Relation between Influenza Virus NS Gene, ds RNA activated Proteinkinase (PKR) and Interferon

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Widmung

Diese Arbeit widme ich meiner Familie, vor Allem meinen Eltern, die durch ihre stete Hilfe wesentlich zum Erfolg beigetragen haben.

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Abstract

Influenza A/PR8/34 virus non structural protein 1 (NS1 protein) is known as a virulence factor capable of antagonizing antiviral response in host cells. In the present study, we investigated whether influenza A virus lacking NS1 gene (delNS1) is able to trigger elevated type I and III IFN serum levels when compared to wild type virus (PR8). C57BL6 mice were immunized by three different administration routes; intranasal, intraperitoneal and subcutaneous of at least two different doses of delNS1 and PR8. Serum samples were collected after 8 and 16 hours post immunization and serum concentration of type I/III IFNs measured by reporter cell based bioassay capable of detecting all antiviral IFNs simultaneously. We furthermore determined the influence of ds RNA activated Proteinkinase (PKR) upon viral infection. For this reason, PKR knockout mice (PKR KO mice) and C57BL6 wild type mice were immunized intraperitoneally with two different doses of delNS1 and PR8 virus and the IFN concentration was measured in serum samples collected 8 and 16 hours post infection.

DelNS1, lacking ds RNA binding domain located in NS1 protein, thus unable to sequester ds RNA, induced significantly higher levels of IFN in C57BL6 mice in a dose dependent manner irrespective of the administration route tested, compared to PR8.

In contrast to C57BL6 mice, significantly lower serum IFN concentrations were detected in PKR KO mice when immunized with delNS1, whereas the IFN concentration induced by the wild type PR8 virus were comparable in both the C57BL6 and PKR KO mice. In addition, we asked the question whether beyond IFN other chemokines and immunstimulating cytokines are also differently regulated in this test system. We found differences in serum concentrations only for KC, IL-5, and MCP1 out of 12 target proteins in total tested which seem to be substantially impaired or delayed in PKR KO mice when compared to wild-type mice.

Kurzfassung (wissenschaftlich)

Influenza A/PR8/34 Virus „Nicht-Strukturprotein 1“ (NS1 Protein) ist ein Virulenzfaktor der fähig ist, antivirale Prozesse der Wirtszelle zu unterdrücken. In dieser Studie wurde untersucht ob Influenza A ohne NS1 Gen (delNS1) erhöhte Interferon Typ I und III (IFN) Serum-Spiegel im Vergleich zum Wildtyp Virus induziert. C57BL6 Mäuse wurden durch unterschieliche Applikationswege intranasal, intraperitoneal und subcutan, mit unterschiedlichen Dosen delNS1 und PR8, immunisiert. Serum wurde 8 und 16h nach Immunisierung gewonnen und mit einem auf Zellen basierendem Reportertest, der alle antiviralen Interferone gleichzeitig messen kann, getestet. Weiters wurde die Rolle von ds RNS aktivierten Proteinkinsae (PKR) bei viraler Infektion untersucht. Aus diesem Grund wurden PKR defiziente Mäuse (PKR KO Mäuse) und C57BL6 Wildtyp Mäuse intraperitoneal mit unterschiedlichen Dosen delNS1 und PR8 immunisiert, Serum nach 8 und 16 Stunden gewonnen, und auf Interferon getestet. DelNS1 fehlt die ds RNS Bindungsdomäne, die im NS1 Protein lokalisiert ist, und deshalb die Fähigkeit ds RNS zu binden. Deshalb konnten signifikant höhere Interferon Serumspiegel, Dosis abhängig, im Vergleich zum Wildtyp PR8, bei allen Applikationsformen gefunden werden.
Im Gegensatz zu C57BL6 Mäusen, konnte in PKR KO Mäusen signifikant reduzierte IFN Serumkonzentration nach Immunisierung mit delNS1 gefunden werden, während PR8 Immunisierung vergleichbare IFN Konzentrationen in C57BL6 und PKR KO Mäusen zeigte.
Es wurden 12 weitere Cytokine und Chemokine auf unterschiedliche Regulation in diesem Testsystem getestet. Deutliche Unterschiede in der Serumkonzentration von KC, IL-5 und MCP1 in PKR KO Mäusen im Vergleich zu Wildtyp Mäusen, zeigen beeinträchtigte oder verzögerte Regulation durch die Abwesenheit von PKR.

Kurzfassung (populärwissenschaftlich)

Influenza A/PR8/34 Virus „Nicht-Strukturprotein 1“ (NS1 Protein) ist ein Virulenzfaktor der fähig ist, antivirale Prozesse der Wirtszelle zu unterdrücken. In dieser Studie wurden Unterschiede in Regulation der Interferonantwort auf virale Infektion zweier Viren mit (Wildtyp) und ohne NS1 Protein (delNS1) in C57BL6 Mäusen untersucht. Weiters wurde der Einfluß von PKR auf die Interferonantwort mittels PKR defizienten Mäusen im Vergleich zum C57BL6 untersucht.
Es konnte gezeigt werden, daß delNS1 signifikant höhere Serum Interferonspiegel in C57BL6 Mäusen als der Wildtyp Virus, abhängig von Dosis und Verabreichungsart der Viren, stimuliert. PKR defiziente Mäuse zeigten einen signifikant reduzierten Serum Interferonspiegel als C57BL6 nach Immunisierung mit delNS1, was den Einfluß von PKR auf die Regulation der Interferonantwort auf virale Infektion unterstreicht. Weiters konnten Hinweise darauf gefunden werden, daß PKR und NS1 eine Rolle bei der Regulation weiterer immunologischer Parameter KC, IL-5 und MCP1 spielen.

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1 Abbrevations

RNA	Ribonucleic acid
mRNA	messenger RNA
NP	Nucleoprotein
NA	Neuraminidase
vRNA	viral Ribonucleic Acid
ORF	Open Reading Frame
MAVS	Mitochondrial Antiviral Signaling Protein
RNPs	Ribonucleoprotein
MHC	Major Histocompatibility Complex
CTL	Cytotoxic T Lymphocytes
IAM	Institute of Applied Microbiology
ds RNA	double stranded RNA
RIG-I	Retinoic Acid inducible Gene I
2’-5’ OAS	2',5'-Oligoadenylate Synthetase
RNase L	Ribonuclease Latent
PI3K	Phosphatidylinositol-3-Kinase
eLF4GI	Eukaryotic Translation Initiation Factor 4GI
PABI	Poly(A)-Binding Protein I
IFN	Interferon
TLR	Toll Like Receptor
PAMPs	Pathogen Associated Molecular Patterns
IRF3	Interferon regulatory factor 3
NF-kB	Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells
IPS-1	Interferon Promoter Stimulator 1
VISA	Virus induced Signal Adaptor
STAT	Signal Transducers and Activators of Transcription
TYK	Tyrosine Kinase
JAK	Janus-Kinase
Mx	Myxovirus Resistance Protein
PKR	ds RNA activated Proteinkinase
eIF-2α	Eukaryotic Initiation Factor 2 alpha
KCS	Kinase conserved Sequence

ISRE	Interferon Stimulated Responsive Element
GTP	Guanosine-5'-Triphosphate
GDP	Guanosine Diphosphate
ELISA	Enzyme-Linked Immunosorbent Assay
DPBS	Dulbecco's Phosphate Buffered Saline
ATCC	American Type Culture Collection
PFU	Plaque Forming Units
EDTA	Ethylenediaminetetraacetate
FCS	Fetal Calf Serum
HiFCS	Heat inactivated FCS
DTT	Dithiotreitol
TRIS	Tris(hydroxymethyl)aminomethane
S-PE	Streptavidin Phycoerythrin
RPM	Revolutions per Minute
IP	Intraperitoneal
IN	Intranasal
SC	Subcutaneous
TPCK	1-Tosylamido-2-phenylethyl chloromethyl ketone
HUGO	Human Genome Organisation
aa	Amino Acids
kD	kilo Dalton
CO2	Carbon Dioxide
L-M cells	Mouse Fibroblasts
IFN	Interferon
TLR	Toll Like Receptor
PAMPs	Pathogen-Associated Molecular Patterns
IRF7	Interferon Regulatory Factor 7
LPS	Lipopolysaccharides
hpi	hours post infection

2 Introduction

2.1 Influenza an overview in brief

Fever, cough, body aches, fatigue and headache, having symptoms like these it is most likely that you are a typical case of the flu.

Influenza, common known as “the flu”, is an infectious disease caused by viruses belonging to the family of the **Orthomyxoviridae**.

During seasonal epidemics influenza is often mixed up with the common cold an influenza like illness caused by different pathogens like variacella, parainfluenza or respiratory syncytial virus.

“Influenza enters the host through the mucosa of the upper respiratory tract, but exerts most of its pathologic effects in the lower respiratory tract.” (M. Vajdy Immunity against mucosal Pathogens 2008)

Transmission of influenza mainly occurs via aerosols emerging from infected mammals by coughs and sneezes. Healthy individuals can also be infected via the faecal oral route following contact with influenza bearing nasal secretions, faeces or blood by touching their eyes, nose and mouth.

Each year seasonal influenza affects 5-15% of the population in the northern hemisphere. [www.euro.who.int/influenza].

Influenza can be a severe disease in high risk groups like elderly, children and immunocompromised patients, resulting in pneumonia which can be fatal. Total fatal cases range from 250 000 to 500 000 per year during an epidemic and up to millions during a pandemic outbreak. (www.euro.who.int/influenza).

Seasonal epidemic Influenza strains share a certain sequence homology, but constantly mutating viral genomes generate new variants of antigens which can evade pre-existing immunity of the host this and makes him susceptible to infection with the new variant of influenza, this process is called **antigenic drift**.

Therefore the world health organisation monitors circulating influenza strains worldwide and predicts each year the predominant strains for the following year vaccine composition.

Antigenic drift is the cause for the emerging of slightly different influenza viruses, while antigenic shift is the reason for the arising of viruses containing novel surface glycoprotein.

The base of **antigenic shift** lies on the one hand within the broad host spectrum like humans, birds and pigs and on the other hand the segmented nature of the influenza genome. A new strain of influenza can arise if a host infected by two different influenza strains resulting in reassortment of segments resulting in a maybe more or less virulent influenza strain. There is no doubt that this can be the first event for a new pandemic.

In recent history man had to deal with several pandemics, the most famous and lethal pandemic occurred in 1918 “The Spanish flu” killing tens of millions of people, later pandemics were not so extreme severe (Johnson et al 2002[1]).

The world health organisation and the scientific community had learned their lesson in providing fast and accurate diagnostic tools for monitoring influenza worldwide.

And providing tools for treating patients down with influenza like Amantadine, Rimantadine, Tamiflu and Relenza interfering with the replication cycle of the virus, and immunological tools like humoral or mucosal immunisation stimulating a specific immune response to desired strains of influenza for preventing sickening.

2.2 Orthomyxoviridae

2.2.1 Characteristics, structure and taxonomy

The family of orthomyxoviridae contains five members [International Committee on Taxonomy of Viruses] Influenza A, B and C, Thogotovirus and Isavirus. Recently a sixth member has been discovered (Presti et al 2009) [2].
The characteristics of orthomyxoviridae are their segmented single stranded negative RNA genome and their viral envelope.
RNA has been termed negative, because of its function to serve as template for the transcription of viral messenger RNA, by convention mRNA is plus stranded.
Influenza A, B and C can be distinguished on the basis of antigenic differences between their nucleocapsid (NP) and matrix (M) proteins.
Influenza A viruses are further divided into subtypes based on the antigenic nature of their hemagglutinin (HA) and neuraminidase (NA) glycoproteins (Fields, Virology)

Virion structure and composition

The filamentous appearing virion size ranges between 80 and 120nm. The envelope is derived from the cell where the virus replicated. Usually Orthomyxoviridae consist of ~ 1% RNA, 70% protein, 20% lipid and ~ 9% carbohydrate.

2.3 The Genome of Influenza A

While Influenza A and B genome consist of eight segments, Influenza C only contains seven segments, lacking neuraminidase.

“The first 12 nucleotides at the 3´end and the first 13 nucleotides at the 5´end of each vRNA are conserved in all eight ssRNA segments.” [Fields Virology]
3´and 5´ends of RNA segments form a circular conformation named panhandle structure.

Segment Nr	Nucleotides	Encoded Polypeptide	Polypeptide Lenght [aa]	Approx. no of molecules/virion
1	2341	PB2	759	30-60
2	2341	PB1	757	30-60
3	2233	PA	716	30-60
4	1778	HA	566	500
5	1565	NP	498	1000
6	1413	NA	454	100
7	1027	M1	252	3000
		M2	97	20-60
8	890	NS1	230	
		NS2	121	130-200

Table1: Segment organisation and encoded proteins for influenza A/PR/8/34.

2.3.1 Segment 1, 2 and 3: The Polymerase Proteins PB2, PB1 and PA

Segment 1,2 and 3 encode for PB2, PB1 and PA , named upon their characteristics on IEF gels. PB2 and PB1 show basic and PA shows acidic behaviour.
Altogether polymerase proteins consume ~40% of the coding capacity of Influenza A. After synthesis in the cytoplasm, the PB proteins are transported to the nucleus.

It was generally believed that synthesis of the viral vRNA is the only function of proteins encoded in segment 1,2 and 3, until in search for alternative reading-frame peptides encoded by influenza A virus, PB1-F2 was discovered in some influenza strains, first published being responsible for cell death (Chen et al) [3]. Recent published data shows that PB1-F2 also inhibits the induction of type I interferon at the level of the MAVS Adaptor Protein (Varga et al) [4]. The fact that pandemic strains emerged in 1918, 1957 and 1968 share PB1-F2 protein, revealed a new major virulence factor.

2.3.2 Segment 4 Hemagglutinin HA

The predominant surface glycoprotein of the influenza virion was named after its biochemical property to agglutinate erythrocytes.

Specific binding of hemagglutinin (HA) to sialic glycoprotein present on the surface of eukaryotic cells is the initial step of influenza reproduction cycle.

HA mediates fusion between viral envelope and the endosomal membrane resulting in internalisation into a cellular endosome, this process is so called endocytosis. Increasing acidification of this vesicle leads to a conformational change in HA releasing the viral nucleocapsid into the cytoplasm.

Influenza C virions, lacking NA, possesses the HEF protein named after its functions **h**emagglutination, **e**sterase- and **f**usion activity, unifying HA and NA functions.

HA is the major target for neutralising antibodies, recently a clone was isolated recognizing all 16 HA subtypes of influenza A, protective in mice and ferrets (Corti et al 2011) [5]

2.3.3 Segment 5: Nucleocapsid Protein NP

Inside the influenza virion particle the NP protein forms together with the viral RNA segments the so called RNPs. Beneath other functions like transport of the incoming viral RNA to the nucleus it appears that the major function of NP is the encapsidation of the viral segments (Ye et al 2006) [6]).
Furthermore NP contains three distinct immunodominant T-cell epitopes (Townsend et al 1986 [7]), presented via MHC, recognized by cytotoxic T lymphocytes leaving an opportunity for an universal influenza vaccine.

2.3.4 Segment 6: Neuraminidase NA

The second surface protein of the virion is neuraminidase (NA).
Its biological activity lies in catalysing the hydrolysis of terminal sialic acid residues from the new formed virions and the host cell receptors. This is a crucial event for the mobility of virus particles through the respiratory tract and the elution of virions from the infected cell.
Neuraminidase inhibitors like Tamiflu and Relenza interfere the hydrolysis of the terminal sialic acids and the new virions stick to the host cell losing their ability to infect new cells.

2.3.5 Segment 7: Matrix Proteins M1 and M2

The proteins M1, the matrix protein, and M2, which shows ion channel activity, are encoded in the open reading frame of segment 7 of influenza A virus.

M1protein is most abundant and located on the inside of the viral lipid envelope.
M2 ion channel activity results in acidification inside the virion particle and dissociation of M1from the RNPs, during virus entry into the host cell.

2.3.6 Segment 8: Non structural Proteins NS1 and NS2

The open reading frame of segment 8 encodes for two non structural proteins NS1 and NS2 also known as NEP.
NS2 has been shown to possess a nuclear export signal and was renamed describing its function from NS2 to **n**uclear **e**xport **p**rotein NEP mediating the nuclear export of viral ribonucleoprotein (O'Neil et al 1998 [8]).

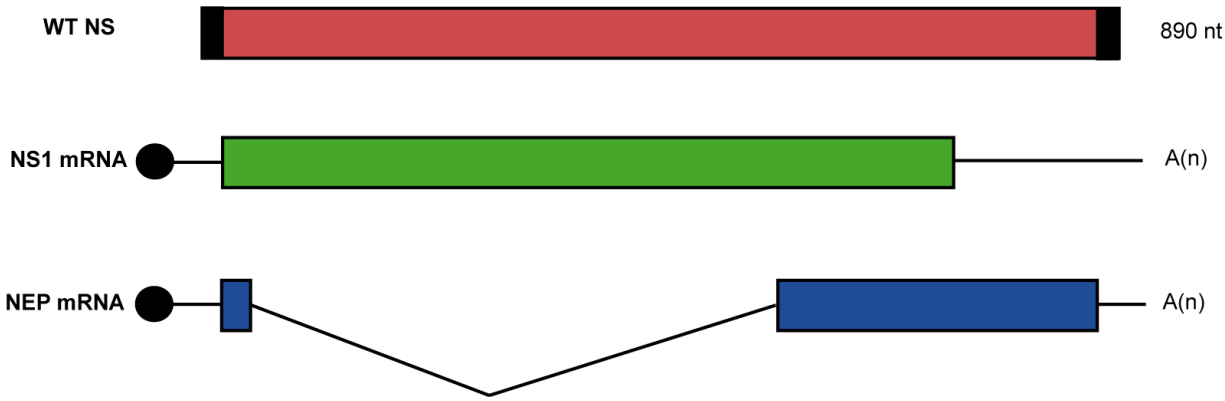


Fig1: Segment 8 of Influenza and derived vRNA. Source Garcia-Sastre et al 1998 [10].

In contrast NS1 is a multifunctional protein, the first NS1 deletion mutant was generated in 1998 by reverse genetics at the IAM by the influenza research group of Prof Muster described as a deletion mutant capable to replicate in interferon deficient systems (Egorov et al 1998 [9]; Garcia-Sastre et al 1998 [10]).

Since then much has been discovered about the versatile nature of the NS1 protein. Pubmed shows about 177 citations for this initial publication.
Recent reviews summarize major functions for NS1 protein until now (Kochs et al 2007 [11], Hale et al 2008 [12]). And there are still interaction partners with unknown function.

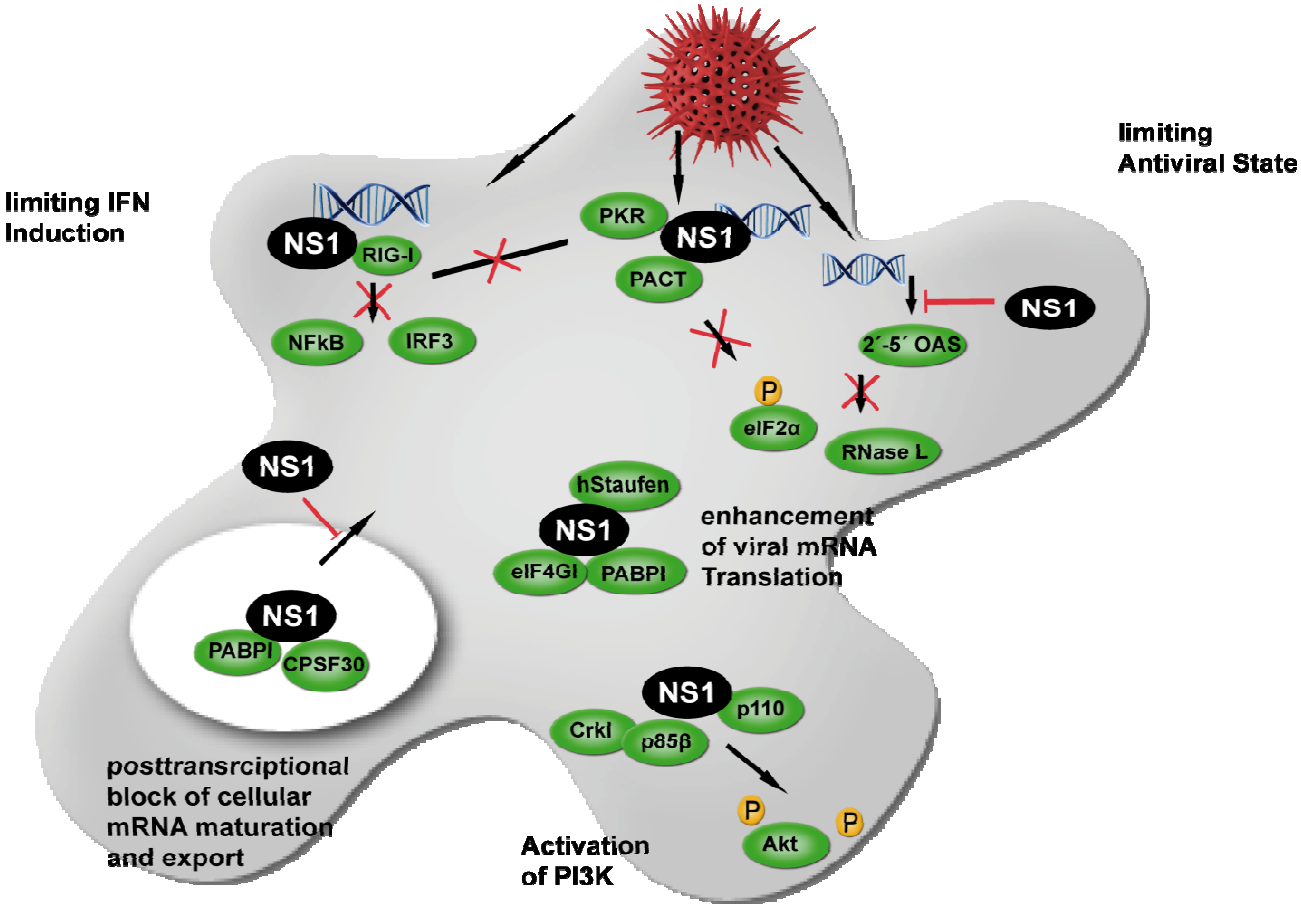


Fig 2: Overview of NS1 involved cellular processes. Modified source Hale et al 2008 [12].

i) Limiting IFN Induction by sequestration of ds RNA (Donelan et al 2003 [13]) preventing activation of RIG-I (Pichlmair et al 2006 [14]) and 2'-5' OAS / RNase L cascade (Min et al 2006 [15]).

ii) Binding to PKR and preventing activation of transcription factors like IRF3 (Talon et al 2000 [16]), NF-kB (Wang et al 2002 [17]) and ATF-2/c-Jun (Ludwig et al 2002 [18]).

iii) Activation of PI3K (Hale et al 2006 [19]).

iv) Interaction with eLF4GI and PABPI to enhance translation of viral mRNA (Burgui et al 2003 [20]).

v) Inhibiting mRNA processing and export of polyadenylated cellular mRNA (Nemeroff et al 1998 [21]).

2.4 Interferon

Interferon (IFN) was first published in 1957] (Issacs et al[22], [23]) and specified as an activity secreted by virus infected cells of preventing further infection of cells exposed to it (Levy et al 2001 [24]), (Lindenmann et al 1982 [25]).

Its common sense that IFNs consists of three major classes, determined by the type of receptor trough which signal transduction is mediated.

Interferon type I: Type I IFNs bind to specific cell surface receptor named IFN-α receptor (IFNAR1and IFNAR2). Members of this class are IFN-α with 13 subtypes and a single member each of IFN-β, IFN-κ IFN-ω and IFN-ε.

Interferon type II: There is only one member in this group IFN-γ which binds to the IFN-γ receptor (IFNGR1 and IFNGR2)

Interferon type III: IFN λs were discovered 2002 (Kotenko et al [26]) ,first designated as IL-29, IL28A and IL-20B by HUGO and renamed as IFN- λ1, IFN- λ2 and IFN- λ3. Members of this group bind to a receptor consisting of IL10R2 and IFNLR1.

Another characteristic of IFNs beside antiviral properties is signalling trough JAK-STAT (Janus kinases signal transducers and activators of transcription) pathway.

2.4.1 Detection of viral infection and induction of interferon

Mammalian cells offer a variety of sensors to detect viral infection. These sensors can be divided into to two classes dependent on their localisation.

2.4.1.1 Extracytoplasmatic pathways and sensors

The Toll – like receptor (TLR) family is designed by nature, part of the innate immune system, to detect pathogen associated molecular patterns (PAMPs) (Medzhitov et al 1998 [27]). Once activated following binding to PAMPs TLRs induce multiple genes trough their Toll / interleukin 1 receptor domains (TIR) including interferon.

TLRs can be divided in two groups the first with TLR3 detects ds RNA, a spin off product during viral replication cycle, on a rather unspecific level.

The second group with TLR7, TLR8 and TLR9 detects viral product on a more specific level.
In context of influenza TLR 7 and TLR8, recognize single stranded RNA, and TLR3 play an important part of the innate immune system.

TLR 7/8/9/3 can be found in the endosomal compartments of a cell.

TLR7/8/9 signal transduction leading to IFN production is mediated by MyD88 (myeloid differentiation primary response protein) which interacts with the TIR domain of the TLRs, the interleukin-1 receptor-associated kinases IRAK1 and IRAK4 factor 6 (TRAF6). This results in downstream activation of IRF7, and of the IKKα/β/γ and the MAPK cascades, leading to NF-κB and AP-1 activation (Garcia Sastre et al 2006 [28])

“IRF7 is functionally similar to IRF3, but IRF3 leads only to induction of IFNβ, whereas IRF7 additionally induces IFNα genes” (Garcia Sastre et al 2006 [28]).

“TLR 7/8/9 and IRF7 appear to be constitutively expressed in only a subset of cells, the plasmocytoid dendritic cells (PDCs)”, which outperform by high IFN production in the early IFN response (Garcia Sastre et al 2006 [28]).

2.4.1.2 Cytoplasmatic pathways and sensors

There are two cytoplasmatic sensors involved for the detection of viral infection RIG-I (Yoneyama et al 2004[29]) and mda5.
Both belong to the RNA helicases and get activated by getting in contact with viral ds RNA [13]. The activation subsequently involves MAVS (also known as Cardif, IPS-1 or VISA) thus activating IKKε and TBK1 resulting finally in phoshorylation of IRF3. Activated IRF 3 together with CBP/p300 NF-κB and ATF2/- Jun induce in the nucleus transcription of interferon genes.

Secreted interferon can now establish an antiviral sate in by standing cells.

2.4.2 Interferon: Signal transduction an induction of gene expression

Mechanism of receptor dependent activation

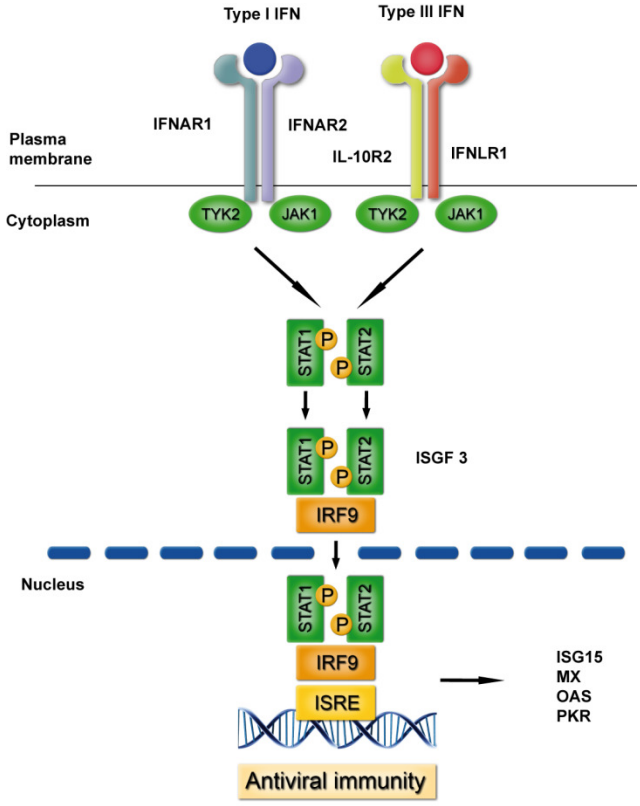


Fig 3: Schematic of Jak/STAT pathway modified. Original Sadler et al 2008 [30].

Activation of cytokine receptors and subsequent phosphorylation of STAT proteins is caused by ligand receptor interaction following receptor dimerisation.

IFN type I receptor composed of IFNAR1 and IFNAR2 and type III receptor composed of IL-10R2 and IFNLR1, are connected with JAK1 and TYK2.

The JAK (Janus-Kinase) family contains JAK1, JAK2, JAK3 and TYK2 (tyrosine kinase2).

Binding of IFN I and III to its cell surface receptor results in dimerisation and activation of JAK tryrosine kinases.

The STAT family contains seven members: STAT1, 2, 3, 4, 5A, 5B and STAT6.

STATs when phosphorylated by JAKs, dimerize and translocate to the nucleus where gene transcription is activated (Shuai et al 2003 [31]).
Interaction of STAT1 – STAT2 complex with IRF9, a DNA binding protein, generates the transcription factor ISGF3, capable of binding to DNA and facilitating transcription of ISREs (interferon stimulated responsive elements).

STATs share high homology in several regions including SH2 domain responsible for activation and dimerisation, a DNA binding domain, and a carboxy terminal transactivation domain (Shuai et al 2003 [31])..

Action of IFN I and IFN Type III are similar except prevalence of receptor complexes. While IFNAR1/2 receptor is widely expressed on almost every nucleated cell, IL28α subunit, which is crucial to express functional IL-28R complex “seems to be cell type restricted” (Mordstein et al 2008 [32]) (Sommereyns et al 2008[33]).
The spectrum of cells which respond to IFN-λ is poorly defined yet. Available data suggests that “epithelial cells are the main target of IFN-λ in the mouse”. (Mordstein et al 2008 [32]).

2.4.3 Antiviral pathways induced by Interferon

STAT signalling induces multiple genes including ISG15, OAS, Mx and PKR which represent the major antiviral pathways.

2.4.3.1 ISG15

Jak STAT signalling leads to transcription of 300 interferon stimulated genes (ISGs). Some ISGs employ pattern recognition receptors (PPRs) sensing viral molecules.
ISG15 was identified many years ago as a ubiquitin homologue and reaction was named as ISGylation (Blomstrom et al 1986 [34]; Loeb et al 1992[35]). ISG15 acts similar to ubiquitin. ISGylation is a 3 step sequential enzymatic reaction involving UBE1L, UBHC8 and HERC5.
Unlike ubiquitylation , ISGyaltion “does not promote degradation of the target protein”, its rather a modulation of enzyme properties (Sadler et al 2008 [30]).

It has been shown that ISG15 prevents degradation of IRF3 to enhance antiviral response (Lu et al 2006 [36]).

Influenza NS1A and NS1B bind to human ISG15 interfering with ISGylation, while NS1B does not interact with mouse ISG15 (Versteg et al 2010[37]).

2.4.3.2 OAS/ RnaseL

Induced by IFN, OAS were first identified as a group of proteins, that generate low molecular inhibitors of cell free protein synthesis (Kerr et al 1977 [38]). OAS gets activated by dsRNA an intermediate of viral replication.
The unique function of activated OAS is to synthesize 2´-5´linked phosphodiester bonds which subsequent activate RnaseL (Kerr et al 1978[39]).
RnaseL cleaves any RNA in the cytoplasm including vRNA resulting in inhibition of protein expression.

2.4.3.3 Mx

The mouse Mx family contains 2 members Mx1 found in the nucleus, and Mx2 localized in the cytoplasm. Mx1 and Mx2 are GTPases, guanine hydrolysing proteins involved in sequestering viral RNPs to subcellular compartments.
Most laboratory mouse strains lack a functional Mx and are therefore more susceptible to influenza than wild type strains (Haller et al 1986[40]).
A reasonable model for action of Mx proteins could involve trafficking or transcriptional activity of influenza RNPs (Weber et al 2000[41]).

2.4.3.4 PKR

PKR is an interferon inducible kinase sensing stress signals like LPS, ceramide, heat shock and viral infection.

The structure of this 515aa protein shows an N terminal dsRNA binding regulatory and a C terminal kinase domain (Williams et al 1999 [42]).

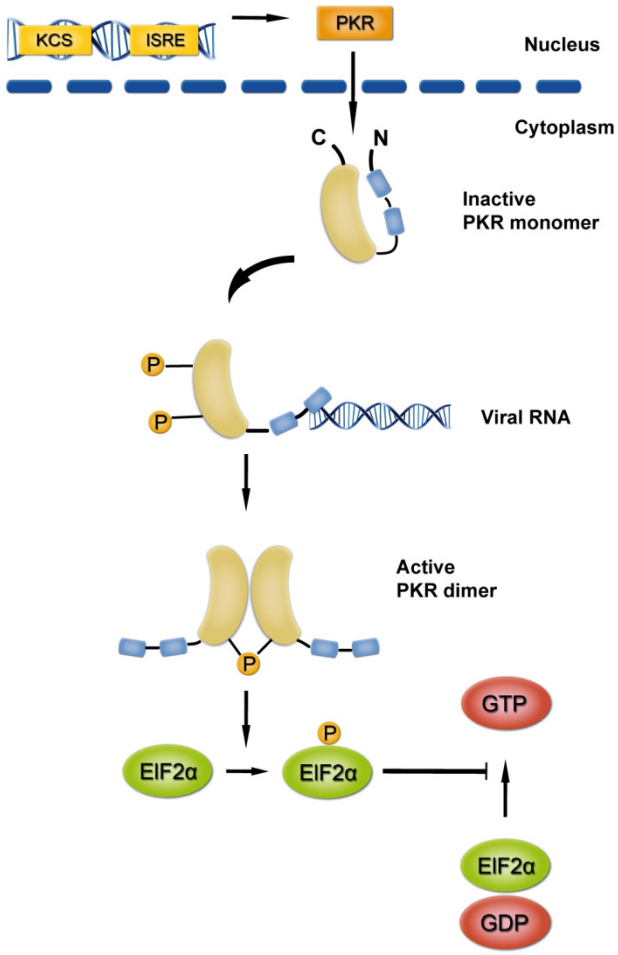


Fig 4: Action of PKR. Original Sadler et al 2008[27].

PKR is a serine/threonine protein kinase, constitutively expressed at low levels.

PKR is induced by IFN under control of KCS and ISRE and accumulates in cytoplasm and nucleus, resting to be activated by contact to viral RNA or other activating compounds. Activated PKR monomers are phosphorylated and associate to dimers. Then EIF2α is phosphorylated, stopping recycling of GDP, which blocks protein synthesis, inhibiting viral replication.

3 Aim of this work

Starting to work with influenza in the research group of Prof Dr Thomas Muster I was involved in a project investigating the relation of the non structural protein 1 (NS1) protein and double stranded RNA activated protein kinase (PKR) (Bergmann et al 2000 [43]) mentioned in the acknowledgements.

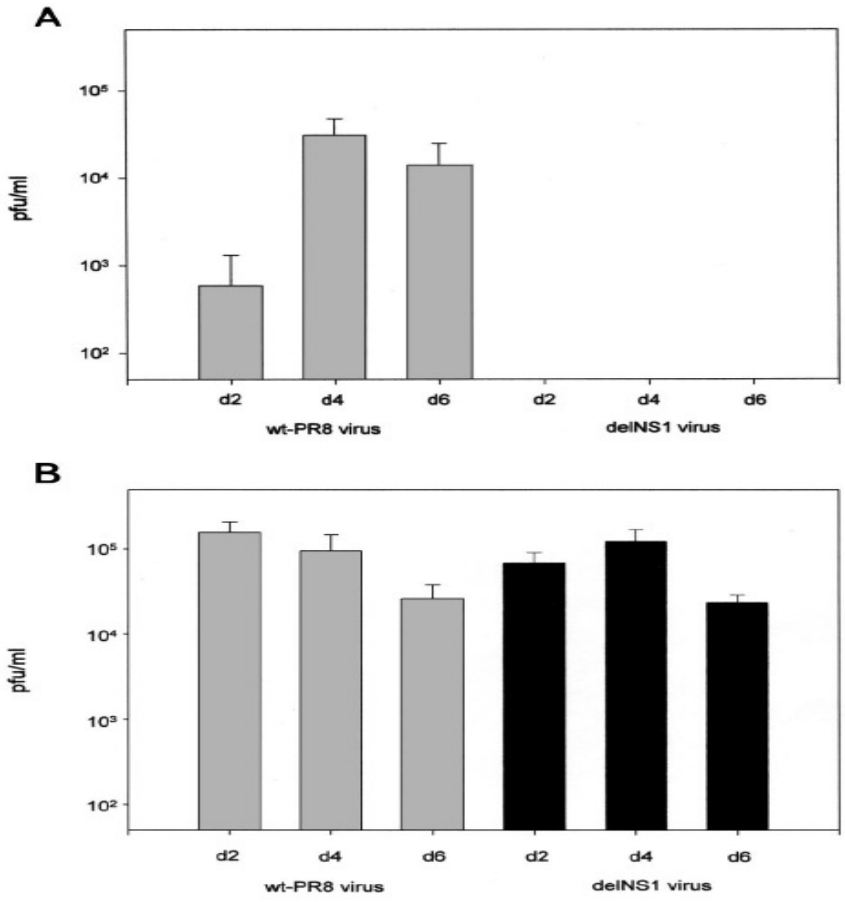


FIG. 2. Titers of influenza PR8 and delNS1 virus in $PKR^{+/+}$ and $PKR^{-/-}$ mice. Female mice at 7 to 9 weeks of age were used for infection with 10^5 PFU of wt PR8 or delNS1 virus. Virus was applied intranasally in a volume of 50 µl under ether anesthesia. To determine viral replication in the respiratory tracts, three mice of each group were sacrificed at day 2, 4, or 6 after inoculation. Lungs were removed and homogenized in 3 ml of phosphate-buffered saline. The quantity of virus in homogenates from each mouse was determined by titration on Vero cells. Virus titers are expressed as the number of PFU per milliliter of tissue extract. (A) Titers in $PKR^{+/+}$ mice. (B) Titers in $PKR^{-/-}$ mice. The standard error of the mean is indicated.

Fig 5: Source Bergmann et al [43]

We observed that PKR KO mice were permissive for PR8 delNS1 virus; so the question appeared: Why they are permissive?

The fact that PR8 delNS1 was capable to replicate in interferon deficient systems made obvious that IFN plays a major role.

The immunogenic feature of IFN to act like an adjuvant made it even more attractive in testing different routes of application and pharmacokinetics and dynamics (Proietti et al 2002[44]).

To investigate the biological activity of induced IFNs following viral infection we utilized a Luciferase Bioassay. The key component of this assay is a reporter cell line which was generated at the Lab of Adolph G at Böhringer Ingelheim and was kindly provided to us.
The basic protocol for Luciferase Assay was kindly provided by Kichler A Genethon France.

The Luciferase Bioassay was established and optimized for sensitivity, seed density of cells, incubation time of samples, signal noise ratio, spike recovery and robustness.

First data based on this Bioassay was published by Ferko B (Ferko et al 2004[45]) measuring IFN in secretions of mice mentioned in the acknowledgements.

A Bioassay for type I and III IFN possesses major advantages, of detecting biological active IFNs while ELISA measures protein content only.
Moreover by this assay the synergistic activity of all 13 known members of IFN-α, and three members of IFN λ family can be determined simultaneously in one assay, whereas ELISA fails to detect all members of IFN-α and IFN-λ in one approach.

Once having this large pool of mouse samples we decided to take a look beyond IFN, to get an idea what else is differently regulated comparing PR8 wt and PR8 delNS1 viruses in wild type and KO mice test systems.

For this reason, we decided to use bead based assays, which are basically an ELISA on a bead, revealing the great advantage of this test system, measuring multiple parameters simultaneously.

The current project was also a logistic challenge, since mouse experiments were conducted at Biotest (Czech Republic), the bioassay was performed at the Lab of Joachim Seipelt (Biocenter, Vienna), the Luminex analysis carried out at the Department of Dermatology (AKH, Vienna) and the viral tissue culture was maintained at the Lab of Prof Michael Bergmann (Department of Surgery AKH, Vienna).

This project was part of the EU Project FLUVACC and a report was generated and filed to the commission.

4 Interferon and beyond

For determination of cytokines – lymphokines in mouse serum a multiplex panel containing 16 components from R&D system was used: CCL2/JE, GM-CSF, IFN-γ, IL-1β/IL-1F2, IL-2 ,IL-4 ,IL-5 ,IL-6 ,IL-10 ,IL-12 p70,IL-13 ,IL-17 ,KC ,MIP-2 , TNF-α/TNFSF1A and VEGF.

Regulated parameters, which are KC, JE MCP1, IL-5 and IL-6, are described in brief in the following section.

4.1 KC (CXCl1)

KC (keratinocyte attractant) or CXCL1 is a member of CXC cytokine family. First identified as a PDGF inducible factor on mouse fibroblasts (Cochran et al 1983 [46]). KC expression following LPS stimulation was detectable on peritoneal and lung macrophages, vacsular smooth cells and endothelial cells.

Members of the CXC chemokines (chemotactic cytokines) are potent attractants and activators of immunological active cells. KC primarily acts on neutrophils.

Mouse KC is a 96aa immature protein, removal of the N-terminal 19aa sequence, leaves a 77aa mature protein (Oquendo et al 1989.[47]).

Signal transduction is mediated trough IL8R, which is composed of a α and β subunit (Lee et al 1995 [48]).

4.2 JE/MCP1 (CCl2)

Mouse JE/ MCP1 is a 148aa precursor and a 125aa mature protein, belonging to the family of C-C chemokines of immunregulatory cytokines (Rollins et al 1988[49]).

Major biological functions are chemoattractant and activator for lymphocytes and monocytes, and regulation of Th1/Th2 differentiation (Luther et al 2001 [50]).

JE/ MCP1 shares functional and structural features with the MCP- Eotaxin subfamily.

Following stimulation with virus, LPS and cytokines JE MCP1 is expressed by a variety of cells including macrophages, mast cells, endothelial cells and fibroblasts (Deshmane et al 2009 [51]).

Signaling of JE/ MCP1 involves CCR2, a G protein coupled receptor, strong expressed on monocytes.

JE/MCP1 has been reported to be involved in disease patterns like asthma, atherosclerosis and multiple sclerosis ([52]Daly et al 2003; Aukrust et al [53]).

4.3 IL-6

Mouse IL-6 is a 221aa immature protein ,containing a hydrophobic signal sequence, once cleaved a 187aa mature protein is derived.(Chiu et al 1988 [54]). Variability in glykosylation is the reason for molecular a mass range from 22 to 27kD.

Pleiotrophic is the most abundant word in IL-6 publications, although members of the IL-6 family share a helical cytokine structure. Further family members are , leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), IL-11 and cardiotrophin-1 (CT-1) (Hibi et al 1996 [55]).

Interleukin 6 is multifunctional protein involved in hematopoiesis, acute phase reactions and immune response.

IL-6 production is enhanced by various stimuli like antigenic stimulation, bacterial and viral infection.

Furthermore IL-6 has been reported to be involved in disease patterns like trauma, inflammation and autoimmune diseases (Van Snick et al 1990 [56])

IL-6 signaling depends on a functional receptor, which is composed of an IL-6 and a gp130 transmembrane glycoprotein. While IL-6R is restricted to a limited number of cell types including hepatocytes, leukocytes and lymphocytes, gp130 is widely expressed extending the range of cell types able to respond to IL-6.
Complexes of soluble IL-6R and IL-6 can eclict response from gp130 bearing cells lacking IL-6R, this process is called transsignaling (Jones et al 2005[57] [58]).

Immunoglobulin production of B cells is dependent on IL-6 among other chemokines (Kishimoto et al 1978 [59]).
Mucosal IgA response in IL-6 KO mice was strong reduced, effect reversed by IL-6 administration (Ramsey et al 1994[60]).

4.4 IL-5

Mouse IL-5 is a 133 immature protein containing a hydrophobic leader sequence, after cleavage a 113aa mature disulfide linked protein containing two identical polypeptides is derived (Takatsu et al1988 [61]).

IL-5 is produced by T lymphocytes and activated mast cells and eosinophils.

Mouse IL-5 promotes B cell differentiation outside, and proliferation of progenitor B cells inside the bone mark. Immunoglobulin IgM, IgG1, IgA, and IgE production and secretion is enhanced.

IL-5 contains two subunits α and β. Alpha is responsible for binding while beta is the signaling unit which also interacting with IL-3 and GMCSF (Takaki et al 1990 [62]).

5 Materials and Methods

5.1 Plaque Assay

5.1.1 Reagents:

Aim V Medium, liquid (Research Grade) GIBCO Cat. No. 31035-025
Glutamine GIBCO Cat. No. 25030164
Pen Strep GIBCO Cat. No.15140163
Trypsin EDTA GIBCO Cat. No. 25200056
DPBS without Ca, Mg GIBCO Cat. No. 14190144
$NaHCO_3$ Merck 106323
DMEM 10x, PROMOCELL, # C71197
DEAE-DEXTRAN, Sigma,# D9885
AGAR, Sigma,# A9915
Trypsin TPCK treated Sigma T1426

5.1.2 Cells: Vero cells (ATCC CCL-81)

● Vero cells were cultivated in AIM V medium supplemented with 5mM glutamine and AB mix in a humidified 5%CO2 incubator at 37℃.

● Confluent cell monolayer were washed twice with DPBS and trypsinized at 37℃ until starting of detachment was visible.
● Trypsin was removed and cells were detached by agitating the flask.
● Cells were aspirated by gentle pipetting and seeded into 6 Well plates.
● Cells are incubated in a humidified 5%CO2 incubator at 37℃ until monolayer gets confluent
● Virus stocks were thawed and a 1:10 serial dilution in AIM V was performed.
● Confluent cell monolayer was washed twice with DPBS appropriate virus dilution was applied and incubated 30min at RT.
● Meanwhile top agar was melted mixed with overlay medium and tempered at 40℃ in a water bath.

Composition of Overlay Mix

Mix1:
12ml Medium
+1,5ml 10x DMEM
+1ml $NaHCO_3$ saturated

12ml Mix 1
+0.25ml DAE-DEX 1%
+24µl Trypsin (5µg/ml)
+12ml AGAR 1,2%

● Remaining AIM V medium was removed and 2ml/well overlay mix was applied.
● Plates were incubated in a Laminar flow hood until overlay mix got solid and then incubated head first in a humidified 5%CO_2 incubator at 37℃.
● Plates were examined two and three days following virus application, plaques were counted and virus titer was calculated.

5.2 Interferon response in mice

5.2.1 Purpose

Determination of Interferon type I and III serum levels following virus administration.

5.2.1.1 Background: Generation of PKR knockout mice

Blastocytes, injected with embryonic stem cells carrying PKR mutation, were implanted into ICR foster mice.
“The resulting male chimeras gave germline transmission of the mutated PKR allele when mated with C57BL/6J females, and heterozygous mice (Pk,D/+) were intercrossed to generate homozygous offspring (Pk,%o). The genetic background of these animals was a mixture of the strains 129/Sv(ev) X C57BL/6J.” (Yang et al 1994[63]).

In this study the interferon response of C57BL6 and PKR mice to the delNS1 will be compared to its wild type respectively.

Furthermore a dose and administration dependent response is investigated.

The result will show the influence of PKR to the different virus types.

5.2.2 Justification

Interferon response is a hallmark during influenza infection and a reliable parameter for monitoring immunogenicity.
To asses influence of NS1 in interferon response in vivo, wild type virus Influenza A/PR/8/34 (H1N1) and NS1 deletion mutant Influenza A/PR/8/34 delNS1 (H1N1) will be tested.

In view of their genetical background, the influence of PKR on interferon response can be determined by comparing PKR knock out with wild type mice, C57BL6.

5.2.3 Experimental design

To investigate interferon response in C57BL6 mice each virus of virus was administered via 3 routes. intranasal (50µl), subcutaneous (100µl) and intraperitoneal (500µl).
To check dose dependency in C57BL6 vehicle and 3 different concentrations of each virus were used. $1*10^8$ PFU/ml, $1*10^7$ PFU/ml, $1*10^6$ PFU/ml and vehicle

The influence of PKR in interferon response was investigated by comparing interferon levels in C57BL6 mice with PKR knockout mice. Due to an expected lower interferon response in PKR KO mice only high doses of delNS1 and PR8 are tested.

$1*10^7$ PFU/ml
$1*10^6$ PFU/ml
vehicle

Virus was thawed, diluted and administrated.

A zero pool, which reflects the base IFN level, was generated by random selection of 20 C57BL6 and 5 PKR KO mice representative to the population and serum was prepared.

To address this questions blood samples were taken from retro orbital plexus at 8 and 16 hours post infection, minimum 50µl serum was prepared, aliquoted and frozen.

Serum samples were frozen and stored at -80°C till further processing.
Transport of virus and blood samples was organized by GNN.

Determination of serum interferon type 1 via Luciferase Bioassay was carried out at Vienna Biocenter.

5.2.4 Animals

5.2.4.1 Specifications and source of the animals

Table 2: Characterisation of experimental animals used in the study.

Species 1, strain	C57BL6
Supplier	Biotest
Number of animals sex	120 / female
Age	7 – 14 weeks
Species 2, strain	PKR Ko
Supplier	ÖZBT
Number of animals and sex	35/ female
Age	7 – 14 weeks

5.2.5 Immunisations, Viruses, Dose levels, Animal Allocation and Sampling

5.2.5.1 Experiment 1

Viruses: Influenza A/PR/8/34 (H1N1) **[Pr8]**
Influenza A/PR/8/34 delNS1 (H1N1) **[delNS1]**

Mice: C57BL6 total 120 animals (7-14 weeks old)

Immunisation route:

Intranasal	50µl/virus
Subcutaneous	100µl/virus
Intraperitoneal	500µl/virus

Animal allocation to test groups:

group	dose level pfu/ml	no. animals
1	10^8	5
2	10^7	5
3	10^6	5
5	vehicle	5
	total	**20**

Samples: Retroorbital blood samples (<200µl) were processed to obtain approximately 50µl serum. Blood collections were performed 8 and 16 hours post infection.
Baseline (zero) sample: Pool of serum samples collected before virus application.

5.2.5.2 Experiment 2:

Viruses: Influenza A/PR/8/34 (H1N1) **[Pr8]**
Influenza A/PR/8/34 delNS1 (H1N1) **[delNS1]**

Mice: C57BL6 total 35 animals (7-14 weeks old)
PKR KO total 35 animals (7-14 weeks old)

Immunisation route:
Intraperitoneal 500µl/virus

Animal allocation to test groups:

group	dose level pfu/ml	no. animals
1	10^8	5
2	10^7	5
3	vehicle	5
	total	**15**

Samples: Retroorbital blood samples (<200µl) were processed to obtain approximately 50µl serum. Blood collections were performed 8, and 16 hours post infection.
Baseline (zero) sample: Pool of serum samples collected before virus application.

5.2.6 Animal maintenance and environmental control

Table 3: Animal maintenance

Hygiene	Standard laboratory conditions
Room number	Separate rooms for each virus
Light	uncontrolled
Cages	Standard cages
Bedding material	JELUXYL SAWI
Feed	complete pellet diet
Water	drinking water ad libitum
Identification	Individual cage numbers
Acclimatisation	7 days

.

5.2.7 Vehicle

SPG, pH 8.0 5.7.05 EM and 19.10.05

Composition:
0.218 M Sucrose,
0.0038 M KH_2PO_4,
0.0072 M K_2HPO_4,
0.0049 K-Glutamate

Experimental documentation

Documentation in Lab – Notebooks.
Data sheets at Biotest – copies at GHB.
Biotest – Study report about animal work.

Data Analysis

Calculation of interferon will be performed by regression with Tablecurve.

5.3 Bioassay for mouse Type I+III Interferon

5.3.1 Introduction

Biological assays for IFNs traditionally measure their antiviral activity in cultured cells. These assays usually comprise several steps: establishment of cell monolayer in microtiter plates, exposure of the cells to interferon, infection with challenge virus, staining and washing of the cell layers, followed by spectrophotometric or visual detection.

Altogether, bioassays often last between two and four days, require frequent handling and employs challenge virus as class II etiological agent.

Immunoassays, on the other hand, while offering numerous advantages in term of simplicity, turnaround time, and precision, do not necessarily reflect the biological activity of an interferon preparation, particularly when natural mixtures of IFNs have to be assayed.

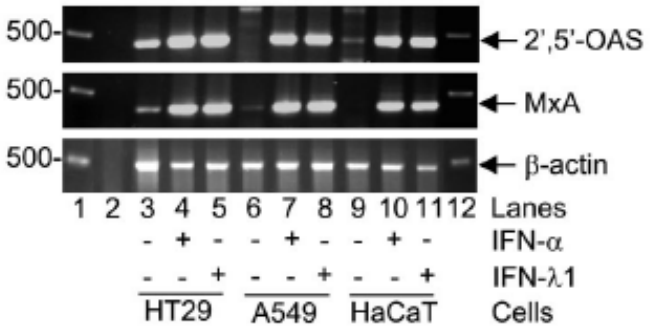


Fig 6: Source Kotenko et al 2002 [26]. RT PCR controlling response of IFN α and IFN λ1 regulated genes in epithelial cell lines HT29, A549 and HaCaT cells.

5.3.2 Summary:

A Bioassay for murine type I IFN was developed by Böhringer Ingelheim, based on a recombinant murine L-M cell clone carrying a Mx promotor/luciferase reporter gene construct (Weissmann et al 1990[64]).
The luminescence signal shows a dose dependent increase after treatment of the cells with IFN. On preformed cell monolayer, the assay can be completed within 8 hours and requires minimal handling.

5.3.3 Generation of the reporter cell line

Murine L-M cells (ATCC CCL-1.2) were transfected by electroporation with a reporter plasmid containing the firefly luciferase gene under the control of a 1,6 kb BamH1 fragment of the Mx gene, containing the promotor and IFN sensitive regulatory elements (Hug et al 1988 [65]).

A clone was selected based on optimal signal-noise ratio and grow properties.

Kindly provided by G. Adolf (Böhringer Ingelheim).

5.3.4 Reporter cell line cultivation

Medium 199

Medium199 (Gibco)	9,82g
NaHCO3	2,20g
Bactopeptone	5,00g
MEM 100xVitamin solution	10ml
1M HEPES	24ml
	ad 1000ml

Assay Medium

Medium 199	900ml
Penicillin, Streptomycin	5ml
L-Glutamine	10ml
HiFCS	100ml

Culture Medium

Assay Medium	1000ml
Ampcotericin B (Severa)	10ml
G-418 Geneticin (Gibco)	8ml

PBS/ 2mM EDTA

EDTA	0,745g
PBS	ad 1000ml

Assay Plates

96well plates Costar 3610 white with clear bottom.

5.3.5 Stock solutions

5.3.5.1 ATP

ATP concentration was 40mM in water. Aliquots were stored at–20℃.

5.3.5.2 Luciferin solution

D-Luciferin salt 167 was microM in water. Aliquots were stored at–20℃.
Note: always protect from light.

5.3.5.3 Lysis buffer

8mM $MgCl_2$
1mM DTT
1mM EDTA
1% Triton (v/v)
15% Glycerol
25mM TRIS – Phosphate Buffer pH 7,8.

$MgCl_2$ was added at the end. Aliquots were stored at–20℃.

5.3.5.4 Assay Buffer:

8mM $MgCl_2$
1mM DTT
1mM EDTA,
15% Glycerol
25mM TRIS – Phosphate Buffer pH 7,8.

$MgCl_2$ was added at the end. Aliquots were stored at–20℃.
ATP was just added before the assay is made the final concentration was 2mM.

5.3.6 Luciferase Assay

●Cells were cultured in Culture Medium.
●20 000 – 50 000 Cells/ well were seeded in 200µl Assay Medium to an Assay plate and incubated over night.
●Serial 1:2 dilutions were made by using a 96well round bottom dilution plate.
● Sera and interferon standard were diluted in Assay Medium and transferred to assay plate.
● Plate was incubated minimum for 6 hours.
● Lysis buffer, Assay Buffer, ATP and Luciferin solution were thawed and allowed to reach room temperature.
● ATP was added to Assay Buffer just before the assay was made the final concentration was 2mM.
● Luciferin was added to Assay Buffer containing ATP (1:1) just before the assay was made (Assay Mix).

● A 96 Well Luminometer Mediators PHL was used for measurement.

Instrument settings: Dispenser 1: 100µl (Lysis Buffer)
Dispenser 2: 100µl (Assay Mix)
Wait Time: 10 s
Lag Time: 2s
Int. Time: 10s

● Plate was washed with PBS/ 2mM EDTA 150µl / well.
● PBS/ 2mM EDTA was removed carefully.
● Assay plate was applied to Luminometer for measurement.

5.4 Luminex

5.4.1 Principle of Test

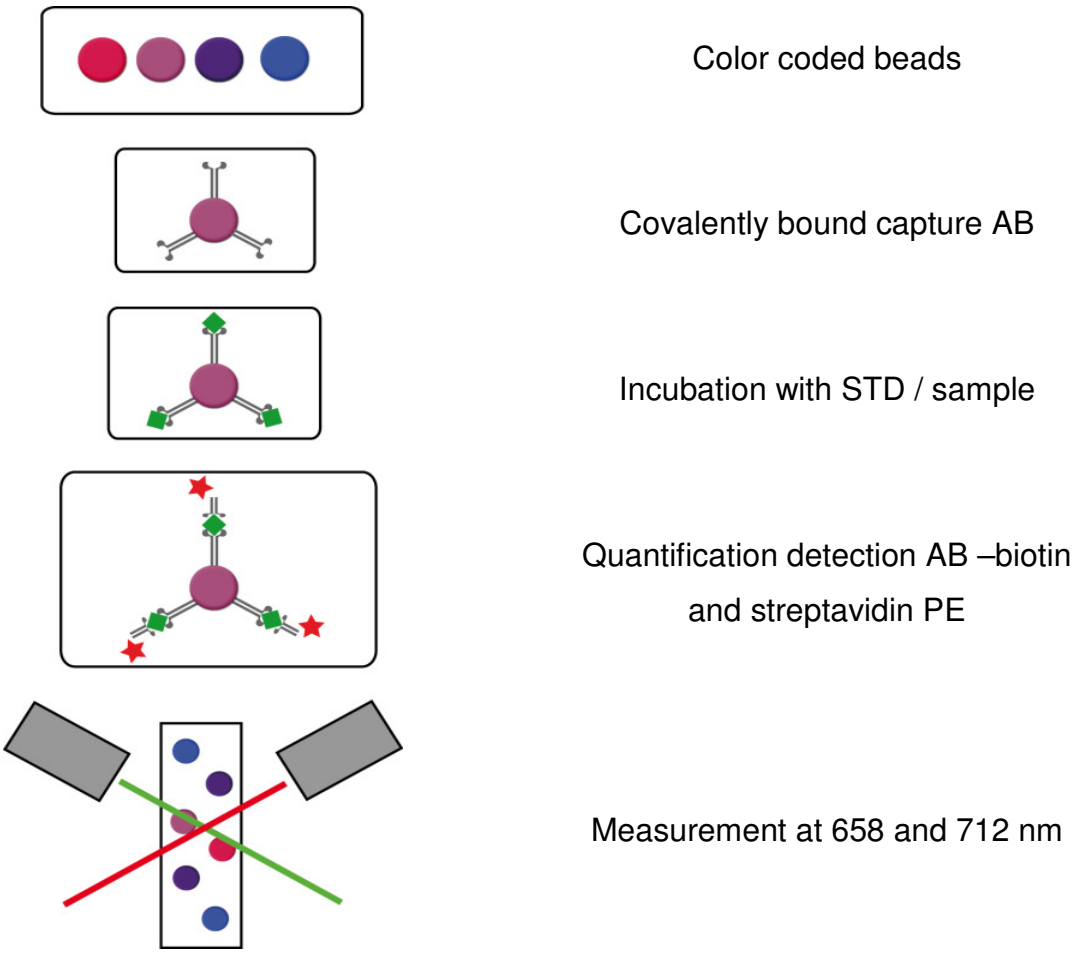


Fig 7: Luminex analysis workflow.

The Luminex technology platform offers a tool for simultaneous determination of any antigen desired.

This method is basicly an ELISA on a bead offering the advantage to combine tens of different analytes in one panel.

Internally labeled beads are covalently coupled to a capture antibody.

Following incubation with sample a second biotinylated detection antibody is added.

For quantification streptavidin-phycoerythrin (S-PE) is used.(Fulton et al., 1997 [66]; Oliver et al., 1998 [67]; Swartzman et al., 1999 [68]; Martins, 2002 [69]).

5.4.2 Equipment

Plate Shaker Titramax 1000 Heidoplh
Luminex 100 Analyzer
Vortexer IKA MS3 Basic
Centrifuge Mikro 200R Hettich
Vacuum Suction Unit HLC Biotech Model AC
Pipettors Biohit M300, M20, M100, M200, M1000

5.4.3 Reagents

Standard Cocktail (Part 895814)
Microparticle Diluent (Part 895815)
Calibrator Diluent RD6-40 (Part 895817)
Wash Buffer Concentrate (Part 895003)
Biotin Antibody Diluent (Part 895816)
Streptavidin-PE (Part 892525)
Microplate (Part 640448)

Succrose Sigma Cat.No. S9378
KH_2PO_4 Sigma Cat.No. P0662
K_2HPO_4Sigma Cat.No. 450200
L-Glutamic acid Sigma Cat.No. G1149
NaCl Sigma Cat.No. S5886

5.4.3.1 Preparation of SPGN buffer

6% w/v Succrose
3.8 mM KH_2PO_4
7.2 mM K_2HPO_4
4.9 mM L-Glutamic acid
75 mM NaCl
Adjust pH to 7.5 ± 0.1

5.4.4 Procedure

General Remarks

- Buffers and diluents were allowed to reach room temperature.

5.4.4.1 Sample Preparation and Preparation of Premixed Standard Dilutions

- All samples were kept on ice during all processing steps.
- Serum samples required a 4-fold dilution in Calibrator Diluent RD5K.
- Glass vial containing the lyophilized standard was tapped on a solid surface to ensure the pellet is on the bottom.
- Cytokine Standard Cocktail 1 was reconstituted with 500µl Calibrator Diluent RD5K.
- Vial was gentle vortexed for 1-3sec and incubated on ice for 15min.
- 300µl of reconstituted Standard was pipetted into the working Standard tube. 200µl of the Calibrator Diluent was pipetted into the remaining tubes.
- A 1:3 –fold dilution series was performed Calibrator Diluent served as Blank.

Std. Nr	stock (µL)	diluent (µL)
1	300	0
2	100 of Std. Nr1	200
3	100 of Std. Nr2	200
4	100 of Std. Nr3	200
5	100 of Std. Nr4	200
6	100 of Std. Nr5	200
7	100 of Std. Nr6	200
8	Blank	200

Table 5 : Preparation of Standard.

5.4.4.2 Microparticle mixture preparartion

- Each microparticle concentrate vial was centrifuged for 30 seconds at 1000xg.
- The vials were gently vortexed to resuspend the Microparticles.
- Microparticle concentrates were diluted in the mixing bottle provided.

Wells	Microparticle Concentrates (µL)	Microparticle Diluent (µl)	Total (µL)
96	50	5000	5800

Table 6: Mixing beads.

- Microparticles were protected from light during handling and used within 30 min following dilution.

5.4.4.3 Detection Antibody Preparation

- A 30sec quick spin centrifugation of the detection antibody vial was performed prior pipetting to collect the entire volume at the bottom of the vial.
- Dilute the detection antibody to 1x concentration using detection antibody as described in table 7. Single Detection Antibodies were combined and adjusted to 5800 µL by the appropriate amount of Biotin Antibody Diluent.

Wells	Biotin Antibody Concentrates (µL)	Microparticle Diluent (µl)	Total (µL)
96	50	5000	5800

Table 7: Preparation of Detection Antibody.

5.4.4.4 Preparation of Streptavidin-PE

- A 30sec quick spin centrifugation of the streptavidin –PE vial was performed prior pipetting to collect the entire volume at the bottom of the vial.
- Streptavidin-PE solution was prepared10 min before use. Add 55 µl ST-PE to 5500µl Wash Buffer.

Wells	Streptavidin-PE 100x (µL)	Wash Buffer(µL)	Total (µL)
96	55	5 500	5555

Table 8: Preparation of Streptavidin-PE solution.

5.4.5 Instrument Settings

Probe height setting was adjusted to avoid puncturing the membrane.

- A bead region to each analyte being measured was assigned.
- 50 events per bead set was appointed.
- Minimum events were set to 0.
- Flow rate was adjusted to fast 60µl/min.
- Doublet Discriminator gates were set to 7500 and 15500.
- Median RFU was measured.

5.4.6 Assay Procedure

- Filter plate was Pre-wet by filling with 100µl of Wash Buffer/Well. Solution was removed by vacuum filtration. Filter plate was tapped on paper towel.
- Beads were resuspended by vortexing and 50µl /well were added.
- 50µl Standard or Samples/well was added. Plate was covered with sealer and incubated 3h at RT 500rpm.
- Plate was washed 3 times with 100µl/Well Wash Buffer. Solution was removed by vacuum filtration. Filter plate was tapped on paper towel.
- 50µl of Detection antibody / well was added. Plate was covered with sealer and incubated 1h at RT 500rpm.
- Plate was washed 3 times with 100µl/Well Wash Buffer. Solution was removed by vacuum filtration. Filter plate was tapped on paper towel.
- 50µl streptavidin –PE/ well was added. Plate was covered with sealer and incubated 30min at RT 500rpm.
- Plate was washed 3 times with 100µl/Well Wash Buffer. Solution was removed by vacuum filtration. Filter plate was tapped on paper towel.
- Particles were resuspended with 100µl Wash Buffer/well. Plate was covered with sealer and incubated 2h at RT 500rpm
- Beads were ready for analysis.

5.4.7 Reading and Calculation of Results

- For calculation of results Luminex Data was imported with Master Plex 2.0 software.
- Standard, Blank and unknown Samples were marked in the plate layout sample.
- Concentrations of standard wells were assigned in order to let the software generate a calibration curve. Then samples were calculated utilizing the 5-parameter logistic (Luminex integrated software).

5.4.8 References

Fluorokine MAP, Mouse Multianalyte Profiling Base Kit, R&D Systems.

6 Results

6.1 Virus titres

Prior immunizations virus titres of the produced virus lots were determined by the plaque assay (three independent plaque assays). The mean virus titres are outlined in Table below.

Table 4: Result virus titration on vero cells.

name	prod #	production date	final formulation buffer	titre [pfu/ml]	virus used for infection
delNS1	30	14.10.2005	SPG 5.7.05	1,3 E+8	delNS WVS2 vial# 47/70
PR8	31	14.10.2005	SPG 5.7.05	3,7 E+8	PR8 MVSS vial# 66/81
plaque assay control:			op.: RF	control -3: 63	

6.2 Type I and III Interferon induction in mice

Experiment 1: Dose dependent systemic IFN Induction in C57BL6 mice by the IP route

Mice were infected by three standard routes: intraperitoneal (ip), subcutaneous (sc), and intranasal (in) with delNS1 and wild type PR8 virus. Mice were bled prior immunization, and 8h and 16h post infection. Serum was prepared and frozen.

Equal volumes of serum samples collected from each mouse per group (N=5) were pooled and serum pools were analyzed by the IFN bioassay. As shown in Fig 8, the **intraperitoneal** application of delNS1 resulted in a substantially higher systemic IFN response in C57BL6 mice in a dose dependent manner when compared to wild type virus. Peak IFN levels induced by the delNS1 were found 8h post infection whereas the wild type virus induced significantly lower but comparable IFN serum levels 8h as well as 16 hours post infection (Fig. 8A and 8B).
IFN was undetectable at any time point measured in serum of mice immunized by the **subcutaneous** route with PR8 virus (Fig. 9A and 9B). In contrast delNS1 virus induced high IFN levels in serum of mice 8h post infection (Fig. 9A). These levels dropped by about 50% 16h post infection (Fig 9B).
Upon **intranasal** administration of viruses, IFN serum levels peaked 16h post infection in mice receiving delNS1 (Fig. 10) whereas at this time point no IFN could be determined in serum of mice infected by PR8.

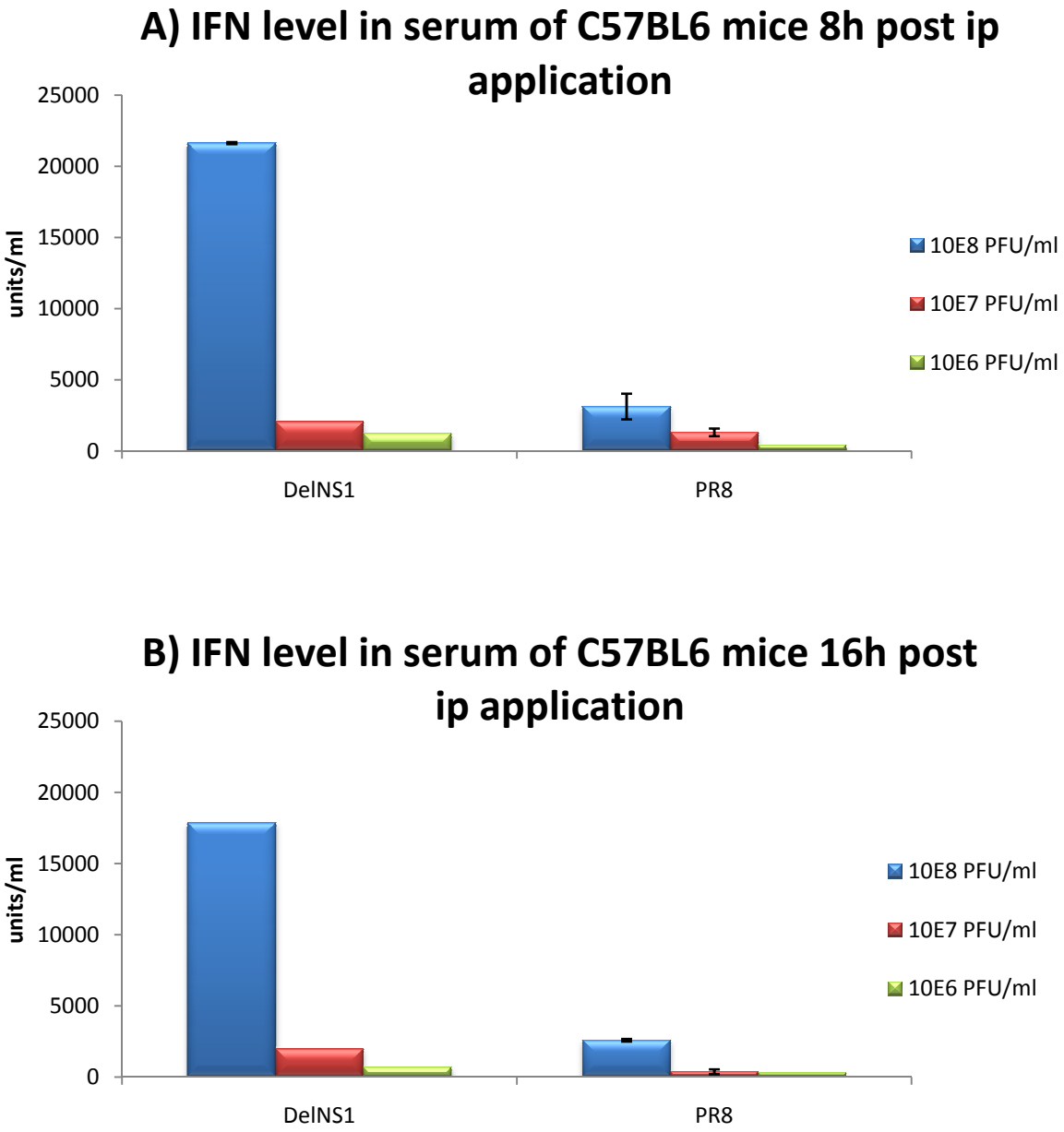


Fig 8: IFN serum levels in C57BL6 mice 8h post intraperitoneal virus application.

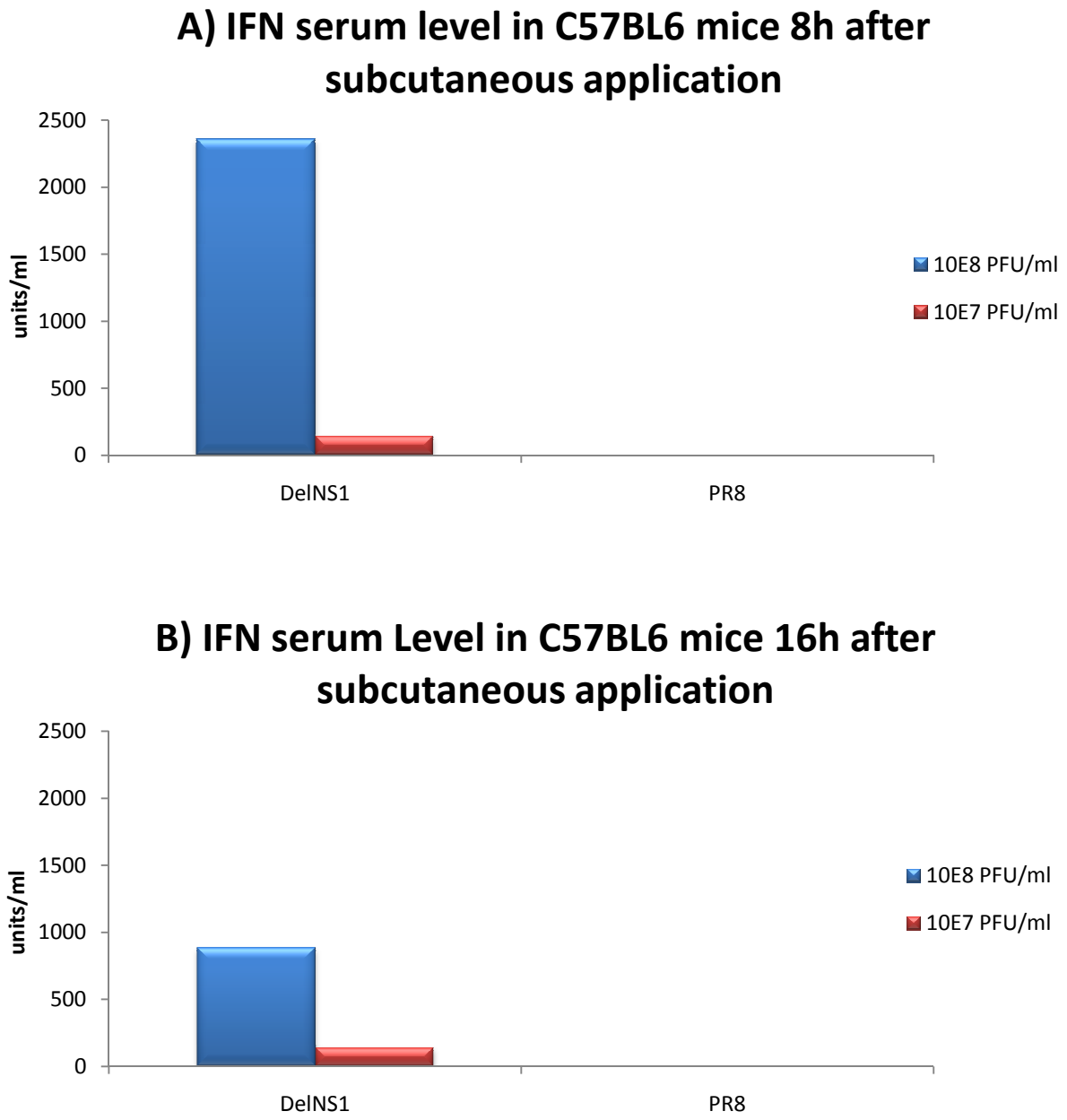


Fig 9: IFN serum levels in C57BL6 mice 8hand 16h post subcutaneous virus application.

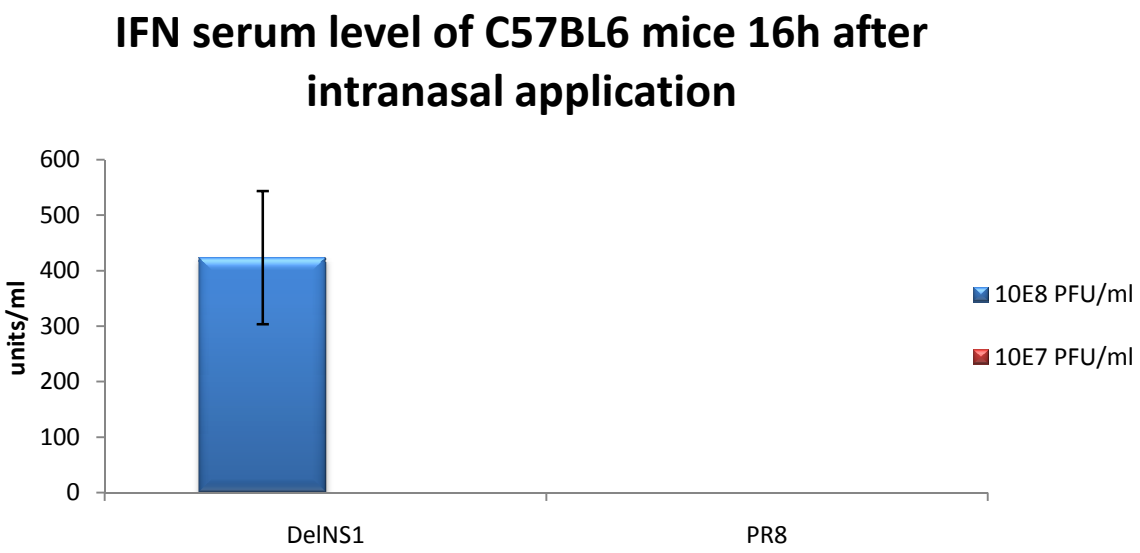


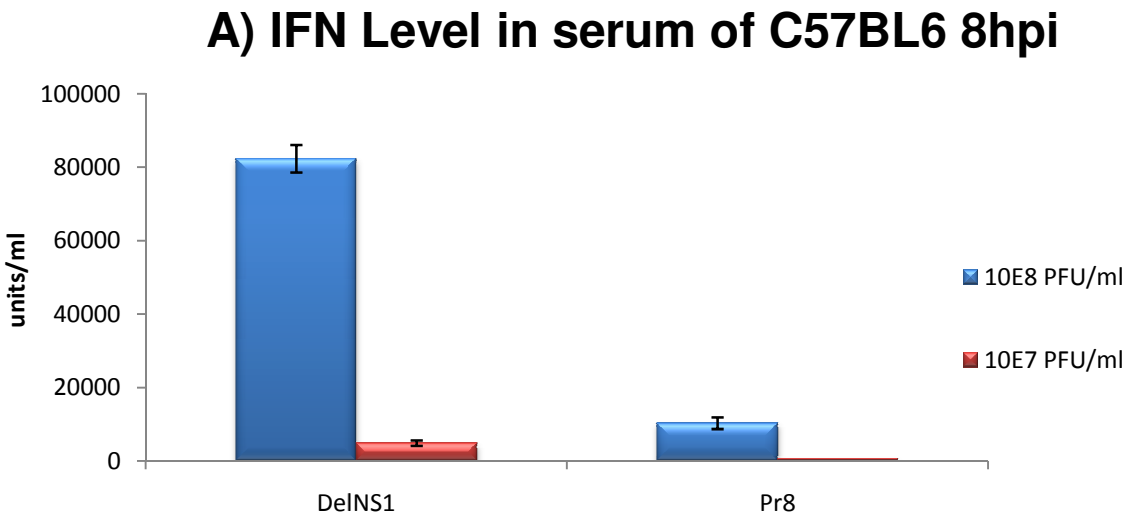
Fig 10: IFN serum levels in C57Bl6 mice 8h and 16h post intranasal virus application.

Comparison of C57BL6 wild-type vs PKR KO mice

In the next experiment mice received intraperitoneally 500µl of the virus suspensions. Mice were bled prior immunization and then again 8 and 16h post infection. Serum was prepared and frozen before assaying. Equal volumes of serum samples collected from individual mice (N=5 per group) were pooled and the serum pools analyzed by the IFN bioassay.

As expected delNS1 induced a high IFN response in C57BL6 mice 8h post infection while wild type virus induced significantly lower IFN levels in C57BL6 mice (Fig.11A). In contrast following intraperitoneal administration of delNS1 serum IFN levels dropped dramatically in PKR KO mice when compared to C57BL6 mice (Fig.11A and 11B).

Interestingly, serum IFN levels of mice immunized intraperitoneally with delNS1 were markedly lower at 16h post infection, than those immunized with the wild type virus which induced comparable concentration of IFN in serum irrespective of mouse strain and time point (Fig.12A and 12B).



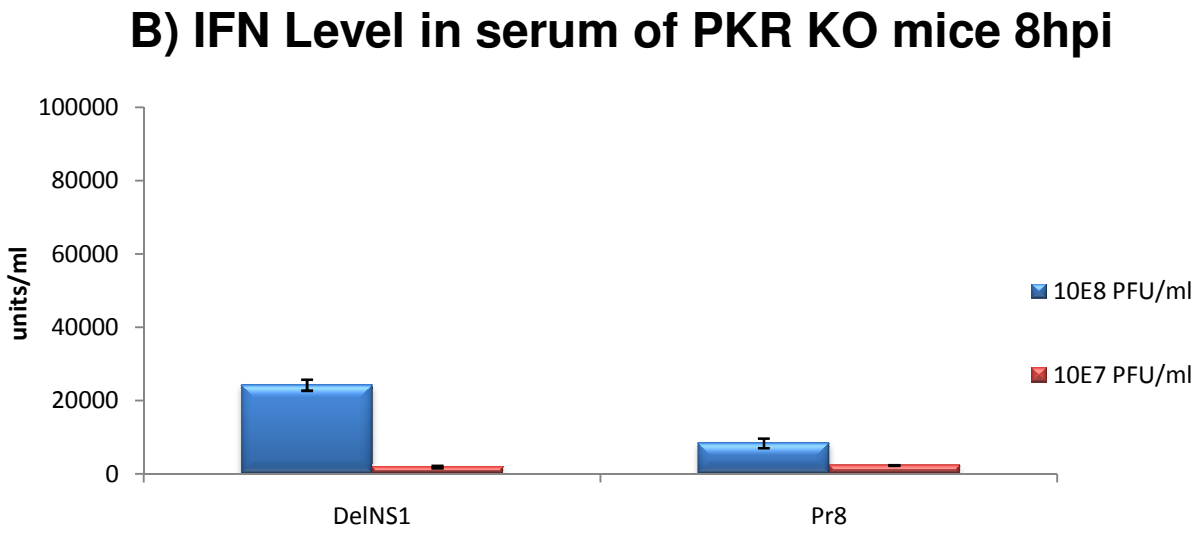


Fig 11: IFN serum levels 8h post intraperitoneal application of virus.

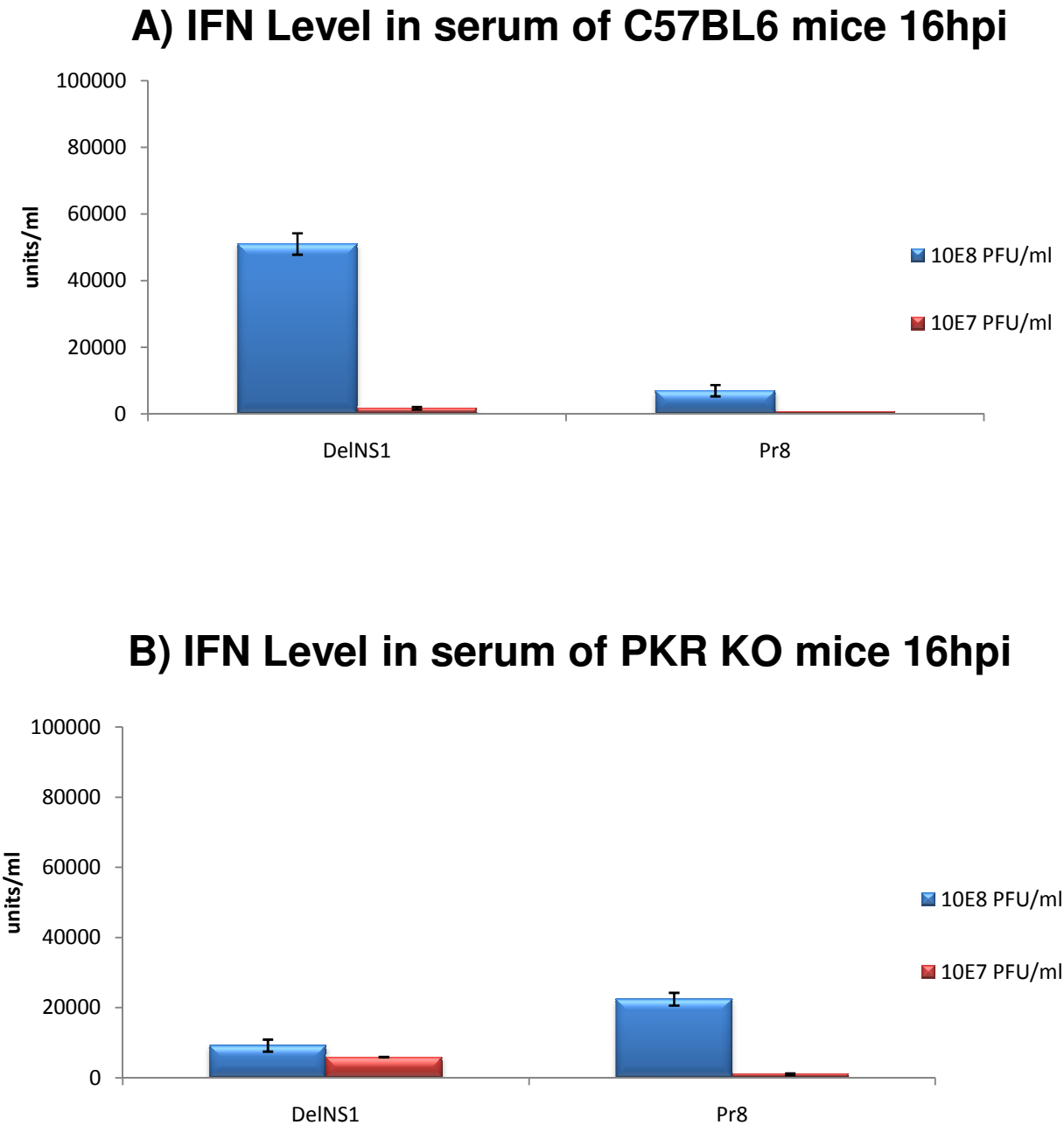


Fig 12: IFN serum levels 16h post intraperitoneal application of virus.

6.3 Determination of chemokines and proinflamatory cytokines in mouse serum samples by the Luminex technology

Group-specific serum pools were analyzed utilizing the Luminex multiplex assay. Detectable serum levels and concentration differences could be determined only for kit components: KC, JE/MCP-1, IL-5 and IL-6.

6.3.1 KC

Serum of 5 mice were pooled and analyzed by Luminex multiplex assay.
Intraperitoneal application of virus in C57BL6 resulted in equal serum levels of KC for high dose (10E8 PFU/ml) virus 8h and 16 h post infection. Differences in KC serum levels could be detected in low dose group where delNS1 induces higher levels of KC 8 and 16h post infection than PR8 (Fig.13A and 13B).

PKR KO mice showed general decreased serum levels of KC compared to C57BL6 mice, noteworthy significant higher levels of delNS1 8h post infection.

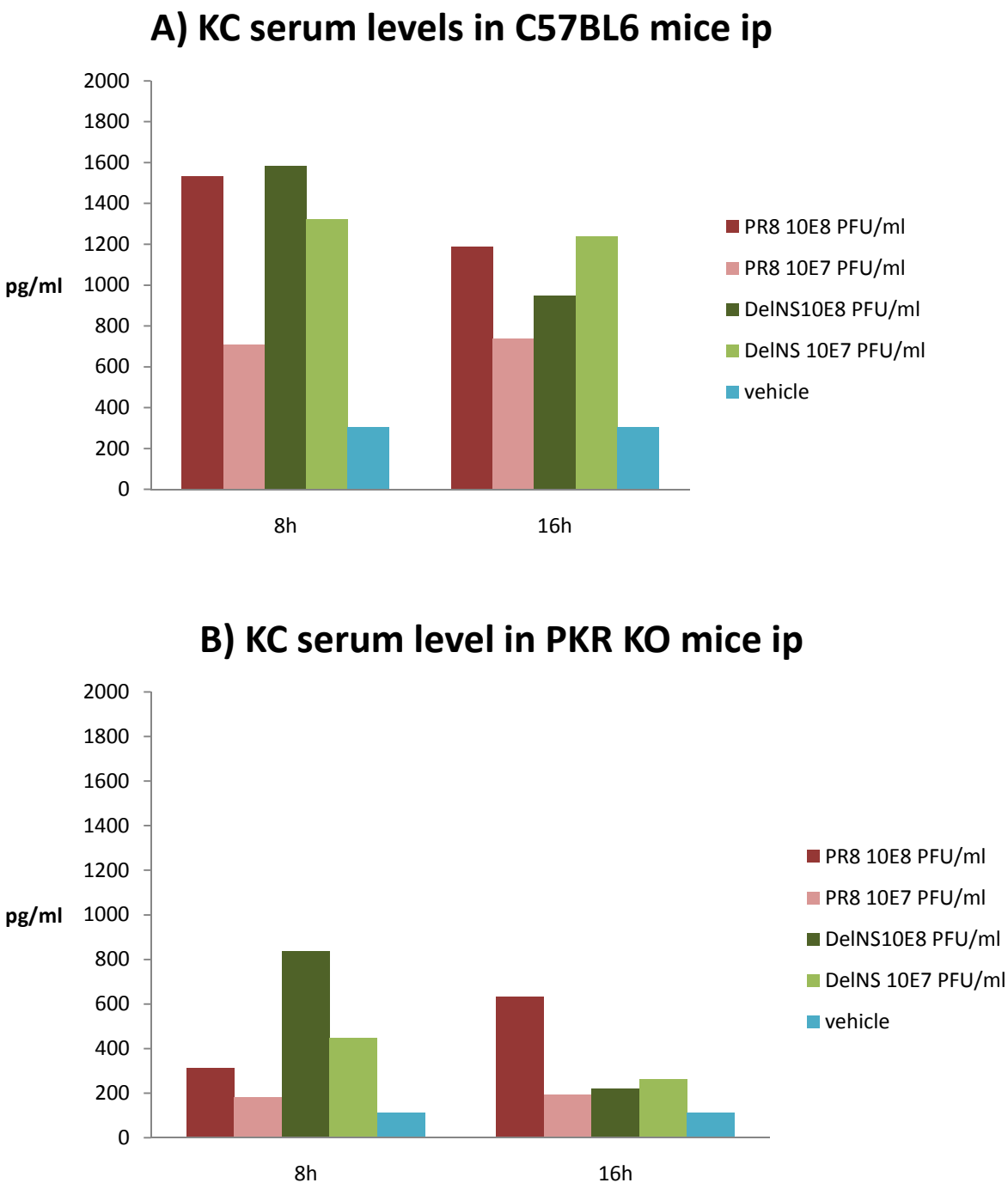


Fig 13: KC serum levels in C57BL6 and PKR KO mice.

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6.3.2 JE/MCP1

In contrast to wild type mice, in PKR KO mice immunized with delNS1, serum levels of JE/MCP1 were at the lower level of detection 8h post infection but the response recovered at 16h (Fig.14B). As PKR KO mice are permissive to wild type and delNS1 virus this 16 h JE/MCP1 serum level is rather induced by virus replication. PKR KO mice showed impaired JE/MCP1 response compared to C57BL6 wild type mice 8h post infection, while 16h values were comparable (Fig 14A and 14B).

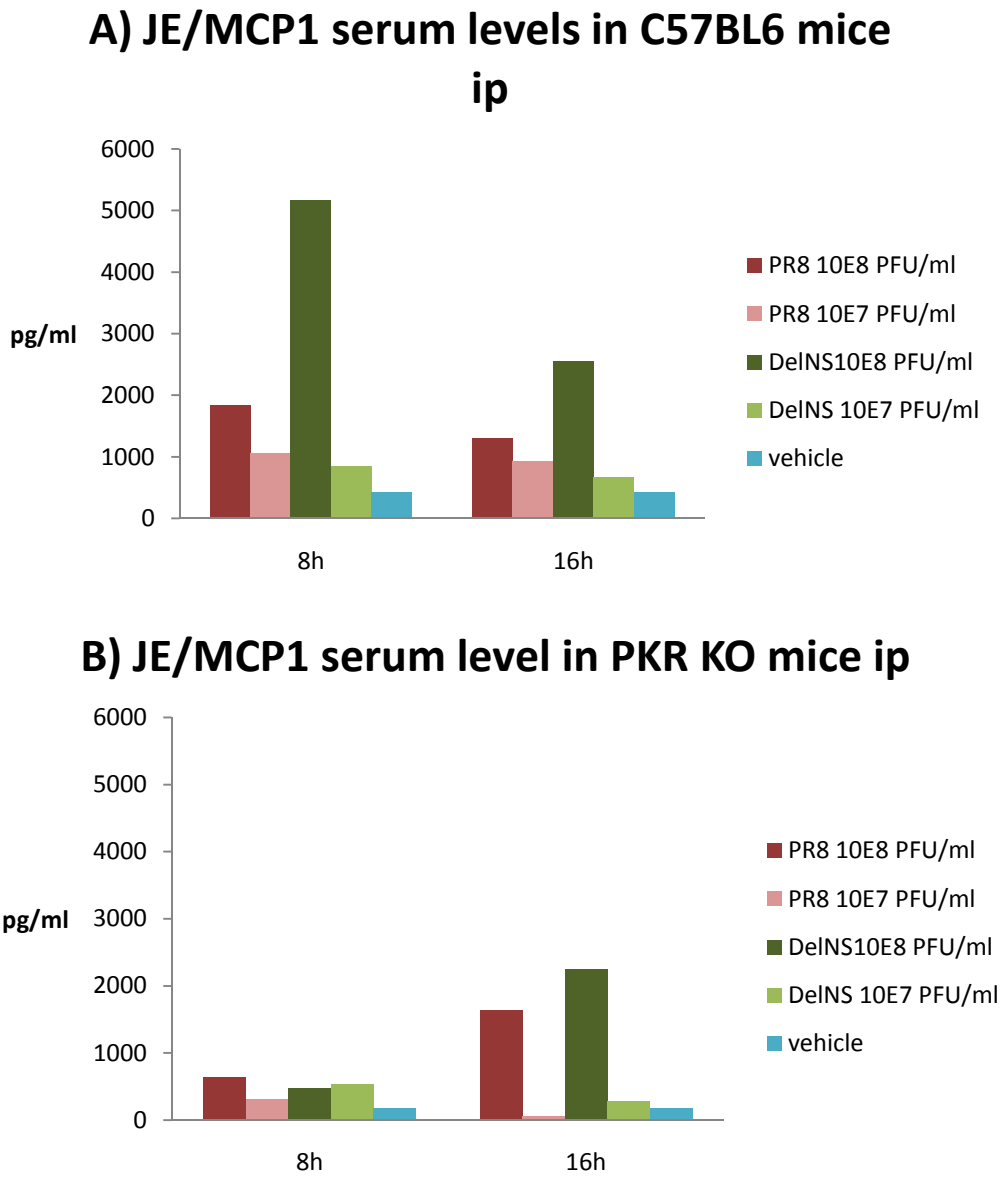

Fig14: JE/MCP-1 serum levels in C57BL6 and PKR KO mice.

6.3.3 IL-5

Intraperitoneal application of delNS1 virus resulted in a higher systemic IL-5 response in C57BL6 mice 8h post infection when compared with wild type PR8 virus. In contrast, wild type virus induces highest IL-5 serum levels in C57BL6 mice 16h post infection.

In PKR KO mice serum levels of IL-5 were low but no significant differences could be detected between delNS1 and wild type PR8 virus 8h as well as 16h post infection, indicating that IL-5 response is impaired in PKR KO mice.

As C57BL6 mice are permissive to wild type virus, increased IL-5 values 16h post infection are most probably due to progressing replication of wild the type virus.

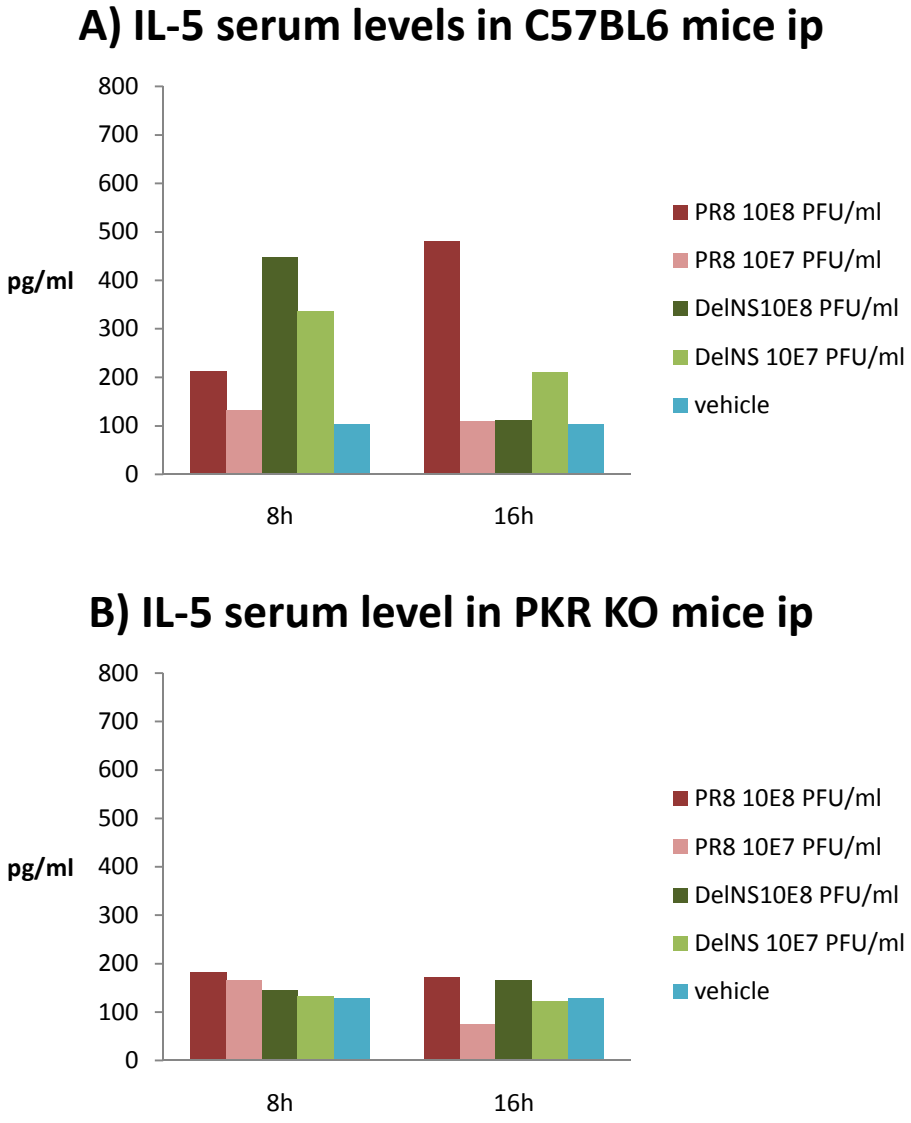

Fig 15: IL-5 serum levels in C57BL6 and PKR KO mice.

6.3.4 IL-6

Next, serum pools were analyzed for the presence of IL-6 by the Luminex multiplex assay. Intraperitoneal application of delNS1 virus resulted in a higher systemic IL-6 response in C57BL6 mice 8h post infection when compared with the wild type virus (Fig.16A).

Wild type PR8 virus induced higher IL-6 serum levels in PKR KO mice than delNS1 16h post infection, most probably due the progressive virus replication (Fig 16A and 16B). No significant differences in IL-6 serum levels could be detected when delNS1 was applied to wild type or PKR KO mice. This fact indicates that PKR is not directly involved in the IL-6 induction pathway.

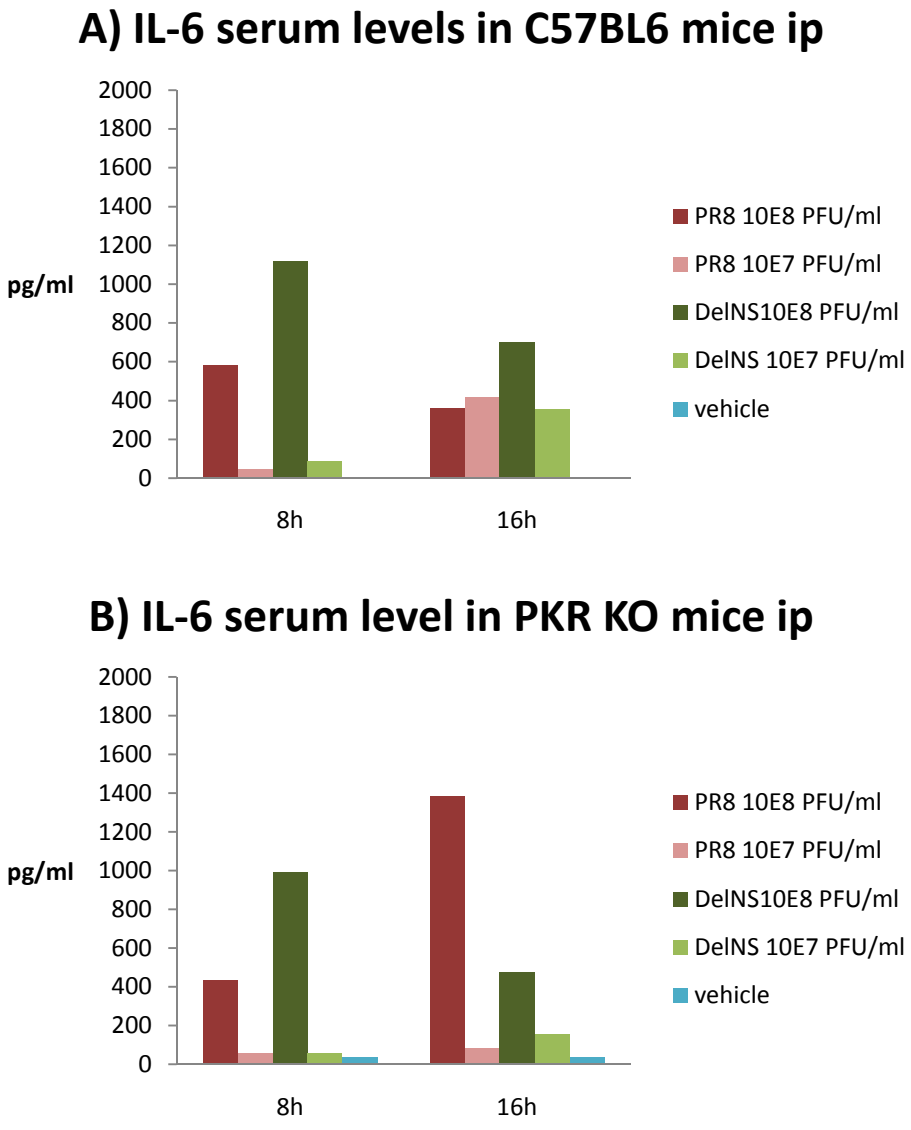

Fig 16: IL-6 serum levels in C57BL6 and PKR KO mice.

7 Discussion

NS1 deletion mutant delNS1 induces higher systemic levels of antiviral IFN than its wild type counterpart PR8 in a dose dependent manner. The highest IFN response was elicited following intraperitoneal route of delNS1 virus administration followed by the intranasal and finally the subcutaneous route. When PR8 virus was applied s.c. and i.n. only extremely low or undetectable levels of IFN could be determined in the systemic compartment. In contrast delNS1 virus which is unable to replicate in IFN-competent cells is capable of inducing high IFN serum levels irrespective of the administration route due to missing NS1 protein translation and thus abrogation of the NS1 protein mediated function to suppress the IFN response machinery or other innate signalling pathways within the infected cells.

“Double stranded RNA is a potent inducer of IFN” (Jacobs et al 1996 [70]).

NS1 protein sequesters ds RNA, preventing activation of PKR (Bergmann et al 2000 [43]).

NS1 protein has also been shown to inhibit synthesis of IFN, interfering with activation of IRF3 and NF-κB (Talon et al 2000 [16]; Wang et al 2000 [17]).

We have shown that IFN levels in PKR KO mice infected with delNS1 are dramatically reduced compared to C57BL6 mice due to the absence of PKR, which plays a key role in the IFN triggering pathway and eliciting of an antiviral state. We also observed increased IFN serum levels in PKR KO mice infected with the wild type PR8 virus 16h post infection A reasonable explanation for this finding is most probably the progressive replication of the wild type virus in PKR KO mice and an increased sensitivity of delNS1 to IFN when compared to the wild type virus (Ferko et al 2004 [45]).

The observed residual IFN response to delNS1l infection in PKR KO mice is most likely mediated trough TLRs.

Besides the high level of type I/III IFNs we could also determine chemokines and immunomodulating lymphokines in serum of mice infected with delNS1.

Intraperitoneal application of delNS1 or PR8 virus to C57BL6 mice resulted in comparable serum levels of KC at high virus dose (10E8 PFU/ml) 8h and 16 h post infection. Differences in KC serum levels were detected in low dose group (10E7 PFU/ml) of mice immunized with delNS1 which induced higher levels of KC 8h and 16h post infection than did wild type PR8 virus.
In general, PKR KO mice showed decreased serum levels of KC compared to C57BL6 mice, But interestingly, significantly higher levels of delNS1 induced KC could be determined 8h post infection in PKR KO mice. Our data indicate that PKR KO mice exhibit a reduced KC response to influenza virus infection.
Published data described pulmonary levels of KC following influenza infection (Hamelin et al 2010 [71]. Respiratory dendritic cells subsets have been reported to produce elevated KC levels following viral infection in vitro (Hao et al 2008 [72]). Elevated levels of KC, among other cytokines, in mice infected with PR8 bearing NS from H5N1/97 have been associated with increased severity of disease (Lipatov et al 2005 [73]).
Next chemokine which is most probably associated with PKR was JE/MCP-1. Intraperitoneal application of delNS1 virus resulted in higher systemic JE/MCP1 response in C57BL6 mice 8h and 16h post infection when compared to wild type PR8 virus. In contrast, PKR KO mice showed impaired response of JE/MCP1 8h post infection, only highest doses of delNS1 and PR8 induced JE/MCP1 serum levels 16 h post infection. Our results indicate that MCP1 response seems to be impaired or delayed in PKR KO mice. This chemokine possesses important immunemodulatory functions as an activator for lymphocytes and monocytes, and regulation of Th1/Th2 differentiation (Luther et al 2001 [50]). Mice lacking MCP1 showed higher viral loads, decreased body weight, decreased leukocyte count in infected lungs and pulmonary IgA levels were decreased (Dessing et al 2007 [74]).
DelNS1 virus induced also higher levels of IL-5 and IL-6 early after infection (8 h post infection) than did the wild type virus in C57/BL6 mice. On the other hand, delNS1 and wild type virus induced low but comparable serum IL-5 levels in PKR KO mice 8h

and 16h post infection, indicating that IL-5 response is impaired in PKR KO mice. Serum levels of IL-5 and IL-6 increased with progressive wild type virus replication. IL-5 response in C57BL6 mice has been described following low dose intranasal influenza A/PR8/34 virus application, IL-5 peaked 7days post infection (Buchweitz et al 2007[75]).
Both ILs are involved in induction of B-cell response.
For example, immunoglobulin production of B cells is dependent on IL-5 among other chemokines (Kishimoto et al 1978 [59]). Mucosal IgA response in IL-6 KO mice was strong reduced and the effect reversed by IL-6 administration (Ramsey et al 1994[60]). IL-6 production was found to be increased in murine epithelial tracheal cells infected with influenza WSN with impaired RNA binding domain compared to wild type virus (Newby et al 2007 [76]).

In conclusion this study shows the immunestimulatory potential of an intranasal influenza virus vaccine candidate delNS1 which is capable of, eclicting multiple innate immune responses, underscoring its immunstimulating properties.
These data confirm that replication-deficient DelNS1 influenza vaccine technology is a precious tool for generating an innovative immunogenic and safe influenza virus vaccines due so called “self-adjuvant effect” based on an early trigger of innate IFN and other chemokines as well as immunemodulatory cytokine response.
Our results underline the role of NS1 protein as a virulence factor capable of “hiding” the virus replication from the innate immune system due to sequestering of ds RNA resulting in induction of low IFN levels in hosts. Furthermore, the key importance of PKR in IFN signaling pathway could be confirmed in vivo. PKR obviously plays a role also in induction of certain chemokines and cytokines. The delNS1 genetic engineering platform possesses a great potential as a technology tool for a new generation of influenza vaccines combining the highly desirable vaccine properties of high immunogenicity, replication-deficiency and therefore safety.

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