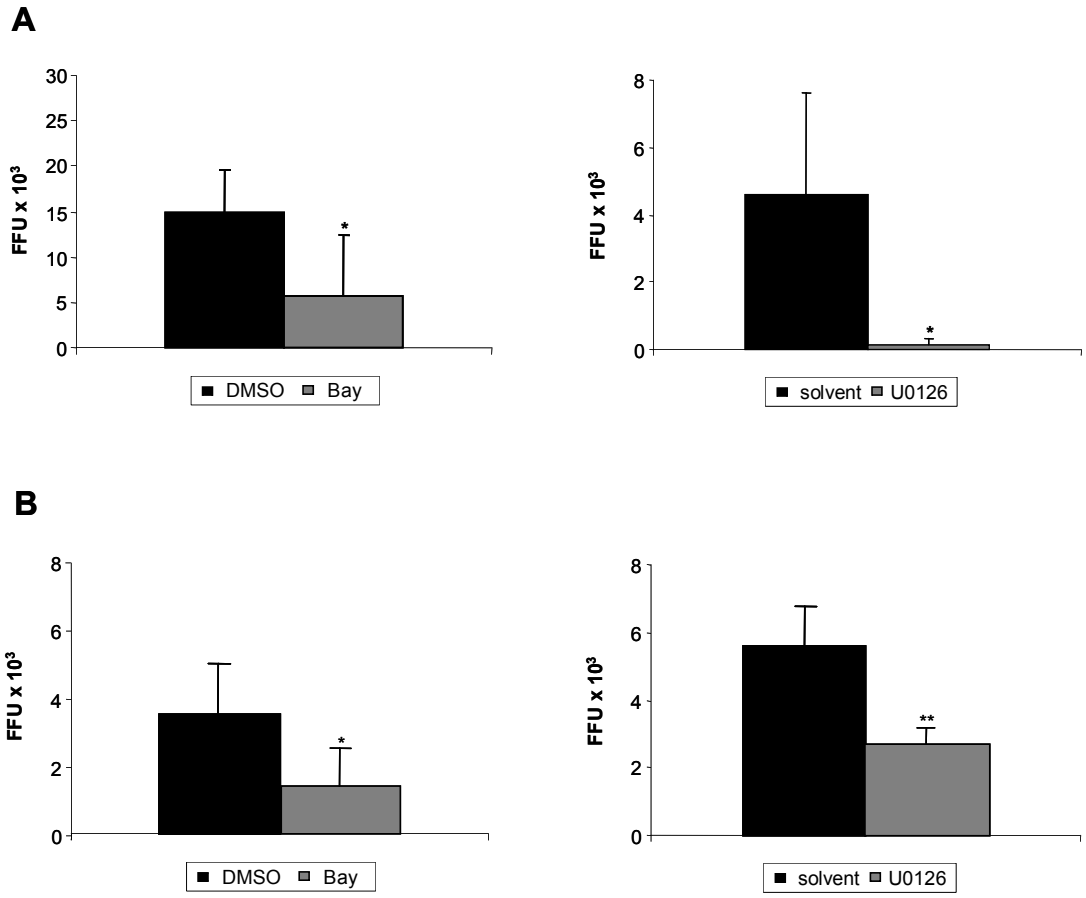

Figure 3.14: Virus titres in mice treated with inhibitors. Mice were infected intratracheally with 500 PFU of PR8 in 70µl in PBS (filtered) or mock infected with 70µl PBS. Solvent (DMSO or DMSO/CremEL), Bay or U0126 were administered intraperitoneally, daily starting at -24h (before infection). Mice were sacrificed on day 2 (**A**) or day 5 (**B**) p.i., and BAL performed. Virus titres were assayed by FFU assay. p values (* < 0.5; ** <0.01; *** < 0.001) of treated mice are given in comparison to mock (solvent) treated mice. All groups were assayed in quadruplicate (day 2) or triplicate (day 5).

3.14.2 Bay 11-7082 and U0126 can decrease virus induced cytokines in C57BL/6 mice

The next step was then to compare the different cytokine/chemokine levels in BAL from mock infected versus PR8 infected mice, treated with solvents or with inhibitors. TNF-α is mainly produced by macrophages and monocytes and it has been previously cited to be an important cytokine induced in IAV infections [126, 248, 289], for this reason I wanted to include this cytokine analysis in the mice experiments.

Both TNF-α and IFNβ were assayed by commercial available specific mouse ELISA kits and the KC, MCP-1, IL-6 and RANTES with cytokine immunoarray kits.

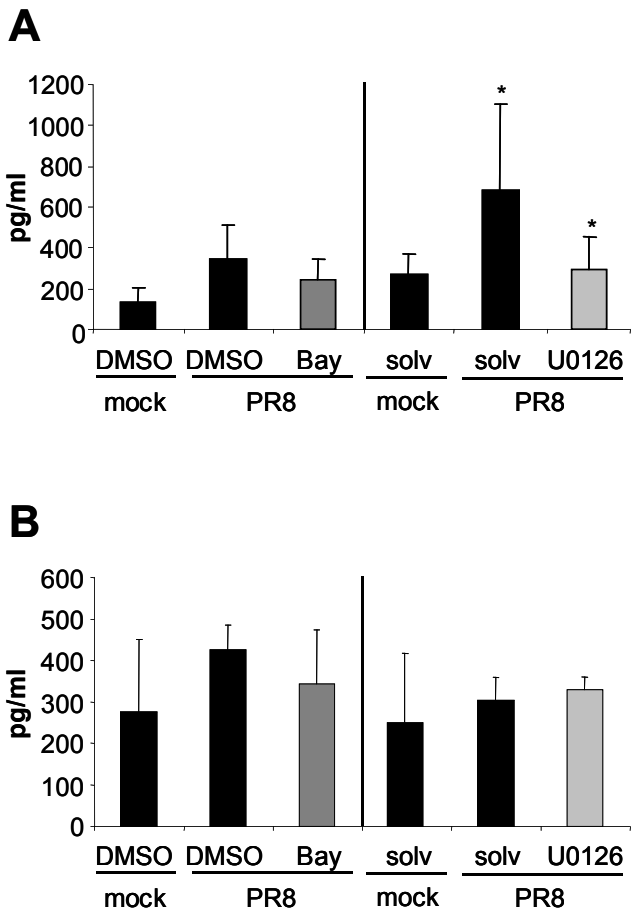

Figure 3.15: TNF-α results in mice treated with inhibitors. Mice were infected intratracheally with 500 PFU of PR8 in 70µl in PBS (filtered) or mock infected with 70µl PBS. Solvent (DMSO or DMSO/CremEL), Bay or U0126 were administered intraperitoneally, daily starting at -24h (before infection). Mice sacrificed on day 2 (**A**) or day 5 (**B**) p.i. and BAL performed. TNF-α was measured using a commercial available ELISA kit. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to mock vs. infected or infected (–) inh. vs. infected (+) inh. treatment. All groups have a number of 3 mice with exception to mock groups of day 5, which were 2 mice per group.

As depicted above (Figure 3.15) at day 2 there was an observed increase in TNF-α in infected, solvent treated mice as compared to mock infected, although more apparent in the solvent (DMSO/CremEL) than in the DMSO group. Both inhibitors however were able to decrease TNF-α production, but the U0126 seemed to be more

effective than the Bay, and reduced TNF-α to almost baseline levels. There was no significant increase or decrease observed when comparing the groups at day 5.

Results from IFNβ analysis (Figure 3.16) both at day 2 and day 5, show a significant increase in infected, solvent treated mice as compared to mock infected.

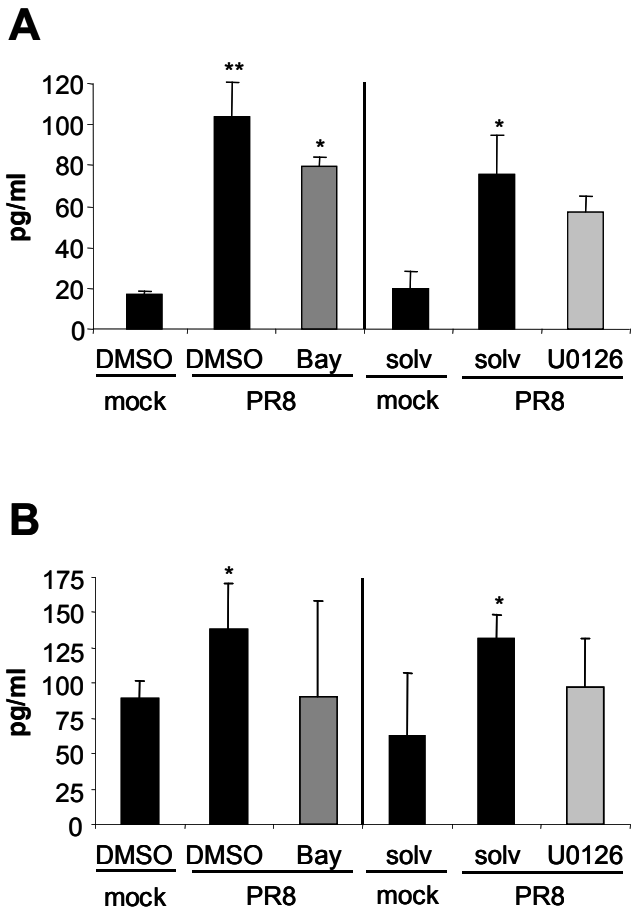

Figure 3.16: IFNβ results in mice treated with inhibitors. Mice were infected intratracheally with 500 PFU of PR8 in 70µl in PBS (filtered) or mock infected with 70µl PBS. Solvent (DMSO or DMSO/CremEL), Bay or U0126 were administered intraperitoneally, daily starting at -24h (before infection). Mice were sacrificed on day 2 (**A**) or day 5 (**B**) p.i. and BAL performed. IFNβ was measured using a commercial available ELISA kit. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to mock vs. infected or infected (–) inh. vs. infected (+) inh. treatment. All groups have a number of 3 mice with exception to mock groups of day 5, which were 2 mice per group.

Only the Bay inhibitor treated mice, however, had significantly lower induction amounts of this cytokine. This "protective" effect of Bay, although not significant was

still able to reduce IFNβ mean values almost to background levels at day 5. The standard deviations were sometimes pretty high due to the relative amount of mice numbers per group, but the tendency of Bay to reduce IAV-induced IFNβ was noted. There was no significant difference between the solvent (DMSO/CremEL) group and the U0126 at both day 2 and 5, nevertheless a tendency was evident.

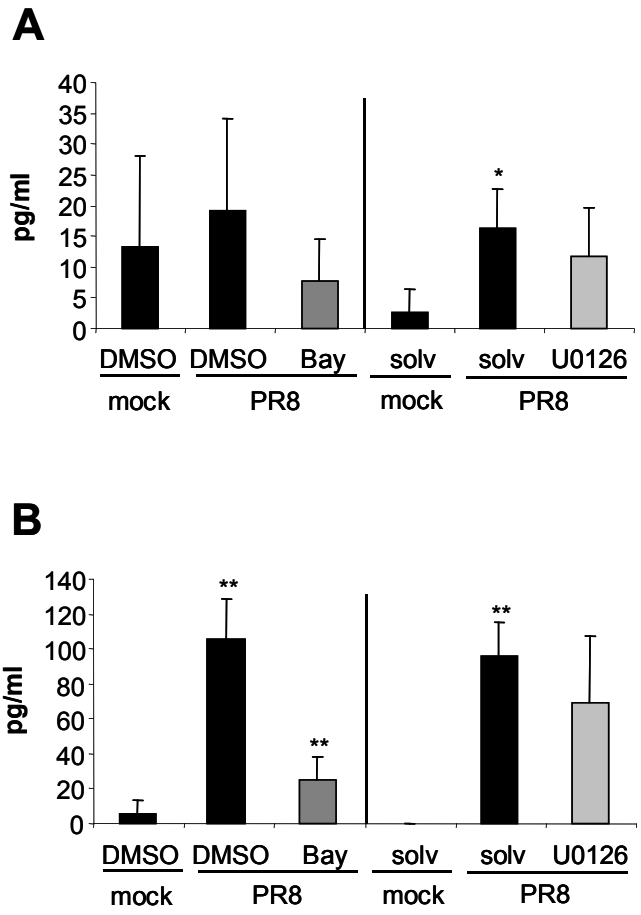

Figure 3.17: KC (A) and MCP-1 (B) results in mice treated with inhibitors. Mice were infected intratracheally with 500 PFU of PR8 in 70µl in PBS (filtered) or mock infected with 70µl PBS. Solvent (DMSO or DMSO/CremEL), Bay or U0126 were administered intraperitoneally, daily starting at -24h (before infection). Mice were sacrificed on day 5 days p.i., and BAL performed. Cytokines were measured using the multiplex bead immunoassay. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to mock vs. infected or infected (–) inh. vs. infected (+) inh. treatment. All groups have a number of 3 mice with exception to mock groups of day 5, which were 2 mice per group.

KC analysis (Figure 3.17 (**A**)) from day 5, showed a significant increase in infected, solvent (DMSO/CremEL) treated mice as compared to mock infected, whereas the

DMSO group only showed a slight tendency for increase. Although there was no apparent significant decrease upon inhibitor treatment in both groups (Bay and U0126) the tendency for reduction was also noted.

For MCP-1 (Figure 3.17 (**B**)) the infected groups showed a significant increase in production of this cytokine as compared to mock groups. Bay inhibitor treatment also seemed to effectively reduce this induction, as compared to the infected DMSO treated group. For the U0126 a tendency was also noted here.

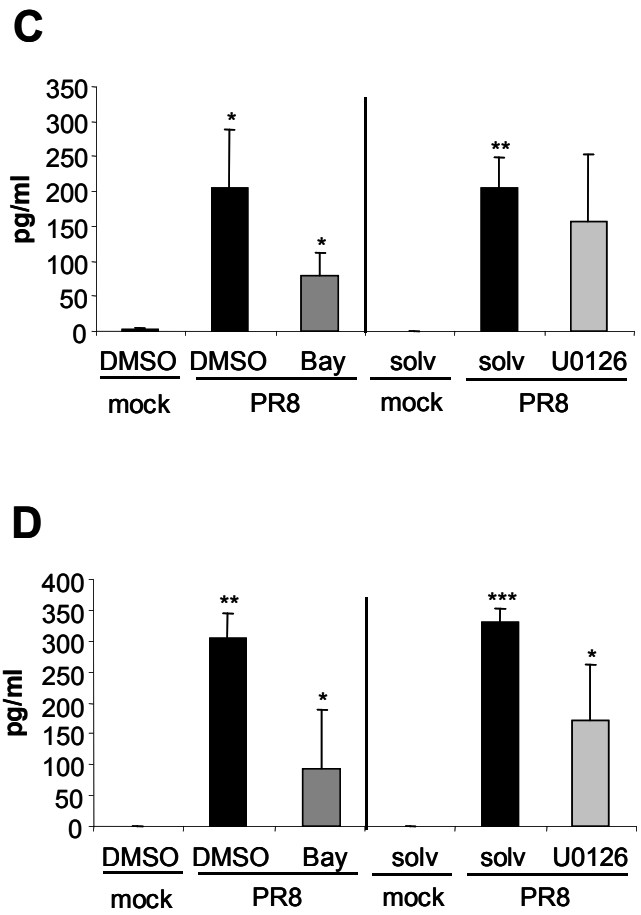

Figure 3.18: IL-6 (C) and RANTES (D) results in mice treated with inhibitors. Mice were infected intratracheally with 500 PFU of PR8 in 70µl in PBS (filtered) or mock infected with 70µl PBS. Solvent (DMSO or CremEL/DMSO), Bay or U0126 were administered intraperitoneally, daily starting at -24h (before infection). Mice sacrificed on day 5 days p.i., and BAL performed. Cytokines were measured using the multiplex bead immunoassay. p values (* < 0.5; ** <0.01; *** < 0.001) are given in comparison to mock vs. infected or infected (–) inh. vs. infected (+) inh. treatment. All groups have a number of 3 mice with exception to mock groups of day 5, which were 2 mice per group.

Both IL-6 (**C**) and RANTES (**D**) (Figure 3.18) were up-regulated on day 5 in infected mice as compared to mock controls. However, whereas in the case of IL-6 (**C**), only the Bay group showed a significant decrease in production of this cytokine compared to the DMSO infected group, in the case of RANTES (**D**) both inhibitors (Bay and U0126) demonstrated a significant effect on reducing this cytokine induction.

Generally both inhibitors showed a tendency for reducing IAV-induced cytokine production in C57BL/6 mice. In the case of Bay treatment it was effective for attenuation of most cytokines, although less effective on TNF-α. U0126 on the other hand was more significant at decreasing TNF-α, but always showed a tendency at reducing all other cytokines tested (see table of results 3.1).

Both inhibitors were also similarly effective at reducing virus titres *in vitro* and *in vivo*, with U0126 being slightly better than Bay.

Taken together both pathways have been demonstrated at being important for both virus replication as well as host cell virus-induced defence mechanisms (in the form of induced cytokine expression), and treatment with specific NF-κB as well as Raf/MEK/ERK inhibitors (Bay and U0126, respectively) were shown to be effective at simultaneously reducing virus titres and virus-induced cytokine production (see table of results 3.1.).

Systems	Inhibitors	IL-8/KC	MCP-1	IL-6	RANTES	IFNβ	TNF-α
A549 cells	Bay	+	++	++	+++		
	U0126	+++	++	++	++		
primary AECs	Bay	+	++	+++	+	+	
	U0126	+	+	+++	-	+	
Mice C57BL/6	Bay	~	++	+	+	+	~
	U0126	~	~	~	+	~	+

Table 3.1.: Summary of reduction of cytokine/chemokine by the specific inhibitors

+++ extremely effective; ++ very effective; + effective; ~ tendency; - no effect

4. Discussion

Influenza A viruses are highly contagious RNA viruses and in humans the main targets are the epithelial cells of the respiratory tract [25]. In general influenza viruses cause acute infections which generally lead to a self-limiting disease [109], and do not spread further than the respiratory tract [110, 111]. Symptoms such as chills, fever, aches, malaise, sore throat, nasal congestion and pulmonary complications are common [113, 114]. However, other possible risks of complications and death are greatly increased in young children and older people [109, 111, 116, 117]. Reports of highly pathogenic influenza virus (HPIV) infections however, have shown more severe symptoms including progressive primary or secondary viral pneumonia [109, 118], extensive pulmonary oedema, acute respiratory distress syndrome (ARDS), alveolar haemorrhage, lymphopenia and multiple organ failure [109, 119]. Severe lung inflammation due to cytokines (i.e. TNF-α, IFNα/β, IL-1, IL-8 and IL-6), has been associated with the pathogenesis of the disease and are mainly responsible for the systemic symptoms observed, like fever and myalgias [114, 115, 119, 121, 125-127, 286].

Despite large immunisation programs, influenza A virus is still a considerable cause of morbidity and mortality worldwide and responsible for major epidemic outbreaks of influenza every year [290]. They have been responsible for the most devastating pandemic outbreaks of the last century, in 1918, 1957 and 1968, costing millions of human lives [3, 126, 291]. Avian Influenza virus (AIV) has also had a severe impact on the poultry industry worldwide which has resulted in serious economic loses.

Although it was previously accepted that AIV could not directly infect humans the emergence of HPAIV of the H5 and H7 subtype and specially outbreaks of the subtype (H5N1), which have crossed the species barrier into humans with high case fatality rates, has brought about deep concerns and the need of more readily available and effective anti-viral therapies [126, 290, 292-296].

Antiviral drugs amantadine and rimantadine, M2 ion channel protein inhibitors, reduce the duration of symptoms of clinical influenza, but the rapid appearance of drug-resistant variants coupled to major side effects have been reported [12, 277, 278, 297]. Innovative sialic acid analogues, neuraminidase inhibitors, zanamivir and oseltamivir, have shown prophylactic and therapeutic effects, however due to the extremely high mutation rate of influenza viruses, inevitable virus resistance will

appear as has already been reported [106, 275]. Vaccination is yet another option, but again, due to constant changes in viral proteins this calls for annual adaptation and in the possible surging of a pandemic situation, the appropriate vaccines would not be produced in time [232, 279] to avoid a major disaster.

In view of these alarming reports, an understanding of the molecular mechanisms responsible for virus-induced inflammatory responses and viral replication in the lung could provide new approaches for therapeutic targeting during an influenza virus infection [12, 232].

As previously mentioned, influenza A virus (IAV) is capable of inducing activation of many different signalling pathways, in infected cells such as the MAPK family members [133, 134], including the classical Raf/MEK/ERK (MAPK) signalling cascade, the NF-κB, the Jun N-terminal kinase (JNK) and the p38 [128]. Also other pathways have recently been found to also be activated by IAV such as PI3K and IRF3/7 [281]. Most of these pathways have been reported to act as antiviral mechanisms by promoting host immune responses (i.e. JNK, NF-κB, IRF3/7, etc) [133, 232] whereas others are mainly/also linked in support of efficient virus replication (Raf/MEK/ERK and also NF-κB) [128, 132, 138, 182, 186, 234].

Taking all this into account, the present work aimed to deal with these known facts and propose to modulate two pathways, the NF-κB and the Raf/MEK/ERK, essential for virus replication and cytokine expression, by using specific inhibitors, with the intent of simultaneously reducing virus titres and virus-induced cytokines. The purpose was to broaden the available knowledge on therapeutic strategies to control and cure influenza virus-induced disease.

4.1. Bay 11-7082 can inhibit virus-induced NF-κB activation in A549

Infection of cells with IAV has been reported to lead to activation of NF-κB pathway either by over-expression of viral proteins such as the viral haemagglutinin (HA), nucleoprotein (NP) or M1 proteins [222, 223] or by the virus itself [232]. Also, studies using influenza virus-induced NF-κB promoter-luciferase-reporter gene assays showed decrease in activity in cells co-expressing trans-dominant negative mutants of IKK2 or IκBα, upstream activators of NF-κB [138]. Many reports on influenza-induced activation of NF-κB have been correlated to increase in expression of pro-inflammatory factors, either by over-expression of chemokines such as IL-8 and

RANTES [135, 232] or in another study by enhanced NF-κB binding to NF-κB elements of the promoters of IFNβ and IL-29 genes [233], or by virus-induced IFNβ promoter studies [71, 138]. Since many antiviral and pro-inflammatory cytokines are known to be controlled by the NF-κB transcription factor [128, 189-192, 222] this pathway seems to play a role in the immune response, in the context of influenza virus infection. However, recent studies have shown that pre-activated NF-κB in cells led to enhanced influenza virus replication, whereas in cells where NF-κB was impaired, virus titres were significantly lower, demonstrating that the activation of this pathway is also somewhat important for efficient viral propagation [138, 139, 234].
In view of this I set out to modulate this pathway with the aim of reducing both virus replication as well as virus-induced cytokine production simultaneously.
Having ensured that treatment with Bay 11-7082 (25μM) inhibitors did not affect A549 cell viability (Figure 3.1), tests on the NF-κB activity, using a specific oligonucleotide ELISA based method, demonstrated that both FPV (Figure 3.2.1(**A**)) and PR8 (Figure 3.2.1(**B**)) infection lead to NF-κB activation in these cells and that this up-regulation could be effectively reduced by the specific IKK-inhibitor (Bay) (70% reduction in FPV and 80% in PR8 infected cells). This stood in agreement with the published articles mentioned above.

4.2. Bay 11-7082 can inhibit virus titres in *in vitro* cell cultures as well as *in vivo*

Although there is consensus in that, infection of cells with IAV virus leads to NF-κB activity, even though the virus NS1 protein can limit this activation [71], the same does not apply to the effects of this activity on virus replication. There have been contradictory reports on the importance/necessity of NF-κB activity for efficient influenza virus propagation [138, 139, 232].
Using the A549 system, in the current study, with the specific IKK inhibitor (Bay at 25μM) (Figure 3.2.2) it was clear that the inhibition of this pathway greatly attenuated virus replication. This was true for both the avian (FPV – 50% reduction) and human (PR8 – 80% reduction) viruses analysed in these experiments.
Some of the reports already mentioned above have also dealt with NF-κB inhibitors in their systems. Nimmerjahn et al. (2004) has previously shown by using another NF-κB inhibitor (Bay 11-7085), that A/FPV/Bratislava virus titres are greatly reduced in A549 cells. Bernasconni et al. (2005), on the other hand, using the A/WSN/33

influenza virus and an alternative NF-κB inhibitor, a cyclopentenone prostanoid (delta^{12}-PGJ$_2$), the drug was unable to show a decline in virus titres. Mazur et al. (2007), very recently has also demonstrated using the acetylsalicylic acid (ASA) and Bay 11-7085, as NF-κB inhibitors, in A549 cells and different influenza viruses that this pathway is required for efficient viral propagation.

A549 cells are human alveolar epithelial carcinoma cells, and are generally considered an artificial system. Therefore I decided to analyse mice primary alveolar epithelial cells (AECs) to get a closer look into a more natural system and also to analyse the correlation between these two *in vitro* systems (A549 and AECs).

After performing viability tests on AECs to determine the non toxic concentration for Bay (15μM) (Figure 3.6) further analysis were conducted by infection and treatment. The tests on AECs infected with FPV or PR8 treated with the Bay inhibitor (15μM) confirmed the earlier results in A549 cells. Analysis of the virus (FPV and PR8) titres from supernatants demonstrated significant reduction (FPV ~ 40% reduction and PR8 ~ 85% reduction) at both 24 and 32h p.i. upon inhibitor treatment, compared to DSMO (solvent) treated cells (Figure 3.8 (**A**) and (**B**)).

The idea that inhibition of the NF-κB pathway could also possibly lead to reduction of virus titres *in vivo*, prompted me to study this model further using C57BL/6 mice. The results clearly demonstrate a significant drop in virus titre (~60%) at day 2 and day 5 p.i. in C57BL/6 mice, treated daily with the Bay inhibitor, when compared to DMSO mock treated (Figure 3.14).

The use of specific NF-κB inhibitors in reducing influenza A virus titres *in vivo* has also just recently been shown by Mazur et al. (2007), using acetylsalicylic acid (ASA). Although in this study I used a different NF-κB inhibitor, Bay 11-7082, the data observed here confirm these newly published results.

Many viruses have been reported to induce NF-κB activation through different mechanisms [129, 193]. Some viruses, such as HTLV can lead to constitutively activated IKK complex and therefore NF-κB activity, shown in chronically HTLV-1-infected myeloid cells [298]. Other viruses, have recognized specific proteins like the LMP-1 oncoprotein of EBV (human γ herpesvirus) and the HBx polypeptide of HBV (hepatitis B virus), which mediate NF-κB activation through IκBα phosphorylation and degradation [299, 300] leading to the clear translocation of NF-κB complexes thereby

trans-activating responsive NF-κB genes. These effects have also been shown to have a supportive effect in the virus replication strategies.

This current report clearly demonstrates, by testing different *in vitro* cell systems as well as *in vivo* mice data that blocking the NF-κB pathway can effectively be used to reduce influenza A virus propagation.

4.3. U0126 can inhibit virus-induced the Raf/MEK/ERK activity in A549 cells

All known MAPK family members have been shown to be activated by influenza virus infection of cultured cells [132-134], amongst these the classical Raf/MEK/ERK (MAPK) signalling cascade, which is of interest to this work. As previously demonstrated activation of this pathway has mainly been linked to efficient influenza virus propagation [132, 182, 186].

In agreement with these earlier findings, the studies here (Figure 3.3.1.A and 3.3.1.B) demonstrate that both avian (FPV) as well as human (PR8) viruses were able to lead to ERK activation in human alveolar epithelial (A549) cells, showing a gradual increase over time, whereas this activity was dramatically decreased (~80%) when administering the specific MEK inhibitor, U0126 (50μM). Previous tests on A549 cell viability (by MTT assay) had demonstrated no toxic effect of treatment with U0126 at 50μM (Figure 3.1).

4.4. U0126 can inhibit virus titres in *in vitro* cell cultures as well as *in vivo*

Previous reports using different cell lines (MDCK and 293T) and different influenza viruses (WSN and FPV), subjected to U0126 MEK inhibitor treatment have demonstrated the importance of this pathway for efficient virus propagation [132]. Also others have shown (in cell lines) through the modulation of this pathway, by using dominant-negative mutants of ERK and Raf, that both influenza A and B replication can be greatly reduced [182]. In contrast cells expressing active mutants of Raf led to enhanced virus production [182, 186].

From the current work it was also evident that inhibition of ERK activity in A549 cells, using the specific MEK inhibitor U0126 (at 50μM), effectively reduced virus titres (FPV and PR8 by ~ 60%) (Figure 3.3.2), as seen in a time course analysis. Results from infected (FPV and PR8) mice primary alveolar epithelial cell (AEC) experiments treated with U0126 (40μM) analysed up to 32h p.i. (Figure 3.8), still stand in

agreement (FPV by ~ 60% and PR8 by ~ 85%) with the effect observed in A549 cells. To add credibility to the notion that the observed effect was not yet another artificial *in vitro* system approach. PR8 infected C57BL/6 mice showed similar reduction in virus titres both at 2 (~ 95%) and 5 day p.i. (50%), when treated daily with U0126 via intraperitoneal injection, compared to mock treated mice.

Previous *in vivo* work with U0126 in a mouse asthma model has reported that this inhibitor reduced ovalbumin (OVA)-induced phosphorylation of ERK and that it could have therapeutic potential for the treatment of airway inflammation [288]. Other studies in mice have also used the U0126 to successfully attenuate the behaviour response to high dose intrathecal morphine [301].

Dependency of virus propagation on the Raf/MEK/ERK pathway has also recently been shown in influenza virus infections *in vivo* using transgenic mice with a constitutively active form of Raf in the alveolar epithelial cells. It was apparent that influenza viruses almost exclusively replicated in cells carrying the transgene as opposed to the wild type animals where influenza viruses replicated mostly in the bronchial epithelial cells [186].

These observed effects are not unique for influenza viruses other viruses have also demonstrated dependency on this pathway for efficient propagation [302]. In a murine coronavirus mouse hepatitis virus (MHV) it was shown that the MEK inhibitor U0126 could reduce virus progeny production in different cells tested [303]. In another study using a different ERK/MAPK inhibitor, PD98059, it was also demonstrated the importance of this pathway for efficient Visna virus replication [302, 304].

In view of these results I could ascertain that the aim of targeting influenza A virus reduction *in vivo* by inhibiting the Raf/MEK/ERK pathway was therefore successfully achieved.

4.5. Bay 11-7082 and U0126 can decrease influenza A virus-induced cytokine production in *in vitro* cell cultures as well as *in vivo*

It has been previously shown that influenza A virus can induce cytokine expression both *in vitro* as well as *in vivo* [120, 238-244, 305]. It has also been postulated that in case of HPAI viruses the increased in observed pathogenesis of the disease could be due to increase and/or deregulated immune responses leading to hypercytokinaemia [111, 114, 115, 119, 120]. It is known that the expression of many

cytokines, including IFNβ, is modulated by NF-κB activation [128, 189-192]. Although the Raf/MEK/ERK pathway in the context of IAV replication is generally linked to pro-viral mechanisms [132, 181], it has been shown in another study that this pathway can also modulate IL-8 cytokine production [180]. We and others have shown that influenza viruses are able to activate both NF-κB as well as Raf/MEK/ERK pathways [132, 139, 181, 231], therefore the aim now was to analyse expression of important cytokines and chemokines linked to immune regulation upon influenza virus infection (IL-8, MCP-1, IL-6, RANTES and IFNβ) and to determine whether these virus-induced cytokine/chemokines could be decreased by application of the specific inhibitors.

Expression profiles of cytokines/chemokines varied depending on the virus used. In A549 cells, FPV had no effect on IL-8, IL-6 and MCP-1, but lead to an increase in RANTES production (Figure: 3.3.1.) whereas PR8 was able to induce IL-8 and IL-6, and suppressed RANTES and MCP-1 expression. What was consistent in all results was that both inhibitors (Bay and U0126) were able to successfully reduce cytokine/chemokine production. Also, in agreement with the report from Kuderer et al. [180], it was evident that IL-8 was mainly dependent on the Raf/MEK/ERK pathway since the U0126 was a better inhibitor than Bay 11-7082.

The results observed in A549 cells are in agreement with previous cell culture studies on BEAS-2B or BEC NCI-H292 (bronchial epithelial cell line) or A549 cells, where IL-8 has been shown to be induced by H3N2 viruses [135, 232, 241]. Also IL-8, MCP-1 and RANTES production have been described upon IVA (H3N2 or H1N1) infection [306-308]. Another study using human middle ear epithelial cells also identified IL-8, MCP-1, RANTES and TNF-α as up-regulated in an influenza virus infection [309].

Still in A549 cells I was unable to detect any measurable amounts of IFNβ upon FPV or PR8 infection. In contrast, the delNS1 virus (a mutant PR8 with deleted NS1 segment), used as a positive control here, was able to significantly induce secretion of IFNβ (Figure 3.4.2). This is in agreement with several reports demonstrating that the IAV NS1 protein has been associated with down-regulation or limited production of IFNβ [66, 67, 137, 230, 246, 310], which at least in part, might be due to limiting NF-κB activation [71].

In primary mice cell (AEC) experiments (Figure 3.9.1) FPV was shown to suppress KC (the mouse analogue for human IL-8), had no effect on MCP-1 or IL-6 secretion but led to a slight increase in RANTES production at 10h p.i.. These results were somehow in agreement with the results see in the A549 tests. PR8, however, was able to increase MCP-1, IL-6, had no effect on RANTES, and decreased KC as compared to mock at 10hp.i.. In the case of PR8 in AECs it was evident that some cytokines were differentially regulated compared to A549 cells where IL-8 was induced and MCP-1 suppressed. Again what was visible in AECs, was that both Bay (IKK inhibitor) as well as U0126 (MEK inhibitor) were able to significantly reduce cytokine production to background levels for both viruses tested, with exception to RANTES. In the case of RANTES, the values identified were almost below the detection limit, proving it difficult to observe any statistical differences.

IFNβ evaluation (Figure 3.9.2) of infected AECs showed that FPV was able to induce significant IFNβ production whereas PR8 lead to a very slight increase. As seen before in A549 cells the delNS1 virus was capable of inducing very high IFNβ secretion (2867pg/ml), again demonstrating the importance of the NS1 protein in viral defence. In this study the Bay inhibitor was the main down-regulator of the viral-induced IFNβ expression, as would be expected, since IFNβ production seems to be regulated by NF-κB, IRF3/7 and AP-1 transcription factors [69, 128, 136].

It is also important to point out that the observed increase in IFNβ in AECs upon viral infection was not in accordance with the results observed in A549 cells, even though the FPV- and PR8-induced cytokine production was relatively low compared to the delNS1 virus control. This alerts to the fact that one should take care to correlate results from “artificial” cell culture experiments to what can actually occur in a natural infection.

In a study, comparing different cell systems (epithelial cells/macrophages) with IAV infection, it was demonstrated that macrophages, in this case, were able to significantly increase IFNβ production as compared to epithelial cells [231]. Other reports comparing H5N1 versus H1N1 in human primary alveolar epithelial cells were able to show very significant increases of IFNβ production from H1N1-infected cells, even though in comparison the H5N1 viruses all led to much higher induction than the H1N1 [240]. Also previous data from Matikainen et al. [246] show that pre-stimulation of A549 cells with IFN-α or TNF-α can induce increase of IAV-induced

IFNβ as well as other cytokines, leading one to acknowledge the idea that in an *in vivo* situation where the environment includes a complex of different cell types and signalling events, the outcome could probably be very different to what one observes in a simple cell culture system, and that extrapolating concluding remarks is very risky.

For this reason *in vivo* studies were conducted in this work to analyse possible differences from cell culture experiments (A549/AECs) and to approach a more "real" situation.

Results from the *in vivo* infection (with PR8 at LD_{50}) showed that, TNF-α (Figure 3.15) at day 2 p.i. was significantly increased in PR8-infected versus mock-infected C57BL/6 mice, whereas there were no significant differences observed at day 5. IFNβ was shown to be significantly increased for both DMSO and solvent treated and infected groups compared to DMSO and solvent treated mock-infected mice, respectively, at day 2 and 5 p.i. (Figure 3.16). All other cytokines tested (KC, MCP-1, IL-6 and RANTES) (Figure 3.17 and 3.18) were found significantly increased in PR8-infected versus mock infected mice at day 5 p.i., with exception to KC in the infected and DMSO treated group, although the tendency to increase was present.

It has to be mentioned that the number of mice used for these experiments was indeed very low (n=3), and in some cases (for day 5 experiments) the control uninfected mice numbers were actually only 2. Due to the obvious ethical reasons, and the fact that these experiments were just to demonstrate a proof of principle, minimal mice amounts were used. For this reason also, the observed standard deviations were pretty high, and as a consequence the statistical analysis of the p-value was sometimes not significant/relevant.

Regarding the inhibitor effects in the *in vivo* study, Bay was able to significantly decrease IFNβ expression, at day 2 p.i., and the tendency at day 5 was still there. It was also able to significantly decrease MCP-1, IL-6, and RANTES at day 5 p.i., and although the decrease for KC was not significant, the trend to decrease was noted. The U0126, on the other hand, showed a significant inhibition on PR8-induced TNF-α release, observed at day 2 p.i., but this effect was lost at day 5, whereas RANTES was significantly reduced at day 5 p.i.. U0126 also demonstrated a tendency for decreasing virus-induced cytokine production for most of the other cytokines

(IFNβ, KC, MCP-1 and IL-6), but due to the limited number of samples analysed, the standard deviations only permit speculation.

One can also speculate on the correlation between virus titres and cytokine induction. In general U0126 seems to reduce virus titres better than Bay, whereas Bay seems to be better at cytokine reduction. This might reflect that most cytokines are NF-κB-dependent, and that the weaker cytokine reduction by U0126 is maybe mostly due actually to the reduction of virus titre.

Reports on influenza A virus induced cytokines are very heterogeneous depending on viruses used, whether the study was conducted using cell lines, primary cells or *in vivo* experiments. Studies from primary cells experiments, whether macrophages, monocytes or epithelial cells have shown all these cytokines/chemokines to be induced by, one influenza virus or another [237-240, 289, 311]. *in vivo* experiments, using different virus strains, have also confirmed induction of all the cytokines studied in this report (IL-8/KC, MCP-1, IL-6, RANTES IFNβ and TNF-α) [242, 254, 312-314].

In my study I have compared different *in vitro* and *in vivo* systems with the same viruses and treatments.

It was interesting to observe different cytokine induction from the different systems (A549/AECs) in this study. For example the IL-8 was found to be up-regulated in PR8-infected A549 cells, whereas in AECs the virus suppressed this cytokine, compared to mock infected cells. Curiously the same effect was observed in other studies where IL-8 was shown to be induced in H3N2 virus-infected A549 cells, [241] and BEAS-2B cells [135], whereas Sprenger et al. [238] and Hofmann et al. [237], showed decrease in IL-8 in human primary monocytes or macrophages, respectively, when using the PR8 virus. In a study conducted in mice Wareing et al. [242] also saw the same suppression or no effect in KC induction upon influenza virus infection.

Studies on the different pathways linked to induction of different cytokine/chemokines, have also shown some interesting data. Production of IFNβ, RANTES, IL-8, IL-6 and TNF-α has been shown to be NF-κB-dependent by different authors using different systems [71, 196, 232, 315]. Whereas IL-8, MCP-1, IL-6 and also TNF-α in other reports, have been linked to the activity of the Raf/MEK/ERK pathway [135, 283]. Still other pathways are also found to be implicated in the production of these cytokines, such as the p38 MAPK in IAV-induced production of RANTES, IL-8 and TNF-α [134, 135, 289, 308]; and the AP-1/JNK pathway in IAV-

induced production of RANTES as well [134, 308] or even specifically the PI3K activation pathway on the influence of RANTES and IL-8 production [135].

In this study, as was expected, the NF-κB pathway was implicated in all the cytokine/chemokines tested, as seen by applying the inhibitor Bay 11-7082 to *in vitro* infected cell cultures (A549/AECs) or *in vivo* (C57BL/6 mice). Generally the Bay inhibitor always demonstrated a stronger effect at decreasing cytokine/chemokine production compared to the U0126 inhibitor, with exception to IL-8 in A549 cells and TNF-α in *in vivo* mice experiments. It was evident that the Raf/MEK/ERK was highly implicated in IL-8 production especially in A549 cells where the U0126 treatment of infected cells led to a very significant reduction of this chemokine. This effect was still apparent in AECs and *in vivo* experiments although not as spectacular. In fact all the cytokines tested in this work were able to be suppressed by inhibiting this pathway (Raf/MEK/ERK). This is the first time that this pathway, Raf/MEK/ERK, has been implicated in cytokine/chemokine production in the context of influenza A virus infection with exception to IL-8 production which had been shown by Guillot et al. [135]. This discovery was also evident in the *in vitro* (A549/AECs) as well as the *in vivo* experiments (C57BL/6 mice).

Altogether, I was able to demonstrate that cytokines/chemokine (KC, MCP-1, IL-6, RANTES, IFNβ and TNF-α) were induced by influenza A virus infection (FPV and PR8) in *in vitro* (A549/AECs) as well as *in vivo* (with PR8) and that this induction could be reduced by the specific IKK- (Bay 11-7082 for NF-κB) or/and MEK-inhibitor (U0126 for Raf/MEK/ERK), in many cases to background levels, in all systems tested. It was also apparent that different systems lead to differential induction of cytokines/chemokines, as seen mainly by IL-8, MCP-1 and IFNβ in A549 cells versus AECs and *in vivo* studies, and in the case of RANTES between *in vitro* PR8-infection and *in vivo* results.

4.6. Both FPV and PR8-induced nuclear RNP export is efficiently blocked by Bay 11-7082 and U0126 in A549 and AECs.

Influenza A virus genome is composed of 8 segments of ssRNAs of negative polarity which are associated to viral polymerases (PA, PB1 and PB2) and nucleoprotein (NP) forming thus the ribonucleoprotein (RNPs). The virus is replicated in the nucleus of the infected cell and late in the replication cycle the viral genome forms the RNP

complexes which have to be efficiently exported from the nucleus into the cytoplasm to be able to be packaged into progeny virions at the cell surface (ref). This step is of course very important in influenza virus replication. As both, NF-κB and ERK pathway activation have been associated with efficient viral nuclear RNP export [132, 181, 234], I decided to confirm these findings in our A549 system, by making use of our specific IKK and MEK inhibitors. Results demonstrate that in A549 cells both avian (FPV) as well as human (PR8) influenza virus showed nuclear RNP export whereas upon inhibitor treatment (with either Bay or U0126), the RNP export was significantly impaired as seen at 10h p.i. (Figure 3.5.1 and 3.5.2). These results add to the already published data, confirming that the activation of both NF-κB as well as Raf/MEK/ERK pathway is necessary for efficient viral nuclear RNP export.

In AECs both FPV and PR8 also showed nuclear RNP export at 10h p.i. but upon inhibitor treatment (with Bay or U0126), the viral RNP was mainly blocked in the nucleus (Figure 3.5.1 and 3.5.2). This excludes the idea that the observed effect in could be an artefact only seen in established cell lines, even though confirmed by others in other cell lines [132, 181, 234]. Since AECs are primary cells one can speculate that this effect reflects the *in vivo* situation.

The mechanism for this effect has been suggested to be partially dependent on the activity of viral nuclear export protein NEP/NS2 [44, 132]. Other findings suggest that this might be dependent on viral induction of the pro-apoptotic factors, such as Caspase 3 [316] and NF-κB-dependent tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and FasL [138]. In support of this NF-κB dependency, Mazur et al. (2007) also just recently showed that ASA (an NF-κB inhibitor) was able to also block virus-induced RNP export from the nucleus of A549 cells.

Although the aim of this study was not concerned with explaining the molecular mechanisms of this effect, it was interesting to observe that during AEC infection studies, cell viability was increased in infected cells treated with the IKK inhibitor (Bay) compared to the infected DMSO treated cells (Figure 3.7 (A) and (B)). This was observed for both viruses (FPV and PR8), which would agree with the above suggestion, that virus-induced NF-κB activity leads to induction of apoptosis, and that by specifically inhibiting this pathway, one can down-regulate NF-κB-dependent virus replication and thereby virus-induced cell destruction.

4.7. Combination treatment does not enhance individual inhibitor-induced decrease in virus titres

With the aim of further reducing virus propagation with combination treatment and thereby possibly reducing the concentration necessary of the individual inhibitors for this effect, studies were conducted in A549 cells and AECs. The idea was that by reducing the amount of inhibitor treatment needed, one would reduce possible intolerance of the drugs or side effects that could later be observed *in vivo* while at the same time still producing the same (or better) effect (reducing virus titres/cytokines). Results from selected non-toxic inhibitor "cocktails" (Bay-15μM/U0126-30μM and Bay-10μM/U0126-30μM) showed no significantly enhanced decrease of virus titres when comparing to results with single inhibitor treatment in A549 cells (Figure: 3.12). This idea was then completely abandoned after results from viability assays in AECs demonstrated a significant toxic effect even when applying low concentrations of both inhibitors (Figure: 3.13).

5. Conclusions

In the present work I show that influenza A virus infection can strongly induce NF-κB as well as Raf/MEK/ERK pathway activity, in human alveolar epithelial A549 cells. The different viruses used in our study avian FPV as well as human PR8 led to different kinetics as well as different activity intensities of these pathways. Inhibition of these pathways using a specific IKK-inhibitor (for the NF-κB pathway) or MEK inhibitor (for the Raf/MEK/ERK pathway) could efficiently reduce this virus-induced activity in A549 cells.

Infection studies using the specific inhibitors Bay and U0126, demonstrated for the first time that these pathways (NF-κB and Raf/MEK/ERK, respectively) are required for efficient virus propagation, not only in human alveolar epithelial A549 cells but also in mice primary alveolar epithelial cells (AECs) and *in vivo* in C57BL/6 mice, as determined in titration by FFU assay.

The role of NF-κB pathway in influenza A virus infection has been a little controversial. In general NF-κB activation is associated with the cellular antiviral response to influenza infection. Aside from this accepted view, it has also been recently considered as a requirement for virus infectivity [138, 139, 234]. Other studies have indicated that viral replication is independent from the ability of the virus to activate NF-κB in infected cells [232].

My data clearly indicates and supports the former idea, that influenza A virus infection not only activates NF-κB transcription factor, but also that this activity promotes efficient virus replication.

As to the Raf/MEK/ERK pathway, studies in influenza A virus infections point to a requirement of activity of this pathway for efficient virus propagation [132, 181, 182, 186], whereas in this study for the first time it becomes evident that this pathway also affects host cell defence mechanisms, and that by inhibiting this pathway it is possible to reduce virus-induced cytokines as well as the predicted virus propagation.

In agreement also with previous studies I could demonstrate that upon virus infection cells will secrete different cytokines and that some of these cytokines can be NF-κB dependently induced (IL-8, KC, MCP-1, IL-6, RANTES and IFNβ) as seen by using the Bay (IKK inhibitor) and/or Raf/MEK/ERK-dependent (IL-8, KC, MCP-1, IL-6,

RANTES and TNF-α). This was apparent in all the models tested here, in the *in vitro* cell cultures and in the mice *in vivo* experiments.
Influenza viruses follow a nuclear replication strategy and therefore late in their replication cycle the viral genome RNP has to be exported from the nucleus into the cytoplasm. Here it was shown for the first time in primary cells, using mice alveolar epithelial cells (AECs), that the viral-induced RNP export from the nucleus was significantly impaired upon treatment with either IKK or MEK inhibitors. Previous studies performed in different cell lines [132, 181, 234] have shown the same effect that was observed here in human alveolar epithelial cells (A549) and in mice primary cells (AECs).

The aim of this study was to modulate these two pathways with the purpose of decreasing virus replication as well as simultaneously decreasing virus-induced cytokine production. It can be safely said that this aim was achieved with success.

It has to be added, however, that the final aim would be to target these pathways for antiviral therapy strategies. Studies conducted with HPIV have shown, in humans, primates and mice, that high viral loads as well as elevated pro-inflammatory factors are central to the pathogenesis observed in these infections [115, 119, 120, 254] and in a H5N1 study in pigs it was demonstrated that this virus was resistant to the effects of interferons and TNF-α [294]. However, a recent report has highlighted the concern of targeting an essential mechanism of the organism in combating the disease, by showing that glucocorticoid (steroids, which show among other functions, suppression of cytokines) treatment (given in the drinking water) of mice infected with HPAIV (H5N1) did not protect against death [317].
In the current report it is apparent that many pathways lead to redundant effects. The idea to target a specific pathway, which is necessary for virus replication as well as for immune regulation, seems logical in view of all the reported studies with HPIV infections. In the *in vivo* context, it is also evident that affecting a specific pathway will not totally block its overall effect on immune response mechanisms, but might be able to attenuate the “cytokine storm” seen with these HPIV strains. The aim here was not to deplete the natural/necessary immune response, but to diminish the virus-induced hyper-induction of the inflammatory response which is a cause of the gravity

of this disease. Further studies could be extremely valuable for fine-tuning the effects of these drugs.

Although targeting a cellular factor brings about concerns as to the possible side effects raised from drug usage, local administration could probably be more, well tolerated [12]. Drugs targeting these pathways are already in current use, such as NF-κB inhibitors like the common aspirin [203, 234, 318], and as for the Raf/MEK/ERK pathway, since chronic activation of this pathway is linked to several cancers [147, 148] there are many drugs under clinical trial for application in cancer therapy [148, 302].

Prospective studies

Many extra experiments could still be done to add to the available data so far achieved in this report, such as: (1) further studies with infected mice using the Bay and U0126 inhibitors, to study survival curves with these inhibitors; (2) attempting other viral strains for infection to compare pathology and cytokine induction in inhibitor treated versus untreated; (3) to analyse lung histology and cell population profiles in infected mice untreated compared to inhibitor treated mice.

Due to the time available further studies will have to be postponed. But the future prospects would be recommended, since the current report shows potential for aiming at influenza virus therapy.

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7. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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M Carneiro de Moura, R Marinho, **R Pinto**, M Santos, "Balance between Th-1 type and Th2-type cytokine response during interferon alpha plus ribavirin combination therapy in chronic hepatitis C". In Ricardo Moreno-Otero, Augustín Albillos, Carmelo García-Monzón, editors. Immunology and the liver: cytokines. Madrid, Acción Médica, 2002:303-12

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R Pinto, "Inhibition of influenza A virus induced signalling: Virus replication and cytokine expression", International PhD Symposium, 21 October 2006, Giessen, Germany
R Pinto, "MAPK-inhibition during Influenza A virus replication and host inflammation in the mouse lung", Second MBML Retreat, 31 July to 2 August 2006, Giessen, Germany
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POSTER PRESENTATIONS:
R Pinto, L Cakarova, S Herold, J Lohmeyer, W Seeger, S Pleschka, "NF-κB and ERK Inhibition Reduces Expression of Pro-Inflammatory Factors and Influenza Virus Propagation Simultaneously", German Congress for Virology, March, 2008, Heidelberg, Germany
R Pinto, L Cakarova, S Herold, J Lohmeyer, W Seeger, S Pleschka, "NF-κB and ERK Inhibition Reduces Expression of Pro-Inflammatory Factors and Influenza Virus Propagation Simultaneously", European Congress of Virology, September,

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R Pinto, L Cakarova, S Herold, J Lohmeyer, W Seeger, S Pleschka, “NF-kB and ERK inhibition modulate expression of pro-inflammatory factors and impair influenza virus propagation” (**poster discussion**), American Thoracic Society (ATS) International Conference, May 18-23, 2007, San Francisco, California, USA
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R Marinho, **R Pinto**, M Santos, M Carneiro de Moura, “Prominent proliferative T-cell response in female patients to structural and nonstructural hepatitis C virus antigens after interferon-alpha treatment”, 50TH Annual Meeting of AASLD, Dallas, USA, November 1999
R Marinho, **R Pinto**, M Santos, M Carneiro de Moura, “Higher NS3 T-Cell Reactivity During Therapy for Chronic Hepatitis C Infection”, 36TH Annual Meeting of EASL, Praga, Chec Republic, April 2001
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