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Recognition of Viruses by the Innate Immune System

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Statement of Collaboration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been clearly indicated.

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Abstract

When a cell gets infected with a virus, the innate immune system swings into action within minutes. The rapid production of pro-inflammatory cytokines and antivirally active type-I Interferons (IFN-α/β) is the most significant mechanism to limit virus spread.

Two conceptually different pathogen recognition mechanisms are known that lead to antiviral responses through production of IFN-α/β: Specialised immune cells possess Toll-like receptors (TLRs), which sense incoming viruses in endosomes. Most other cells rely on the cytoplasmic RNA-helicases RIG-I and MDA5 that sense the presence of viruses within the cell. However, although proteins and signalling networks involved in innate recognition of viruses are well known, the exact molecular details of their interactions with the virus are only marginally understood.

During my PHD thesis I dedicated myself to aid our understanding of virus recognition. I could show that recombinant lentiviruses are weak inducers of IFN-α/β in murine immune cells. Standard preparations of lentiviral vectors, however, are strong activators of the innate immune system. This activity is contained in tubulo-vesicular structures that are present within standard lentiviral preparations and have the ability to activate TLR9. Tubulo-vesicular structures can serve as adjuvant to facilitate adaptive immune responses and may therefore be important when considering lentiviral vectors for clinical applications.

In my second project I focused on cytoplasmic virus recognition. Surprisingly, viral genomic single-stranded RNA from influenza virus can activate the cytoplasmic virus recognition receptor RIG-I. Unlike most cellular RNA species, single-stranded RNA from influenza and other viruses bear a 5' triphosphate group, which marks this RNA as 'foreign' and thereby induces interferon responses. Importantly, influenza virus codes for an interferon antagonist, the non-structural protein 1 (NS1), which forms a complex with RIG-I, suggesting that influenza virus specifically interferes with this pathway.

In conclusion, the innate immune system employs diverse mechanisms to sense the presence of a virus through recognising diverse forms of viral nucleic acid.

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This thesis should be in memory of my beloved father whose creativeness was essential part in all my experiments.

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Table of Abbreviations

2'5'OAS	2'5' oligoadenylate synthetase
AB	Antibody
APC	Antigen presenting cell
BM	Bone marrow
BM-DC	Bone marrow derived dendritic cells; if not stated otherwise BM-DC grown in the presence of GM-CSF
CARD	Caspase activated recruitment domain
cDC	Conventional dendritic cells
CFSE	Carboxyfluorescein
CIP	Calf intestinal phosphatase
CpG	Oligonucleotides containing unmethylated CpG motifs. If not stated otherwise CpG-B
CTL	Cytotoxic T-cell
DAI	DNA receptor and activator of interferon regulatory factors
DNA-PK	DNA-protein kinase
DC	Dendritic cells
dsRNA	Double-stranded RNA
eIF2α	Eukaryotic initiation factor 2α
ELISA	Enzyme linked immuno-sorbant assay
EMCV	Encephalomyocarditis virus
FADD	Fas activated protein with death domain
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Flt-3L	FMS-like tyrosine kinase 3 ligand
Flu	Influenza A virus
FluΔNS1	Influenza A virus lacking the NS1 protein
GCN2	General control nonrepressible protein-2
GFP	Green fluorescent protein
GM-CSF	Granulocyte and monocyte colony stimulating factor

gt	Goat
HIV-1	Human immunodeficiency virus-1
HRP	Horse radish peroxidase
hu	Human
IFN	Interferon
IFNAR	Interferon type-I receptor
IFN-α/β	Type-I interferon
IKK	I kappa B kinase
IL	Interleukin
IPS-1	Interferon beta promoter stimulator-1
ISG	Interferon stimulated gene
IRF	Interferon regulatory factor
LCMV	Lymphochoriomeningitis virus
LCMV-G	Lymphochoriomeningitis virus glycoprotein
LGP2	Laboratory of genetics and physiology-2
LRR	Leucine rich repeat
LV	Lentivirus
LVΔenv	Lentivirus lacking the envelope protein
LVΔRNA	Lentivirus lacking the viral genome
MACS	Magnetic activated cell sorting
MDA5	Melanoma differentiation associated gene 5
MHC	Major histocompatibility complex
MLV-A	Murine leukaemia virus amphotropic envelope protein
MMTV	Mouse mammary tumour virus
MOI	Mulitplicity of infection
mu	Murine
mutNS1	NS1 with two point mutations in the dsRNA binding site (R39A, K41A)
ms	Mouse
MyD88	Myeloid differentiation factor 88
NDV	Newcastle disease virus

NF-κB	Nuclear factor-kappa B
NoCIP	RNA treated with buffers in the absence of CIP
NS1	Non-structural protein 1 of influenza A virus
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PFA	Paraformaldehyde
PIV-5	Parainfluenza virus 5
PKR	dsRNA activated protein kinase R
Poly-I:C	Polyinosinic : polycytidylic acid
PRR	Pattern recognition receptor
RAG-2	Recombination activating gene-2
rb	Rabbit
ReoV	Reovirus
RD	Repression domain
RIG-I	Retinoic acid inducible gene-I
RIP-I	Receptor interacting protein-1
RLR	Rig-like receptor
RSV	Respiratory syncytial virus
rt	Rat
RT-PCR	Reverse transcription PCR
SeV	Sendai virus
SFV	Semliki forest virus
siRNA	Short interfering RNA
SN	Supernatant
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of translation
TBK-1	TANK binding kinase 1
TC	Tissue culture

TCID50	Tissue culture infectious dose 50
THOV-G	Thogotovirus glycoprotein
TIR	Toll/IL1R domain
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF-receptor associated factor
TRIF	TIR-domain containing adaptor molecule inducing IFN-β
TVS	Tubulo-vesicular structures
WNV	West Nile virus
vRNA	Genomic viral RNA
VSV	Vesicular stomatitis virus
VSV-G	Vesicular stomatitis virus glycoprotein

CHAPTER 1: Introduction - mechanisms of virus recognition

1.1. The concept of viral defence

Viruses are obligate intracellular parasites that enjoy the supportive environment of the cell in order to proliferate and spread. This process goes at the expense of the host cell that very often dies as a result of virus infection (Benedict et al., 2002). For this reason it is essential for the host to recognise viruses in order to mount an innate- and adaptive immune response (Haller et al., 2007; Hedrick, 2004; Pichlmair et al., 2004b).

Evolution shaped several lines of defence. Unspecific physical barriers, like the stratum corneum of the skin and secretions containing antimicrobial elements are the first shields used to fend off pathogens. The interferon system constitutes one of the body's main initial modulated responses to viral infections (Samuel, 2001). Alick Isaacs and Jean Lindenmann first described interferon (IFN) 50 years ago as a factor that is generated after virus infection and can confer resistance ('virus interference') to infection with a homologous or heterologous virus (Fig 1.1.1) (Isaacs and Lindenmann, 1957). Although the significance of this discovery was not appreciated initially (Vilcek, 2006), it is now clear that the early immune response to most viral infections relies on a functional interferon system and that mutations in the interferon system in mice or men render individuals highly susceptible to a variety of microbial and viral pathogens (Casrouge et al., 2006; Dupuis et al., 2003; Stetson and Medzhitov, 2006b). To date, we still do not understand all of the diverse functions of IFN, which play important roles in innate- and adaptive immunity, cell differentiation, development, angiogenesis and apoptosis (Samuel, 2001; Stetson and Medzhitov, 2006b; Vilcek, 2006).

Patients receiving IFN as antiviral therapy very often suffer from flu-like symptoms, gastro-intestinal side effects, and neuropsychological-, renal-, dermatological- and

haematological disorders (Dusheiko, 1996; Theofilopoulos et al., 2005; Trask et al., 2000). Clearly, IFN has wide-ranging beneficial and detrimental effects and therefore its induction has to be controlled very tightly, i.e. cells produce no or very little IFN in a quiescent state but have to secrete vast amounts of this cytokine upon encountering a virus. Hence, it is of fundamental importance for the organism to be able to discriminate an invading pathogen from cellular components and subsequently trigger the IFN system. To ensure efficient recognition of pathogen infection, the organism has evolved pathogen recognition receptors that can recognise conserved molecules specific to a given pathogen (Akira et al., 2006; Creagh and O'Neill, 2006; Meylan et al., 2006).

This 'Innate recognition' is not only important for innate resistance to viruses and other pathogens, but also the basis for mounting an adaptive immune response. Charles Janeway originally proposed the existence of 'pathogen associated molecular patterns' (PAMPs) that are associated with, and are particular to any given immunity-inducing microorganism (Janeway, 1989). To induce immunity, PAMPs must be recognised by receptors of the innate immune system, so-called 'pattern recognition receptors' (PRRs), that have the ability to regulate the transcriptome and thereby activate innate- and adaptive immune responses, resulting in enhanced innate resistance and T-cell priming and B-cell activation (Fig 1.1.2) (Janeway, 1989). Meanwhile, the concept of innate recognition shaping adapive immune responses has been proven to be correct (Iwasaki and Medzhitov, 2004; Steinman and Hemmi, 2006). Both, mice and men with genetic mutations in specific PRRs or in the PRR signalling cascade fail to mount efficient innate- and adaptive immune responses when encountering the corresponding pathogen (Lee and Iwasaki, 2007; Oganesyan et al., 2006; Yang et al., 2005; Zhou et al., 2007a). Similarly, viruses that have evolved mechanisms to evade the innate immune recognition system are poor inducers of innate- and adaptive immunity (Fernandez-Sesma et al., 2006). Consequently, to ensure efficient pathogen recognition, our organism is equipped with a vast array of diverse receptors that sense a variety of different PAMPs and have an instructive role in adaptive immunity (Akira et al., 2006; Creagh and O'Neill, 2006; Meylan et al., 2006).

Innate recognition of pathogens is evolutionary ancient and large parts of the innate sensing mechanisms are conserved in various species. One of the best examples of this is the Toll-like receptor (TLR) system. Toll was initially discovered in the fruit fly (*Drosophila melanogaster*) where it serves as a signalling molecule during anti-fungal responses. Later, close homologues of Toll were found in many other metazoan species, including vertebrates, in which a panel of Toll-like receptors serve as innate immune sensors for a variety of pathogens, including viruses (Takeda et al., 2003). More recently another group of PRRs that belong to a family of RNA-helicases was discovered and identified a major player in intracellular recognition of viral pathogens (Meylan et al., 2006; Yoneyama and Fujita, 2007).

The contribution of single pathways adding up to a complete picture of an immune response is complicated as pathogens are sometimes very complex and carry a variety of PAMPs which may stimulate parallel pathways. Virus particles, for instance, differ greatly in their structure: some but not all viruses contain a membranous envelope; others have dense core proteins, although the existence of virus-like particles without core-proteins has also been reported (Harrison, 2001; Rolls et al., 1994). Interestingly, the innate immune system appears to be able to sense all of these distinct particles (Akira et al., 2006; Lechner et al., 2002). Specific interactions between receptors of the immune system and viral proteins do occur (Finberg et al., 2007); however, these might be exceptions and cannot be generalised for a diverse class of pathogens (see later). One common structural feature present in all virus particles is the virus genome consisting of nucleic acid. Virus particles can contain DNA, single stranded RNA or double stranded RNA and, indeed, recent discoveries suggest that antiviral responses are mainly orchestrated through recognition of virus genomes (Bowie and Haga, 2005) or via recognition of by-products of virus replication (Kawai and Akira, 2006; Yoneyama and Fujita, 2007).

My interest lies in the innate recognition of virus infections. I will introduce and discuss the current knowledge on virus recognition, the interferon system and present results I obtained in two projects. The first project deals with Toll-like receptor-mediated recognition of recombinant lentiviruses and revealed an unforeseen mechanism of cytokine induction by gene therapy vectors. The second project focuses on cytoplasmic virus recognition and describes single-stranded

(ss)RNA as an activator of RIG-I, which is a cytoplasmic PRR. A specific modification present on genomic RNA of some viruses renders ssRNA a potent interferon inducer and may constitute the mechanistic basis of the initial discovery of IFN 50 years ago.

1.2. Interferon signalling and function

Since the original description of IFN as antiviral substance, it has become clear that the observed antiviral effect is orchestrated by a family of proteins that share high sequence homology and require similar proteins for downstream signalling. IFNs can be subdivided into three classes, type-I, -II and -III IFN (Pestka et al., 2004; Roberts et al., 1998). Type-I IFNs (IFN-α/β) are encoded from intron-less genes and consist of one IFN-β protein and 13-14 IFN-α subtypes, as well as lesser known IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-τ and IFN-ζ (Pestka et al., 2004; van Pesch et al., 2004). IFN-γ is the only known type-II IFN and is produced by NK cells and activated T-lymphocytes and acts on cells of the immune system. The recently discovered type-III IFN consists of IFN-λ1, -λ2 and -λ3 (also termed IL-28A, IL-28B and IL-29) (Kotenko et al., 2003). Although binding to distinct receptors, type-I and type-III IFNs activate a similar downstream signalling cascade that results in a similar gene expression pattern (Ank et al., 2006; Stark et al., 1998; Zhou et al., 2007b). Type-I and –III IFN can be produced by all nucleated cells although the expression pattern of the different subtypes depends on upstream signalling and, hence, the inducing stimulus (Ank et al., 2006; Pestka et al., 2004) (see later).

After secretion, type-I IFNs bind with various affinities to the two subunits of the type-I IFN receptor (IFNAR) causing heterodimerisation and a conformational change of the intracellular signalling domain. It is believed that the affinities of the IFN-α/β subtypes explain differences in their functions (see later) (Jaks et al., 2007; van Boxel-Dezaire et al., 2006).

Activation of the IFNAR initiates intracellular signalling that involves Janus kinase 1 (JAK1) and Tyrosine kinase 2 (TYK2) that phosphorylate signal transducer and activator of transcription (STAT)-1 and -2 molecules (van Boxel-Dezaire et al.,

2006). Together with Interferon regulatory factor (IRF)-9, STAT1 and -2 form a trimeric complex named Interferon stimulated Gene factor 3 (ISGF3) which translocates into the nucleus and binds to interferon stimulated response elements (ISREs) to drive transcription of interferon stimulated genes (ISGs). Activation of the Jak-STAT pathway initiates transcription of more that 300 genes (Der et al., 1998), including enzymes, transcription factors, cell surface glycoproteins, cytokines and other factors that still await elucidation (Der et al., 1998). Functionally, ISGs include proteins that display antiviral activity, are involved in interferon signalling or act as PRRs (e.g. RLRs and TLRs) (Fig 1.2.1). Furthermore, IFNAR signalling facilitates DC and NK cell activation and therefore is involved in induction of adaptive immunity (Le Bon and Tough, 2002; Stetson and Medzhitov, 2006b) (Fig 1.2.1).

Gene products with antiviral activity include the Fv or tripartite motif protein (TRIM) 5α, both of which interfere with viral proteins (Bieniasz, 2004) and are thought to sequester viral proteins and thereby inhibit virus trafficking within the cell. Similarly, the orthomyxovirus resistance (Mx) proteins appear to recognise virus nuclecoproteins or ribonucleoprotein complexes and sequester them into perinuclear complexes, thereby limiting virus assembly (Haller et al., 2007; Kochs et al., 2002). An increase in expression of the eIF2α kinases, the dsRNA activated protein kinase R (PKR) and general control nonrepressible-2 (GCN2), leads to increased apoptosis and a block in protein translation (Garcia et al., 2007; Samuel, 2001). Similarly, 2'5' Oligoadenylate synthetase (2'5'OAS) is highly upregulated after IFN-α/β treatment. RNAse L an endoribonuclease that is activated by 2'5'OAS and ISG20, a 3'-5' exoribonuclease, can lead to induction of apoptosis and reduce the amount of viral and cellular RNA (Bisbal and Silverman, 2007; Espert et al., 2003). Importantly, some of the expressed genes (e.g. PKR, GCN2 and 2'5'OAS) need to be activated by viral nucleic acid before they can execute their function, which assures that possible detrimental effects are kept in check (Berlanga et al., 2006; Williams, 1999). The orchestrated expression of antiviral and cytostatic proteins is instrumental in reducing virus replication and thereby preventing virus spread. These mechanisms may even be effective enough to clear virus infections in a non-cytolytic manner (Guidotti and Chisari, 2001). Mice or humans lacking

functional proteins involved in IFNAR signalling are highly susceptible to virus infections (Dupuis et al., 2003; Durbin et al., 1996; Muller et al., 1994).
The IFN response can be seen as a self-amplifying circuit, which explains reduced production of IFN-α/β in the absence of functional IFNAR signalling (Haller et al., 2006) (Fig 1.2.1). However, PRRs are of central importance as they initiate IFN-α/β in the first place, thereby kick-starting the entire system.

1.3. Toll like receptors are bona-fide PRRs

TLRs are evolutionary conserved PRRs that bind a variety of PAMPs (TABLE 1).
Finding PRRs in mammals proved to be difficult, but was eventually boosted by the discovery of Toll, a protein in the fruit fly, *Drosophila melanogaster*. Toll was initially identified as a product involved in dorsoventral polarity in the fruit fly's development (Lemaitre et al., 1995) and later found to have a pivotal role for anti-fungal responses in flies (Lemaitre et al., 1996). Medzhitov and Janeway identified a gene in mammals that showed high sequence homology to *Drosophila* Toll and encoded a transmembrane protein with an extracellular domain containing a leucine-rich repeat (LRR) and an intracellular signalling domain with high homology to the interleukin (IL)-1 receptor (Medzhitov et al., 1997). Furthermore, they showed that a constitutively active protein induces pro-inflammatory cytokines such as IL1, IL6 and IL8 and therefore argued that human Toll (later called TLR4) represented the first described PRR linking the innate- and adaptive immune system in vertebrates (Medzhitov et al., 1997).
To date 11 TLRs are known in humans (Table 1) (Akira et al., 2006) but many more exist in animals lacking an adaptive immune system, such as the sea urchin, which possesses 222 TLRs (Rast et al., 2006). TLRs are type-I integral membrane gycoproteins characterized by extracellular domains carrying a varying number of LRRs and a cytoplasmic signalling domain that is homologous with that of the IL1R, also called Toll/IL1R domain (TIR) (O'Neill and Bowie, 2007).
Most TLRs are localised on the plasma membrane facing the extracellular milieu (Table 1) (Takeda et al., 2003). A subset (TLR3, -7, -8 and -9) however is located in the endosomes of specialised immune cells (Fig 1.3.4), and these TLRs screen

incoming material for pathogens. This subset of TLRs seems to be of special importance for recognition of viruses and shares the ability to sense nucleic acid that can lead to IFN-α/β induction (Bowie and Haga, 2005; Wagner, 2004). For this reason I will mainly concentrate on endosomal TLRs in this introduction.

Table 1: Toll-like receptors, their localisation, agonists and signalling molecules

	localisation	agonist	adaptor
TLR1	cell surface	**bacteria**: triacyl lipoproteins	MyD88/MAL
TLR2	cell surface	**bacteria**: lipoproteins, peptidoglycan, lipoteichoic acids; **fungi**: zymosan, mannans; **viruses**: glycoprotein of Measles virus, HSV-1	MyD88/MAL
TLR3	endosome	**viruses**: dsRNA in virally infected cells, poly-I:C, poly-I	TRIF
TLR4	cell surface	**bacteria**: lipopolysacharide; fungi: mannans; **viruses**: glycoproteins of MMTV and RSV	MyD88/MAL TRIF/TRAM
TLR5	cell surface	**bacteria**: flagellin	MyD88
TLR6	cell surface	**bacteria**: diacyl lipoproteins	MyD88/MAL
TLR7	endosome	**viruses**: nucleotide analogs, ssRNA	MyD88
TLR8	endosome	**viruses**: nucleotide analogs, ssRNA	MyD88
TLR9	endosome	**bacteria**: unmethylated DNA motifs; **viruses**: DNA	MyD88
TLR10	cell surface	Unknown	Unknown
TLR11	cell surface	**parasite**: profilin-like molecule	MyD88

1.3.1. Activation of endosomal TLRs by their ligands and consequences for the cell

The N-terminus of TLR3 is the only endosomal TLR ectodomain that has thus far been successfully crystallized (Choe et al., 2005). The LRRs form a horseshoe-like structure that was initially thought to provide a binding-pocket for the ligand at its concave site (Choe et al., 2005). Surprisingly, extensive mutagenesis analysis suggest that dsRNA binds on the lateral side of the TLR3 ectodomain (Bell et al., 2006). Binding of a TLR3 agonist results in receptor clustering and induces a conformational change that brings the two cytoplasmic TIR domains in close

proximity, thereby initiating downstream signalling events via TIR-domain containing adaptor molecules (Bell et al., 2006; O'Neill and Bowie, 2007). A recent report shows that TLR9 may work in a similar manner: binding of its ligand (unmethylated DNA) changes the conformation of latently forming homodimers and this unleashes signalling capacity (Latz et al., 2007). Interestingly, binding of DNA without stimulatory activity does not result in a conformational change in TLR9, indicating that binding of a receptor cannot be equalised with its activation.

Cells prominently expressing endosomal TLRs include antigen-presenting cells, i.e. Dendritic cells (DC), macrophages and B-cells (Iwasaki and Medzhitov, 2004; Reis e Sousa, 2004). Stimulating TLRs on these cells results in expression of co-stimulatory molecules and cytokines that ultimately shape the adaptive immune response and instruct naïve CD4 T-cells to differentiate into effector T-cells (Fig 1.1.2).

For DC, it was shown that TLR activation results in massive cytoskeletal rearrangement. An initial boost of endosomal uptake, which can be understood as a 'sampling process' of the environment, is followed by a phase characterised by a tremendous reduction in endocytosis and in maturation of the DC (West et al., 2004).

1.3.2. Signalling events as a result of TLR activation

TLRs activate the same signalling molecules that are used for IL1R signalling through a conserved Toll/IL1 Receptor (TIR) domain (Kawai and Akira, 2007)(Fig 1.3.1). Five adaptor molecules are known to signal downstream of TLRs (O'Neill and Bowie, 2007). These are Myeloid differentiation factor 88 (MyD88), TIR-domain containing adaptor molecule inducing IFN-β (TRIF), MyD88 adaptor-like (MAL) protein (also called TIR adaptor like protein (TIRAP)), TRIF-adaptor related adaptor molecule (TRAM) and sterile α- and armadillo-motif-containing protein (SARM). Usage of different combinations of adaptor proteins partly explains differences in cytokine production after TLR triggering (O'Neill and Bowie, 2007).

Upon TLR stimulation, MyD88 interacts with the cytoplasmic portion of all TLRs, with the exception of TLR3. TLR3 and TLR4 can signal in MyD88 deficient cells, consistent with the fact that those receptors use an alternative pathway via TRIF (see below). For TLR2 and TLR4, MAL/TIRAP is necessary to bridge the association

between MyD88 and the TLR (O'Neill and Bowie, 2007). The recruitment of MyD88 results in formation of a complex with IL1R associated kinase-1 (IRAK-1) and IRAK-4 (Suzuki et al., 2002). Recently IRAK-2 was shown to be essential part of the same complex (Keating et al., 2007). The MyD88-IRAK complex engages TNFR associated Factor 6 (TRAF6) (Kawai et al., 2004), which in turn activates the NF-κB and Janus kinase (JNK) signalling cascade and Interferon regulatory factor (IRF) 7 and/or IRF5 (Honda et al., 2005b; Takaoka et al., 2005) through additional signalling molecules (Chen, 2005). These events eventually result in binding of activated transcription factors to the corresponding NF-κB, AP1 and IRF-binding sites and lead to the expression of IFN-α/β and pro-inflammatory cytokines such as IL12p40 and IL6. In most cell types a lack of IRF7 severely impairs IFN induction and mice lacking IRF3 and IRF7 do not produce any detectable IFN-α/β (Honda et al., 2005b). An exception are conventional DC (cDC) which rely on IRF1 (Negishi et al., 2006; Schmitz et al., 2007). The production of pro-inflammatory cytokines appears to be a common feature of TLRs but only activation of few TLRs can elicit IFN-α/β (Uematsu and Akira, 2007).

TLR3 and TLR4 use a MyD88 independent TRIF- or TRAM-mediated pathway whereas for TLR7, -8 and -9 MyD88 is required (O'Neill and Bowie, 2007). After activation, TLR3 directly recruits TRIF to its TIR domain (Hoebe et al., 2003; Yamamoto et al., 2003) whereas TLR4 requires a bridging molecule, namely TRAM (Fitzgerald et al., 2003b). However, in both cases TRIF eventually interacts with receptor-interacting protein 1 (RIP1), which activates the NF-κB pathway (Meylan et al., 2004). TRIF can also recruit TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) via TRAF3 (Oganesyan et al., 2006). This recruitment results in the phosphorylation of IRF3 and -7, which form hetero- or homodimers and translocate into the nucleus to bind and activate the IFN-promoter (Honda and Taniguchi, 2006).

TLR7 and -9 mediated IFN-α production is restricted to plasmacytoid dendritic cells (pDC) (Uematsu and Akira, 2007). This subset of DC has also been described as interferon producing cells (IPC) due to the vast amounts of IFN-α/β produced after virus infection (Liu, 2005). The exact mechanism for the restricted expression of

IFN-α is not entirely understood as cDC also express TLRs and can produce high amounts of pro-inflammatory cytokines and IFN-β in response to TLR9 agonists (Negishi et al., 2006; Schmitz et al., 2007). Furthermore, pDC and cDC use a MyD88 dependent signalling pathway, which, in pDC, suggests either a bifurcation of signalling events downstream of this molecule or engagement of additional molecules beside MyD88 (Negishi et al., 2006; Schmitz et al., 2007). In case of pDC, TLR7/9 triggering leads to formation of a complex consisting of MyD88, IRAK-1, IRAK-4, TRAF-3 and -6, TBK1 and IRF7 (Uematsu and Akira, 2007).

Recently, IκB kinase-α (IKKα) has been identified as a key player for IFN-α production by pDC after TLR7 and -9 stimulation (Hoshino et al., 2006). IKKα seems to be a unique requirement for TLR-mediated IFN-α production in pDC, as this protein is not required for secretion of other cytokines expressed after TLR stimulation or for IFN-α/β production elicited by the cytoplasmic virus recognition pathway in other cell types (Hoshino et al., 2006). As IKKα is not unique for pDC and cDC contain both TLRs and IKKα, the unique requirement of IKKα for IFN-α production in pDC does not elucidate why it is that pDC can and cDC cannot produce this cytokine. IFN-α induction also relies on osteopontin (Opn), a phosphoprotein that is necessary for TH1 immune responses. pDC lacking Opn show a defect in expressing IFN-α but produce normal amounts of other cytokines (Shinohara et al., 2006).

Although cDC do not produce IFN-α when stimulated with a TLR9 agoinst, they have recently been found to secrete substantial amounts of IFN-β upon TLR9 stimulation. Surprisingly, IFN-β production in this cell type depends on IRF1 but not on IRF3 or -7 (Negishi et al., 2006; Schmitz et al., 2007).

The discrepancy between pDC and cDC in terms of IFN-α induction has fuelled speculation on the nature of the underlying mechanism. The most widespread explanation for the superiority of pDC is based on the fact that pDC have higher basal levels of IRF7 and therefore may be able to promote IFN-α induction earlier than any other cell type (Izaguirre et al., 2003; Liu, 2005). However, this notion may not be entirely correct as IFN-α/β treatment of cDC induces IRF7 to similar or higher levels than those observed in resting pDC, yet IFN-α/β pre-treatment of cDC

does not promote IFN-α production after TLR7 or -9 stimulation (personal communication Oliver Schulz). Furthermore, pDC stimulated by TLR activation and cDC stimulated via cytoplasmic PRRs can, in principle, produce similar amounts of IFN-α (Diebold et al., 2003) suggesting that signalling molecules necessary for both recognition pathways are not limiting.

It may be that cell biological aspects like differences in endocytosis are the basis for the special ability of pDC to translate TLR9 activation to intracellular signalling leading to IFN-α production (see below).

1.3.3. TLR2 and TLR4 stimulation by viral proteins leads to pro-inflammatory cytokines

Some TLRs can be activated by viral proteins. One example for this is the activation of TLR4 by the RSV-F protein (Kurt-Jones et al., 2000). Compared to wild-type animals, infection of TLR4-deficient animals showed lower numbers of tissue-infiltrating mononuclear cells and decreased IL12 levels and eventually resulted in reduced virus clearance (Kurt-Jones et al., 2000). However, another publication has suggetsted that there is no involvement of TLR4 in the clearance of this virus (Ehl et al., 2004). Therefore the contribution of TLR4 to an immune response against RSV remains controversial.

TLR2-dependent immune responses have been reported for other viruses, such as measles, human cytomegalovirus, murine cytomegalovirus (MCMV), Herpes Simplex Virus 1 (HSV-1), coxsackie virus and Vaccinia virus (VV) (Aravalli et al., 2005; Bieback et al., 2002; Compton et al., 2003; Kurt-Jones et al., 2004; Richer et al., 2006; Szomolanyi-Tsuda et al., 2006). However, there is considerable difference in terms of the relation between TLR2 and these viruses: TLR2 activation seems to be critical to mount an efficient immune response against VV, whereas TLR2 recognition of HSV-1 triggers a vast cytokine response, which is responsible for HSV-1 induced immunopathology linked to morbidity and mortality (Aravalli et al., 2005; Kurt-Jones et al., 2004). Similar to HSV-1, Coxsackie virus activates TLR4 and the resulting pro-inflammatory cytokine burst is linked to cell damage and disease (Richer et al., 2006). Thus, the interaction between a virus and plasmamembrane-bound TLRs does not necessarily result in an advantage for the

host. On the contrary, some reports suggest that TLR – virus interactions can be of benefit for the pathogen: mouse mammary tumour virus (MMTV) envelope protein, for instance, stimulates TLR4 on B-cells or DC (Burzyn et al., 2004; Rassa et al., 2002) and thereby activates B-cell divisions and expression of the MMTV entry receptor CD71, both essential factors for MMTV infection (Otten et al., 2002). Clearly, some viruses hijack cellular 'anti-viral' mechanisms for their own purposes, which make predictions of the benefits of TLR2 and -4 activation by viruses difficult. Therefore, TLR2 and -4 mediated immune responses have to be evaluated separately for any given virus. This may be even more complicated as many *in vivo* experiments can vary considerably depending on the exact experimental procedure used, i.e. virus clearance depends on the mouse strain and age, the infection route, the exact virus isolate and the amount of virus used (Pichlmair et al., 2004a; Zhou et al., 2007a).

1.3.4. Endosomal TLRs mediate IFN-α/β production upon virus infection

Treatment of pDC with a variety of viruses results in production of high amounts of IFN-α/β, a feature that designated pDC as interferon producing cells (Liu, 2005; Siegal et al., 1999). The fundamental basis for this was not known but it appears that pDC have a special ability to link TLR7, -8 and -9 activation in the endosome to IFN-α production and that this is the main reason for their superiority (Kato et al., 2005). TLR3 present in cDC and TLR7, -8 and -9 in pDC are are able to sense nucleic acid that gains access into endosomes (Fig 1.3.4) (Uematsu and Akira, 2007). During their life cycle most viruses enter the endocytic compartment and thereby are believed to deliver TLR ligands, which results in TLR activation and production of IFN–α/β.

Poly-I:C, poly-I and viral dsRNA activate TLR3

It is well established that dsRNA is produced after viral infection. In 1964 David Baltimore showed that the ssRNA containing poliovirus produces vast amounts of dsRNA during virus replication (Baltimore et al., 1964). As dsRNA is uncommon in cells it was soon recognised to be a PAMP for cytosolic virus recognition and was

considered to be the major or even only structure being sensed after virus infection (Field et al., 1967a; Lampson et al., 1967; Merigan, 1970; Tytell et al., 1967). The discovery that the synthetic dsRNA analogue polyinosinic-polycytidylic acid (poly-I:C) induces IFN-α/β supported the notion that long dsRNA serves as determinant for virus infection (Field et al., 1967b).

One protein that recognises dsRNA is TLR3, which is expressed on many cell types although predominantly on cDC (Iwasaki and Medzhitov, 2004; Reis e Sousa, 2004). dsRNA derived from reovirus particles and poly-I:C activate the NF-κB pathway via TLR3 *in vitro* (Alexopoulou et al., 2001) and TLR3 on CD8 positive cDC ($CD8^+$ DC) can recognise dsRNA in virus-infected cells (Fig 1.3.4) (Schulz et al., 2005). Cells infected with EMCV and SFV or loaded with the dsRNA homolog poly-I:C can be phagocytosed by $CD8^+$ DC and stimulate this cell type to produce IFN-β and pro-inflammatory cytokines like IL6 and results in increased ability of the DC to cross prime T-cells (Schulz et al., 2005). Influenza virus, that does not generate detectable amounts of dsRNA (Weber et al., 2006) is a weak inducer of this pathway (Pichlmair et al., 2006) emphasising the notion that TLR3 can recognise dsRNA. More recently it was proposed that mRNA and poly-inosinic acid (poly-I) were sufficient to activate TLR3, suggesting that special forms of ssRNA can contain stimulatory activity (Kariko et al., 2004; Marshall-Clarke et al., 2007).

Several *in vivo* studies have demonstrated that TLR3 is dispensable for the outcome of a virus infection. TLR3-deficient animals show almost unchanged susceptibility to viruses such as vesicular stomatitis virus (VSV) lymphochoriomeningitis virus (LCMV) and reovirus (ReoV) when experimentally infected (Edelmann et al., 2004; Johansson et al., 2007). Interestingly, some DNA viruses appear to activate antiviral responses through TLR3, which is consistent with the notion that DNA viruses commonly produce dsRNA during replication (Colby and Duesberg, 1969; Jurale et al., 1970; Weber et al., 2006). Macrophages lacking a functional TLR3 signalling cascade were compromised in their ability to suppress vaccinia virus replication *in vitro* and infection of TLR3 deficient mice with MCMV resulted in increased viral titres in the spleen, slightly increased mortality, decreased serum levels of IFN-α/β and other cytokines and decreased activation of natural killer (NK) cells (Edelmann et al., 2004; Tabeta et al., 2004). A recent paper by Jean-Laurent Casanova's group

describes TLR3 as an important receptor to prevent encephalitis after HSV-1 infection in human patients, suggesting that TLR3 is mainly involved to control neurotropic viruses (Zhang et al., 2007).

Surprisingly, pathogenesis of some virus infections is reduced in mice that lack TLR3. For instance, TLR3-deficient mice are more resistant to infection with lethal doses of West Nile Fever virus (WNV) (Wang et al., 2004b). This phenomenon was explained by a TLR3-mediated peripheral inflammatory response that results in leakiness of the blood-brain barrier, thereby promoting virus infection of the brain (Wang et al., 2004b). Likewise, infection of mice with Influenza A virus that causes a highly contagious, acute pulmonary disease resulted in pneumonia, which was linked to inflammatory mediators (Le Goffic et al., 2006; Le Goffic et al., 2007). Finally, *tlr3*$^{-/-}$ mice are also more resistant to infection with Punta Toro virus, perhaps because TLR3-deficiency restricts production of proinflammatory mediators, such as IL6, which contribute to immunopathology (Gowen et al., 2006). It has to be noted, however, that experimental infection of mice does not necessarily reflect the situation *in vivo* where limited amounts of virus may not result in exacerbated TLR3 stimulation (Gowen et al., 2006). Moreover, mice used in most experiments are inbred and lack interferon stimulated response genes such as Mx-proteins that are essential to control pathogenicity caused by orthomyxo- and rhabdoviruses (Haller et al., 2007; Pichlmair et al., 2004a).

TLR9 is a receptor for DNA

TLR9 can be activated by unmethylated 2'deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA motifs that are present in bacterial and viral DNA whereas in mammalian genomes such motifs are mainly methylated (Hemmi et al., 2000). Nontheless, cellular DNA can activate TLR9 if the DNA is experimentally delivered into endosomes or if present in immune complexes that can be internalised by Fc-receptor mediated uptake (Barrat et al., 2005; Leadbetter et al., 2002). Although first discussed as a means to recognise bacteria it became clear that TLR9 is important for recognition of DNA viruses such as herpes simplex virus 2 (HSV-2), HSV-1 and MCMV (Hochrein et al., 2004; Iacobelli-Martinez and Nemerow, 2007; Krug et al., 2004a; Krug et al., 2004b; Lund et al., 2003). pDC are the main cell type producing

IFN-α after stimulation with CpG DNA and viruses but other cell types can produce pro-inflammatory cytokines like IL6 (Akira et al., 2006).

Endosomal localisation apparently serves as a mechanism to guarantee specificity of TLR9 activation: extracellular DNA, for instance, is quickly degraded and does not allow activation of TLR9. The endosomal localisation of TLR9 can be seen as mechanism to minimise responses to self-DNA that can be released from cells during necrotic cell death. A chimeric TLR (TLRN9C4) consisting of the ectodomain of TLR9 and the intracellular tail and transmembrane domain of TLR4 can be found at the plasma membrane, facing the extracellular milieu (Barton et al., 2006). Unlike cells expressing wild-type TLR9, cells expressing TLRN9C4 responded to naked DNA added into the culture medium. TLRN9C4 was less effective in recognising HSV-1 and the authors therefore speculated that the endosomal localisation of TLR9 is of importance for both sensing viral pathogens and preventing recognition of self-DNA, which could be produced by bursting cells (Barton et al., 2006).

The exact molecular mechanism of TLR9 recognition was only recently uncovered (Latz et al., 2007). Latz and colleagues could show that binding of agonistic DNA to TLR9 *in vitro* results in a conformational change that initiates downstream signalling (Latz et al., 2007). However, it is still not entirely clear how this works *in vivo* and whether cell type specific TLR9-dependent cytokine expression patterns can solely be explained by conformational change of the receptor. A lot of work has been done with two types of synthetic CpG oligonucleotides that differ in their sequence: CpG-A (also called D-type CpG) with a 3' poly-C sequence and CpG-B (also called K-type CpG) (Klinman, 2004; Krieg, 2002) can both stimulate TLR9, but lead to different cytokine production in pDC. CpG-A elicits IFN-α from pDC, whereas CpG-B promotes lower levels of IFN-α but initiates large amounts of IL6 and TNF-α. Interestingly, pDC retain CpG-A in early endosomes for a longer time than CpG-B, which is transported into lysosomal compartments quickly after uptake (Guiducci et al., 2006; Honda et al., 2005a). Similarly to CpG-B in pDC, CpG-A is transported into lysosomal compartments in cDC. CpG-A complexed with cationic lipids (i.e. DOTAP) is retained in early endosomal compartments of cDC and can elicit a TLR9 dependent IFN-α response (Guiducci et al., 2006; Honda et al., 2005a). This shows

that TLR9 activation can lead to expression of different cytokine patterns depending on the agonist and the cell type used. However, more work is required to understand the mechanistic basis for this and whether this is also the case during a viral infection.

Anti-nucleic acid antibodies, that are present in systemic lupus erythematosus (SLE) patients, can deliver nucleic acid into endosomes through Fc-receptor mediated uptake (Leadbetter et al., 2002). In case of SLE, the constant stimulation of endosomal TLRs (TLR7 and -9) exacerbates this autoimmune disease (Christensen et al., 2005). It remains to be shown, however, whether this is the cause or a consequence of SLE.

In vitro evidence of TLR9 activation and its importance for cytokine production after stimulation with DNA ligands and viruses is clear but its role *in vivo* is less well understood. Human patients with mutations in UNC93B that is involved in recognition via endosomal TLRs are highly susceptible to HSV-1 infection and often die from virus-induced encephalitis, demonstrating a role of the TLR pathway in controlling HSV-1 during a natural infection in humans (Casrouge et al., 2006; Yang et al., 2005). However, IRAK4 that is involved in the TLR7, -8, -9 pathway seems to be redundant to control HSV-1 and other viruses in humans (Yang et al., 2005). Consistent with its function *in vitro*, the adjuvant effect of CpG-containing oligonucleotides to mount an immune response against a co-injected antigen clearly depends on the presence of TLR9 (Klinman, 2004). Furthermore, in an *in vivo* mouse model TLR9 is important in controlling HSV-2 in an intravaginal infection model and mice depleted of pDC are more susceptible to HSV-2 challenge (Lund et al., 2006), in agreement with the notion that pDC recognise HSV-2 via TLR9 leading to production of antiviral cytokines. However, when HSV-1 was injected in the footpad, wild type and *TLR9*$^{-/-}$ mice mounted a similar adaptive immune response and were similarly resistant to the virus (Krug et al., 2004b) consistent with the notion that DNA viruses can be detected by TLR9 independent mechanisms (Hochrein et al., 2004). Further, plasmid DNA clearly activates TLR9 *in vitro*, but when plasmids are used for DNA immunisation TLR9 deficient mice mount an immune response that is indistinguishable from that of control mice (Spies et al., 2003). Clearly, TLR9 dependent immune responses *in vivo* can be seen in some but

not in other experimental models and alternative DNA- and DNA-virus sensing mechanisms are in place.

In conclusion, endosomal recognition of DNA by TLR9 is the current working model that explains how TLR9 expressing cells can sense viral nucleic acid (Fig 1.3.4).

TLR7 and TLR8 are activated by ssRNA

TLR7 and TLR8 show a high degree of similarity to TLR9 and are both located on the X-chromosome. When expressed, both receptors are mainly localized at the endoplasmatic reticulum and at endosomes. The murine version of TLR8 was previously believed to be non-functional or have a non-immune role (Jurk et al., 2002; Ma et al., 2006), although recent evidence from Gorden and colleagues suggests that overexpresion of murine TLR8 in HEK cells and stimulation with a selective TLR8 agonist can lead to activation of an NF-κB promoter (Gorden et al., 2006). Initially, murine TLR7 and human TLR7/8 were found to recognise imidazoquinolins (e.g. R848, Imiquimod, etc) and some guanine nucleotide analogs (e.g. Loxoribine) (Hemmi et al., 2002; Jurk et al., 2002). The discovery that ssRNA of viral and cellular origin can activate TLR7 when experimentally delivered into the endosome led to the supposition that this receptor may have evolved to sense RNA-viruses (Diebold et al., 2004; Heil et al., 2004). Indeed, TLR7 and human TLR8 recognise ssRNA-virus particles such as influenza, VSV or Hepatitis C (Diebold et al., 2004; Heil et al., 2004; Lee et al., 2006; Lund et al., 2004; Triantafilou et al., 2005a; Triantafilou et al., 2005b), and mice lacking the signalling molecule MyD88 are highly susceptible to intranasal infection with the ssRNA virus VSV (Zhou et al., 2007a). In murine pDC, the IFN-α/β response to VSV expressing a glycoprotein mediating fusion at the plasma membrane is reduced, which is consistent with the notion that TLR7 senses viruses in endosomes (Lund et al., 2004). Interestingly, some viruses of the paramyxo- and retrovirus family (e.g. Sendai virus (SeV) and HIV-I) infect cells by fusion at the plasma membrane and one could speculate that this infection route serves as a means to evade innate recognition by TLR recognition. Surprisingly, however, sensing of SeV by murine pDC requires endosomal acidification and TLR7 (Lee et al., 2007; Lund et al., 2004)(see later).

Similarly, it was proposed that IFN-α/β production by pDC treated with HIV-1 was due to TLR7/8 activation (Beignon et al., 2005).

TLR7 recognises RNA that is delivered into endosomes but the molecular details leading to TLR7 activation are only marginally understood. Various studies have proposed the existence of sequence-dependent "immunostimulatory motifs" leading to TLR7 activation (Heil et al., 2004; Judge et al., 2005; Sioud, 2005) but more recently it was demonstrated that three or more uridine molecules on a ribose backbone are necessary and sufficient for TLR7 stimulation (Diebold et al., 2006). However, total RNA or tRNA isolated from mammalian cells only induce minimal amounts of cytokines in DC derived from human monocytes, whereas RNA isolated from *E. Coli* appears to be a very potent TNFα inducer (Kariko et al., 2005). A possible explanation for this reduction of stimulatory potential is that mammalian RNA may be subject to posttranscriptional RNA modifications that are commonly found in mammalian cells. RNA consisting of methylated adenine, cytidine or uridine or thiolated uridine is less potent in activating TLRs (Kariko et al., 2005), which suggests that RNA modifications that evolved in mammalian cells can alter the stimulatory potential of cellular RNA.

As for TLR9, activation of TLR7 can lead to differential cytokine responses depending on the stimulus used. In murine pDC, a RNA homopolymer consisting of uridine nucleotides (poly-U) induces high amounts of IFN-α but little IL6 whereas R848 and Loxoribine elicit much less IFN–α/β but potent IL6 responses (Diebold et al., 2006). Currently it is unknown how this differential cytokine production is mediated but one could speculate that clustering of the receptor or engagement of additional receptors could be involved. In addition, Imidazoquinolins and guanine nucleotide analogs may be engulfed differently than RNA and therefore lead to different responses. Whether similar differences can be found after treatment of pDC with viruses belonging to varying classes is currently not clear.

1.3.5. Cells involved in TLR recognition

Although at first glance it may seem obvious that a cell should posses every means possible to recognise and respond to viral infections, it is not beneficial *in vivo* to be

over-sensitive. Autoreactive B-cell responses resulting in autoimmunity, for instance, are fuelled by the presence of two copies of TLR7 (Pisitkun et al., 2006).
TLR activation differs greatly depending on the cell type being stimulated. Murine $CD8^+$ DC and Langerhans cells, for instance do not express TLR7 and therefore cannot respond to TLR7 ligands. As $CD8^+$ DC are involved in phagocytic clearance of dying cells the lack of TLR7 may constitute a mechanism not to respond to cellular RNA present in apoptotic bodies. The converse argument applies to TLR3 expressed on the same cell types: some virally infected cells contain dsRNA and their uptake activates TLR3 on $CD8^+$ DC (Schulz et al., 2005). Similarly, TLR2 and TLR4 are highly expressed in human macrophages whereas only small amounts of TLR7 and TLR9 are present. In humans, TLR9 is exclusively expressed on pDC and B-cells (Iwasaki and Medzhitov, 2004).
TLR agonists can elicit differential cytokine responses depending on the cell type. As mentioned above, TLR9 agonistic CpG-A mainly elicits IFN-α in pDC, whereas cDC exposed to the same agonist secrete IFN-β, IL6 and other pro-inflammatory cytokines. In cDC this relies on a signalling pathway strongly depending on IRF1 but not on IRF7, whereas pDC need IRF7 but do not require IRF1 (Negishi et al., 2006; Schmitz et al., 2007), suggesting that activation of PRRs is coupled differently to downstream signalling molecules in a cell-type specific manner.
Clearly, the cells of the immune system are specialised to serve the specific needs they are designed for and share the challenge to fight a diverse range of pathogens.

1.4. Cytoplasmic recognition of viruses

The very first description of IFN in 1957 was carried out in chicken egg chorio-allantoic membrane and, as we now know, most likely based on intracellular recognition of a virus: supernatant from chicken egg chorio-allantoic membrane cultures infected with influenza virus contained a factor (interferon) that conferred resistance to infection with a heterologous or homologous virus (Fig 1.1.1) (Isaacs and Lindenmann, 1957). Only very recently, almost 50 years after the original discovery of IFN, was the receptor responsible for IFN induction after influenza virus infection finally identified (see later) (Kato et al., 2006).

One of the central issues following the discovery of IFN was to identify the component the cell recognises during a viral infection. It was hypothesised that such a component differs from cellular components, and is related to the viral infection. Alick Isaacs proposed that nucleic acid that is not self, for instance RNA from a different cell type, can elicit IFN when added to cells (Isaacs et al., 1963). This idea was questioned however (Lampson et al., 1967), as the experiments appeared to be of little reproducibility (Isaacs, 1965). In 1964 David Balitmore described that cells infected with poliovirus, a ssRNA virus, contained large amounts of dsRNA and this depended on virus replication (Baltimore et al., 1964). This phenomenon appeared to be true for a variety of other ssRNA viruses like Tobacco mosaic virus, EMCV, Semliki forest virus (SFV) and Sindbis virus as well as for the dsRNA viruses ReoV and a dsRNA bacteriophage (Friedman and Sonnabend, 1965; Horton et al., 1964; Kaerner and Hoffmann-Berling, 1964; Shipp and Haselkorn, 1964; Stollar and Stollar, 1970) Even DNA viruses like Vaccinia virus and T4 bacteriophage (Colby and Duesberg, 1969; Jurale et al., 1970) generate dsRNA in infected cells. DsRNA fulfilled all the criteria necessary for being a virus specific marker: it has apparent different structural features compared with cellular RNA and is only present in virally infected cells. Furthermore, the IFN–α/β inducing activity was contained in the RNA fraction of virally infected cells and it was resistant to digestion with RNAses specific for ssRNA (Merigan, 1970). After early publications could show that highly purified dsRNA isolated from penicillium funiculosum or virally infected cells had the potential to induce IFN-α/β *in vitro* and *in vivo*, dsRNA was soon established as the predominant or even the only IFN-α/β inducer (Field et al., 1967a; Field et al., 1967b; Lampson et al., 1967; Merigan, 1970; Tytell et al., 1967). This notion was further underlined by the fact that dominant interfering particles from VSV contained dsRNA and were very strong IFN-α/β inducers whereas a ssRNA wild-type virus did not induce IFN-α/β (Marcus and Sekellick, 1977). Following the original description of dsRNA being an interferon inducer, research on this molecule soared and it soon consolidated its central position in the field of interferon research. A protein responsible for recognising cytoplasmic dsRNA and linking this IFN-α/β induction, however, has not yet been found.

1.4.1. Receptors involved in cytoplasmic recognition of viruses

Unlike the expression of TLRs that is restricted to specific cells, cytoplasmic receptors that recognise viruses appear to be expressed in most cell types. Many cellular proteins can sense viral nucleic acid or viral proteins and thereby restrict virus growth. Examples for these are adenine deaminases (ADAR proteins), GCN2, ISG20, Fv- and Mx-proteins (Haller et al., 2007; Samuel, 2001). However, only few proteins have the ability to regulate interferon production and I will concentrate on these within this introduction.

DsRNA dependent protein kinase R (PKR)

As described above, dsRNA was defined as the bona-fide inducer of IFN-α/β after viral infection, and therefore the search for receptors mainly focused on dsRNA-binding proteins. PKR was identified as a protein that is activated by dsRNA (Williams, 1999). This process requires binding of two dsRNA-binding domains, which explains the minimal length requirement of around 60 nucleotide dsRNA (Garcia et al., 2007). Activation results in PKR dimerisation, autophosphorylation and signal transduction to downstream molecules.

Mice with deletions in the dsRNA binding domains of PKR are more susceptible to virus infection (Bergmann et al., 2000; Streitenfeld et al., 2003; Zhou et al., 1999), which is believed to be due to the ability of activated PKR to constitutively phosphorylate the eukaryotic initiation factor 2α (eIF2α) and, leading to a block of protein synthesis (Garcia et al., 2007). *In vitro* PKR has been shown to be pro-apoptotic, probably through activation of the Fas-Associated protein with Death Domain (FADD) pathway and it may be that virus clearance *in vivo* requires pro-apoptotic signals through the activation of PKR (Balachandran et al., 1998).

Cells lacking functional PKR appear to produce less IFN-α/β when treated with poly-I:C (Der and Lau, 1995; Diebold et al., 2003). PKR has been shown to interact with IKKβ and JNK2, both of which are essential for IFN-α/β induction (Chu et al., 1999; Zamanian-Daryoush et al., 2000). PKR can be found in a complex with TRAF3 that is also implicated in IFN-α/β induction (Oganesyan et al., 2006).

Surprisingly, *pkr*$^{-/-}$ cells produce normal amounts of IFN-α/β when infected with some viruses like Newcastle disease virus (NDV), SeV and Influenza virus (Gilfoy and Mason, 2007; Smith et al., 2001)and personal communication, Sandra Diebold). Interestingly, a recent report shows that PKR is required for potent IFN-α/β production in West-Nile virus infected cells and mice, which could suggest that PKR may only sense a specific group of viruses (Gilfoy and Mason, 2007).

DAI and other receptors sense intracellular DNA

DNA is a fundamental entity present in all living organisms and normally tightly packaged either in the nucleus, in mitochondria, in bacteria or within viral structural components. However, any failure of DNA removal e.g. by the lack of DNAse-II results in TLR9 independent activation of innate and adaptive immune responses and can eventually climax in an autoimmune disease (Ishii and Akira, 2006; Okabe et al., 2005), suggesting the existence of additional DNA-sensing mechanisms beside TLR9. The notion that transfected DNA can induce innate immune responses is not new; in 1999, Suzuki and colleagues reported that transfection of DNA into target cells can lead to upregulation of co-stimulatory molecules and expression of low amounts of IFN-β (Suzuki et al., 1999). More recent evidence suggest that cytoplasmic delivery of DNA results in a TLR9 independent induction of IFN-α/β (Martin and Elkon, 2006; Shirota et al., 2006; Yasuda et al., 2005) through the canonical IFN induction pathway involving IRF3 (Ishii et al., 2006; Stetson and Medzhitov, 2006a). B-form DNA, which constitutes the DNA most commonly found in the nucleus, is better recognised than the Z-form DNA that appears to be more common in pathogens. Therefore, cytoplasmic DNA recognition in mammalian cells does not require a specific signature on DNA and a tight spatial separation of the nuclear from the cytoplasmic compartment guarantees unresponsiveness to self-DNA.

One candidate involved in cytoplasmic DNA recognition is Z-DNA binding protein 1 (Takaoka et al., 2007). Exogenous expression of this protein enhances IFN-α/β production in response to B-DNA transfection and HSV-1 infection. In addition, Z-DNA binding protein 1 can form a complex with IRF3. For both reasons this protein was re-named DNA activator of interferon regulatory factors (DAI). siRNA

knockdown of DAI reduces IFN-α/β mRNA in response to B-DNA 3-5 fold as compared to control cells receiving unspecific siRNA. Cells lacking IRF3, however, show a 1000-fold reduction in their response to DNA transfection (Takaoka et al., 2007). Although this may be attributable to insufficient knockdown, it may suggest the presence of other proteins sensing cytoplasmic DNA, and studies with mice lacking DAI will probably clarify this point in the future.

Chu and colleagues reported that immunostimulatory DNA activates bone-marrow-derived macrophages (BMMs) to produce IL6 and IL12 and that this depended on DNA-protein kinase (DNA-PK) (Aderem and Hume, 2000; Chu et al., 2000). DNA-PK is implicated in the DNA damage response pathway and is known to phosphorylate p53, leading to transcriptional activation of proteins involved in cell cycle arrest. Importantly, CpG-dependent activation of BMMs is independent of TLR9, as activation of DNA-PK dependent signalling can also be found in BMMs and MEFs lacking TLR9 (Dragoi et al., 2005). DNA-PK forms a complex with IKKβ and thereby activates NF-κB and AKT signalling. However, whether DNA-PK is involved in cytoplasmic sensing of DNA was questioned by other groups (Hemmi et al., 2003; Shirota et al., 2006).

DNA viruses commonly produce high amounts of dsRNA during replication and therefore would possibly activate both cytoplasmic DNA receptors like DAI and receptors sensing viral dsRNA.

Rig-like Helicases

In 2004, Takashi Fujita's group identified an ATP dependent DExD/H box RNA-helicase, Retinoic acid inducible gene-I (RIG-I), as a protein that is able to bind poly-I:C and induce IFN responses (Yoneyama et al., 2004). RIG-I expression itself is enhanced when cells are treated with IFN-α/β and reducing RIG-I levels by siRNA diminishes the IFN response elicited by NDV. The protein has a very unusual structure as it contains a helicase domain, which has the ability to bind dsRNA, and two caspase activation and recruitment domains (CARDs) (Fig 1.4.1) (Yoneyama et al., 2004). CARDs are known players in signal transduction and are generally composed of six antiparallel α helices that serve as docking platform for homotypic interactions with other CARD bearing proteins (see RLR signalling) (Werts et al.,

2006). Expression of the RIG-I CARD constitutively activates IRF3 and IFN-β expression. The helicase domain has ATP-binding capacity believed to be important in unwinding dsRNA and an amino acid substitution at position Lys-270 (K270A) abrogates this activity and results in a dominant inhibitor. Likewise, RIG-I lacking the CARD or bearing a mutated CARD (T55I), leaving only a functional helicase domain, also acts as a dominant negative protein and suppresses IFN-α/β induction in response to NDV (Yoneyama et al., 2004). More recently it has been shown that a C-terminal repression domain can act as an inhibitor of activation and keeps RIG-I silent in the absence of virus infection (Saito et al., 2007). Binding of an agonist to RIG-I induces protein dimerisation and a conformational change that exposes the CARD (Yoneyama et al., 2004). The identification of RIG-I as a cytoplasmic sensor for viral infection has defined a new family of PRRs that are therefore known as Rig-like receptors (RLRs) (Creagh and O'Neill, 2006).

Two other proteins belong to this family: Melanoma differentiation factor-5 (MDA5) and Laboratory of genetics and physiology-2 (LGP2) are both DExD/H box RNA-Helicases involved in virus recognition (Kato et al., 2006; Rothenfusser et al., 2005; Yoneyama et al., 2005). Like RIG-I, MDA5 bears a RNA-helicase and a CARD (Kato et al., 2006). MDA5 was first implicated in virus recognition as it interacts with the interferon antagonist of Parainfluenza virus-5 (PIV-5) (Andrejeva et al., 2004). It was therefore hypothesised that MDA5 may be involved in PIV-5 recognition. Interestingly, despite the striking similarity to RIG-I, MDA5 has a distinct virus specificity (see below). MDA5 is the only RLR that lacks a C-terminal repression domain and overexpression of MDA5 results in production of IFN-α/β (Andrejeva et al., 2004; Saito et al., 2007).

LGP2 shares high homology with RIG-I but lacks a CARD (Rothenfusser et al., 2005; Yoneyama et al., 2005). Consequently, LGP2 cannot transmit signals to downstream molecules and was thought to act as a down-regulator of IFN-α/β production. In agreement with this hypothesis expression of LGP2 in HEK cells reduced IFN-α/β responses to RIG-I and MDA5 dependent stimuli, as seen for dominant negative RIG-I. Therefore LGP2 was proposed to have regulatory properties, which it may accomplish through sequestering agonists for RIG-I and MDA5 and/or through interfering with components of the IFN-α/β signal

transduction pathway (Rothenfusser et al., 2005; Yoneyama et al., 2005). Consistent with this, mice lacking LGP2 show enhanced production of IFN-β when stimulated with poly-I:C (Venkataraman et al., 2007). However, *in vitro* IFN-α/β levels declined similarly in wt MEFs and MEFs lacking LGP2, suggesting that LGP2 is dispensable for down-regulation of IFN-α/β. LGP2-deficient mice produce more pro-inflammatory cytokines in response to VSV infection and appear to be more resistant to this virus. Surprisingly, however, cytokine expression after EMCV infection is impaired in these mice, which manifests itself in enhanced susceptibility to EMCV. Therefore, it is believed that LGP2 plays important roles in the recognition of EMCV, but may not be mandatory for modulating IFN-α/β expression after infection (Venkataraman et al., 2007).

1.4.2. Interaction of RLRs and their ligands

As described above, dsRNA is thought to be the major agonist for IFN-α/β induction. All RLRs have the ability to bind the dsRNA homolog poly-I:C or dsRNA forming poly-A:U (Rothenfusser et al., 2005; Yoneyama et al., 2005; Yoneyama et al., 2004).

Shizuo Akira's group generated mice lacking RIG-I and MDA5 (Kato et al., 2005; Kato et al., 2006). Surprisingly, these mice showed remarkable virus specificity (Fig 1.4.2): *in vitro*, RIG-I is required for IFN induction in response to viruses like Japanese encephalitis- (JEV), SeV and influenza virus, whereas MDA5 appears to be a specific receptor for picornaviruses, i.e. EMCV and mengovirus (Kato et al., 2006). Furthermore, IFN induction from *in vitro* transcribed dsRNA depended on RIG-I, whereas poly-I:C responses were mediated by MDA5. Similarly to the *in vitro* data, RIG-I deficient mice were highly susceptible to infection with JEV and MDA5 deficient mice succumbed to EMCV as early as mice that cannot respond to IFN-α/β (*ifnar*$^{-/-}$ mice) (Kato et al., 2006). The mechanism underlying this discrepancy was unknown.

As for activation of TLR9 by DNA, the above results clearly show that binding of a ligand is not necessarily sufficient to activate a given PRR. Binding of poly-I:C is not sufficient to activate RIG-I responses and an unknown specificity associated with *in vitro* transcribed dsRNA is required for RIG-I activation. Similarly, MDA5

responses are not simply elicited by dsRNA, as *in vitro* transcribed dsRNA does not activate MDA5. Therefore, PRR binding of a ligand and PRR activation by an agonist need to be considered separately.

1.4.3. Signalling downstream of RLRs

The discovery of RIG-I and MDA5 resulted in unravelling of an entirely new signalling cascade downstream of these proteins. Both PRRs seem to funnel through Interferon-β promoter stimulator-1 (IPS-1), also called MAVS, CARDIF or VISA, which contains a N-terminal CARD that links to the CARDs of RIG-I and MDA5 (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). Interestingly, IPS-1 appears to attach to mitochondria through its C-terminus; this localisation is important as disruption of this association through mutation analysis or proteolytic cleavage by the NS3 protein of Hepatitis C virus can abrogate signalling (Meylan et al., 2005). Overexpression of IPS-1 results in activation of an IFN-β-promoter and NF-κB-promoter and siRNA mediated knock down or a genetic knockout diminishes virus and poly-I:C induced IFN responses (Kawai et al., 2005). Binding of RIG-I to IPS-1 results in the recruitment of molecules that mediate downstream signalling (simplified depicted in Fig 1.4.2). A C-terminal catalytic domain associates with the adaptor FADD and the kinase receptor interacting protein-I (RIP1), transforming growth factor- β- activated kinase 1 (TAK1), IKKα and IKKβ that mediate activation of NF-κB (Balachandran et al., 2004; Kawai et al., 2005; Meylan et al., 2006; Xu et al., 2005). TBK1 and IKKε, both kinases that mediate IRF3 phosphorylation and activation of IFN responses, co-immunoprecipitated with IPS-1 (Meylan et al., 2006; Xu et al., 2005). Additional proteins like TRAF6 or NAK associated protein 1 (NAP1) are also found in the same complex (Seth et al., 2005; Xu et al., 2005) and may be necessary for this interaction. Taking together, IPS-1 activation appears to initiate the formation of an intracellular signalling complex that mediates activation of promoters containing NF-κB and IRF3 and -7 binding sites.

The association with mitochondria and the signalling through FADD, both of which are involved in apoptosis, supports the supposition that there are intervening pathways of IFN induction and cell death (Balachandran et al., 2004; Takahashi et

al., 2006). Although FADD and RIP1 deficient cells are highly susceptible to VSV infection (Balachandran et al., 2004), the role of FADD and RIP1 in IFN-α/β induction is not entirely clear (Kawai et al., 2005).

IFN-α/β induction critically requires Interferon regulatory factor (IRF) 7 and -3 (Honda et al., 2005b; Sato et al., 2000) that are activated by TBK1 and IKKε (Fitzgerald et al., 2003a; Sharma et al., 2003). Loss of IRF7 abolished IFN-α/β induction from both, the TLR and the RLR pathway and therefore IRF7 appears to be a merger of these two pathways (Honda et al., 2005b). IRF3 and -7 are first phosphorylated, then they dimerise, translocate into the nucleus and bind to IRF-binding sites present in IFN promoters (Honda et al., 2006). In addition to the IRF binding site, the IFN-β promoter contains binding sites for ATF-2/cJun (AP1) and NF-κB and the best activation is achieved when all sites are activated in parallel (Honda and Taniguchi, 2006).

1.5. Battle of existence – viruses vs. interferon system

Viruses have to multiply extensively in infected hosts to ensure successful transmission. This is a challenging task, especially in the presence of the powerful innate- and adaptive immune system (see paragraph 1.2.1). The IFN system in particular seems to be a major target of virus anti-immunity mechanisms (Haller et al., 2006; Weber et al., 2004). Viruses can inhibit IFN-α/β synthesis, inactivate secreted IFN molecules, interfere with IFN-α/β signalling or specifically block activation of IFN stimulated antiviral response proteins. Viruses that have lost their ability to block IFN-α/β are mainly non-pathogenic and are sometimes used as vaccine strains (Haller et al., 2006; Weber et al., 2004). Interferon is necessary to prevent mice being infected with Myxoma virus, which may suggest that IFN-α/β contributes to confine virus spread in certain species (Wang et al., 2004a). *In vitro* studies suggest that some interferon antagonists may act in a species-specific manner and thereby contribute to restricted host-range of viral pathogens (Bossert et al., 2002; Hagmaier et al., 2007; Hayman et al., 2007; Park et al., 2003; Wang et al., 2004a). Of particular interest is the activity of the V-proteins of Simian virus 5 and

Parainfluenza virus 5 that can degrade STAT proteins in a species specific manner (Parisien et al., 2002). In the light of recurrent epidemics it may seem as if the body's immune system is insufficiently prepared to resist attacks from viral pathogens, but one has to be aware that only the most sophisticated viruses can successfully battle the immune system. Despite the action of IFN antagonistic mechanisms during most viral infections, IFN suppression is never as severe as a genetic knockout for IFNAR or STAT-proteins, illustrating the remarkably potent ability of the IFN system (Dupuis et al., 2003; Durbin et al., 1996; Muller et al., 1994). Therefore, anti-interferon mechanisms only modify antiviral responses to allow the virus to spread sufficiently.

A summary of anti-IFN mechanisms has been discussed in depth in some recent reviews (Garcia-Sastre and Biron, 2006; Goodbourn et al., 2000; Haller et al., 2006; Hengel et al., 2005; Pichlmair et al., 2004b; Weber et al., 2004), and therefore I will only touch on a few mechanistic details of interferon antagonists.

As mentioned above, viral IFN antagonists can inhibit different levels of the innate immune system. Unspecific mechanisms include a broad shutdown of cellular transcription and translation. The NSs protein of Rift valley fever virus, for instance, can interact with the TFIIH transcription factor, thereby preventing proper assembly of the cellular polymerase II (Le May et al., 2004). This results in diminished mRNA expression including mRNA of IFN-α/β genes. Intriguingly, the virus expresses its own polymerase and therefore does not require the cellular transcription machinery to generate progeny virus. The Matrix (M) protein of VSV also interferes with the transcription factor (TFIID) but in addition blocks mRNA export into the cytoplasm and can inhibit the translation machinery (Hengel et al., 2005). Likewise, subversion of the IFN system is put forward as the major reason for the transcriptional and translational shut off seen in picornavirus- and HSV-I infected cells (Weber et al., 2004). Interestingly, Sindbis virus seems to block the cellular machinery through using the cell's own weapon: the activation of PKR and other eIF2α-kinases blocks protein translation but the viral RNA possesses a 5' hairpin that facilitates initiation of translation through stalling of ribosomes, despite the presence of phosphorylated eIF2α (Ventoso et al., 2006).

Inhibiting cellular protein synthesis cannot be sustained without severely impacting on cellular viability and therefore eventually affecting virus replication. For that reason some viruses may have evolved mechanisms tailored to suppress the IFN system more specifically. dsRNA binding proteins, for instance, are thought to sequester dsRNA and thereby have an anti-IFN function (Garcia-Sastre and Biron, 2006). The Vaccinia virus E3L protein blocks IFN induction and it has been suggested that this is due to the capacity to bind dsRNA through a C-terminal dsRNA-binding site. More recent evidence suggesting that cytosolic DNA induces IFN-α/β may explain the presence of a Z-DNA binding site on the N-terminus of the same protein (Kim et al., 2003). *In vivo* pathogenicity of the Vaccinia virus requires both the N- and the C-terminus of the E3L protein and it would be interesting to know whether the DNA binding motif is responsible for interfering with innate recognition of cytoplasmic DNA (Kim et al., 2003).

Signalling molecules downstream of PRRs are another attractive target to block IFN-α/β induction. The NS3-4A protein of Hepatitis C virus, for instance, can cleave the RIG-I adaptor protein IPS-1 and TRIF that acts downstream of TLR3 (Meylan et al., 2005). Similarly, a variety of viruses belonging to different classes have been reported to interfere with activation of IRF3, suggesting either direct interaction with IRFs or inhibition of upstream kinases. Vaccinia virus expresses B18R, a IFNAR-like molecule that neutralizes secreted IFN-α/β (Symons et al., 1995). More specifically, V proteins of paramyxoviruses can lead to degradation of JAK and STAT proteins thereby alleviating the antiviral action of type-I, -II and -III IFN (Goodbourn et al., 2000).

Another efficient way to block the action of IFN-α/β is to specifically interact with antiviral proteins. As mentioned above, many of these proteins need to be activated by viral RNA and viral antagonists capable of binding dsRNA often inhibit their activation. The E3L protein of Vaccinia and the non-structural protein 1 (NS1) of the influenza virus, for instance, block activation of 2'5'OAS (Xiang et al., 2002). EMCV and HIV induce expression of a cellular RNAse L inhibitor (RLI) (Martinand et al., 1999). HSV-1 and HSV-2 express 2'5' oligoadenylate derivates that bind and de-activate RNAse L (Weber et al., 2004).

PKR is one of the best-studied targets of viruses (Garcia et al., 2007). Poxviral proteins can directly bind and inactivate PKR, and poliovirus leads to PKR degradation. The VAI RNAs of Adenoviruses are believed to inhibit dimerisation of PKR and therefore its activation also. Hepatitis C virus and HIV express pseudosubstrates that inhibit activation of cellular targets of PKR and the Herpes Simplex virus γ34.5 protein dephosphorylates eIF2α (Garcia et al., 2006; Weber et al., 2004). The efforts made by viruses to inhibit PKR suggest that this protein plays a central role in cellular IFN-α/β responses. In fact, PKR has multiple functions beside blocking cellular translation, including induction of apoptosis and activation of cytokine transcription through the NF-κB pathway.

Viruses have co-evolved under the selective pressure of the innate immune system, and therefore understanding their countermeasures can give further insight into cellular processes involved in virus recognition (Hengel et al., 2005). Recent discoveries highlighting divergent recognition processes in virus innate immunity may suggest that viruses have evolved similarly divergent multipotent mechanisms to escape this cellular surveillance. Indeed, some viral proteins interact with the IFN pathway at various points. One such protein is NS1 of influenza virus, a pathogenicity factor that is active in a wide variety of species (Hayman et al., 2006).

A genetic or natural deletion of the NS1 protein results in virus attenuation *in vitro* and *in vivo* (Garcia-Sastre et al., 1998). This attenuation is due to the IFN system, because wt and NS1-deleted Influenza viruses (strain A/WSN/33) can replicate to similar levels in IFN-deficient systems (Garcia-Sastre et al., 1998). Garcia-Sastre and colleagues speculated that the NS1 protein has the ability to sequester dsRNA and thereby blocks IFN-α/β induction (Talon et al., 2000). However, the dsRNA binding capacity could serve multiple purposes as the sequestration of dsRNA could explain the NS1-mediated inhibition of dsRNA binding proteins such as PKR and 2'5'OAS (Bergmann et al., 2000; Hatada et al., 1999; Lu et al., 1995; Min and Krug, 2006). Two point mutations in the dsRNA binding site in the NS1 protein of Influenza A/WSN/33 (NS1 R38A, K41A) result in greater cytokine production than with the wild-type virus (Donelan et al., 2003). A revertant NS1 protein (NS1 R38A, K41A, G42S) that lacks dsRNA binding activity inhibits IFN-α/β induction, which indicates that dsRNA binding is only one feature that modifies innate immune

responses. Recent work from Robert Krug's laboratory suggests that the same amino-acid residues mutated in Influenza A/Udorn/72 inhibit the nuclear accumulation of NS1, and the authors speculated that this altered cellular distribution is the real reason why NS1(R38A, K41A) is inactive (Min and Krug, 2006).

The C-terminus of some NS1 strains impair the post-transcriptional processing and nuclear export of cellular pre-mRNAs (Fortes et al., 1994; Li et al., 2001) through direct interaction with the cleavage and polyadenylation specificity factor (CPSF) (Chen et al., 1999; Nemeroff et al., 1998). Un-processed pre-mRNA is not exported into the cytoplasm, resulting in diminished IFN-α/β expression (Noah et al., 2003).

Clearly, the NS1 protein may serve different functions and may be remarkably flexible in its mode of action. However, this activity depends on the influenza virus strain the NS1 protein derives from as is highlighted in recent reports (Hayman et al., 2007; Kochs et al., 2007). Kochs et al. for instance directly compared NS1 proteins of different virus strains and could show that NS1(A/PR8/34) is more potent in blocking activation of IRF3 than NS1(A/TX/36/91), and that NS1(A/TX/36/91) but not NS1(A/PR8/34) has the ability to interfere with mRNA processing (Kochs et al., 2007).

1.6. Aim of this thesis

IFN-α/β clearly plays a major role in antiviral immunity, yet the mode of its induction is ill understood. I therefore dedicated myself to work on the following two questions, both designed to deepen our understanding of virus sensing:

- TLR recognition takes place in endosomes and therefore I wanted to understand what role the infection route of a virus has on TLR activation. To address this I used recombinant lentiviruses that were pseudotyped with glycoproteins that mediate infection via endosomes or fusion at the plasma membrane. Although I did not succeed in answering the original question, I found that preparations of lentiviruses pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) are strong activators of the innate immune system. Surprisingly, this does not depend on virus particles but on tubulo-vesicular structures that are produced through VSV-G expression. VSV-G on

the surface of those tubulo-vesicular structures mediates uptake and delivery of a TLR agonist into the endosome.

- dsRNA is the proposed activator for cytoplasmic PRRs. However, ssRNA can induce IFN-α/β (Baron et al., 1969), and some viruses do not express detectable amounts of dsRNA. I wanted to understand whether there are cytoplasmic recognition mechanisms that go beyond dsRNA sensing. I found that RIG-I can be activated by single-stranded viral genomic RNA bearing 5'phosphates. The NS1 protein of influenza-A virus counteracts this activation and forms a complex with RIG-I.

1.7. Figures

Figure 1.1.1

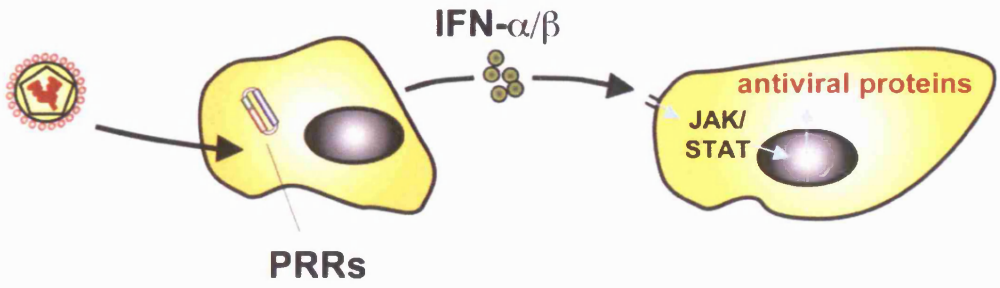


Figure 1.1.1: Innate virus recognition results in IFN production and antiviral resistance

Upon virus infection, pattern recognition receptors (PRRs) alert the cell that a virus is present and this results in the production of pro-inflammatory cytokines including type-I interferon (IFN-α/β). IFN-α/β acts on adjacent cells where it activates the so-called JAK/STAT pathway which increases expression of antiviral proteins that reduce virus proliferation and virus spread.

Figure 1.1.2

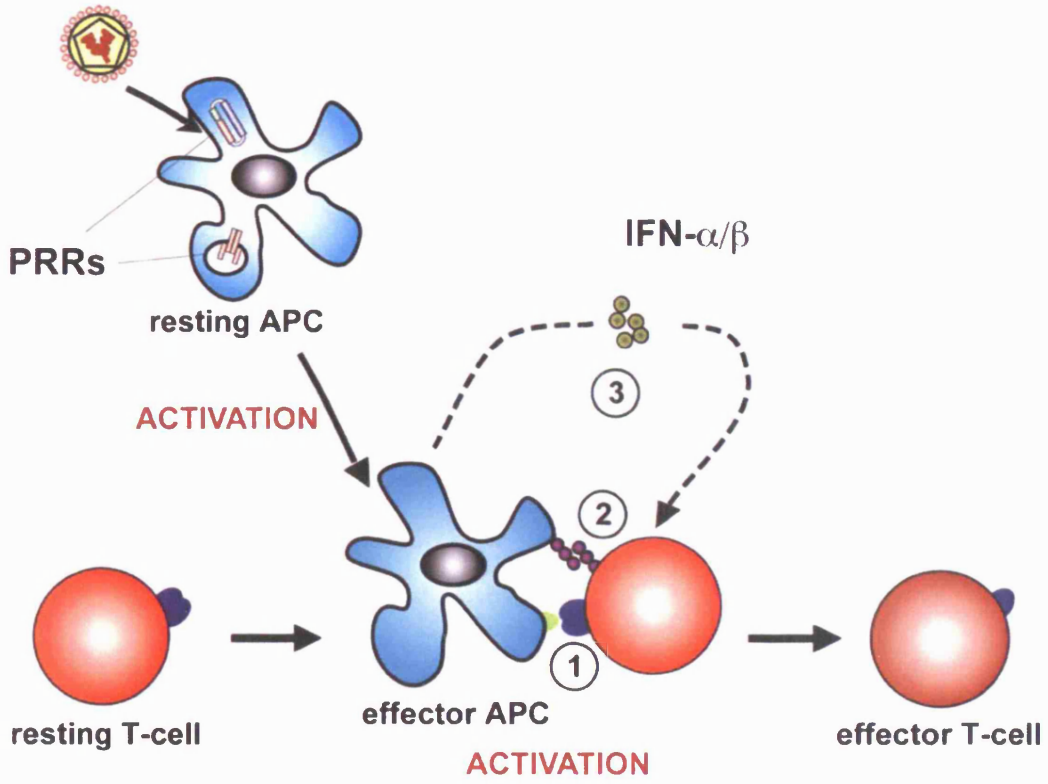


Figure 1.1.2: Innate immune recognition controls adaptive immunity

An antigen presenting cell (APC), e.g. dendritic cell or B-cell, senses the presence of a virus through pattern recognition receptors (PRRs). This leads to APC activation and activated APCs present peptides on MHC-I (green, 1), costimulatory molecules (purple, 2) and produce pro-inflammatory cytokines (green dots, 3). Signals 1, 2 and 3 are necessary for activation of resting T-cells to gain effector function.

Figure 1.2.1

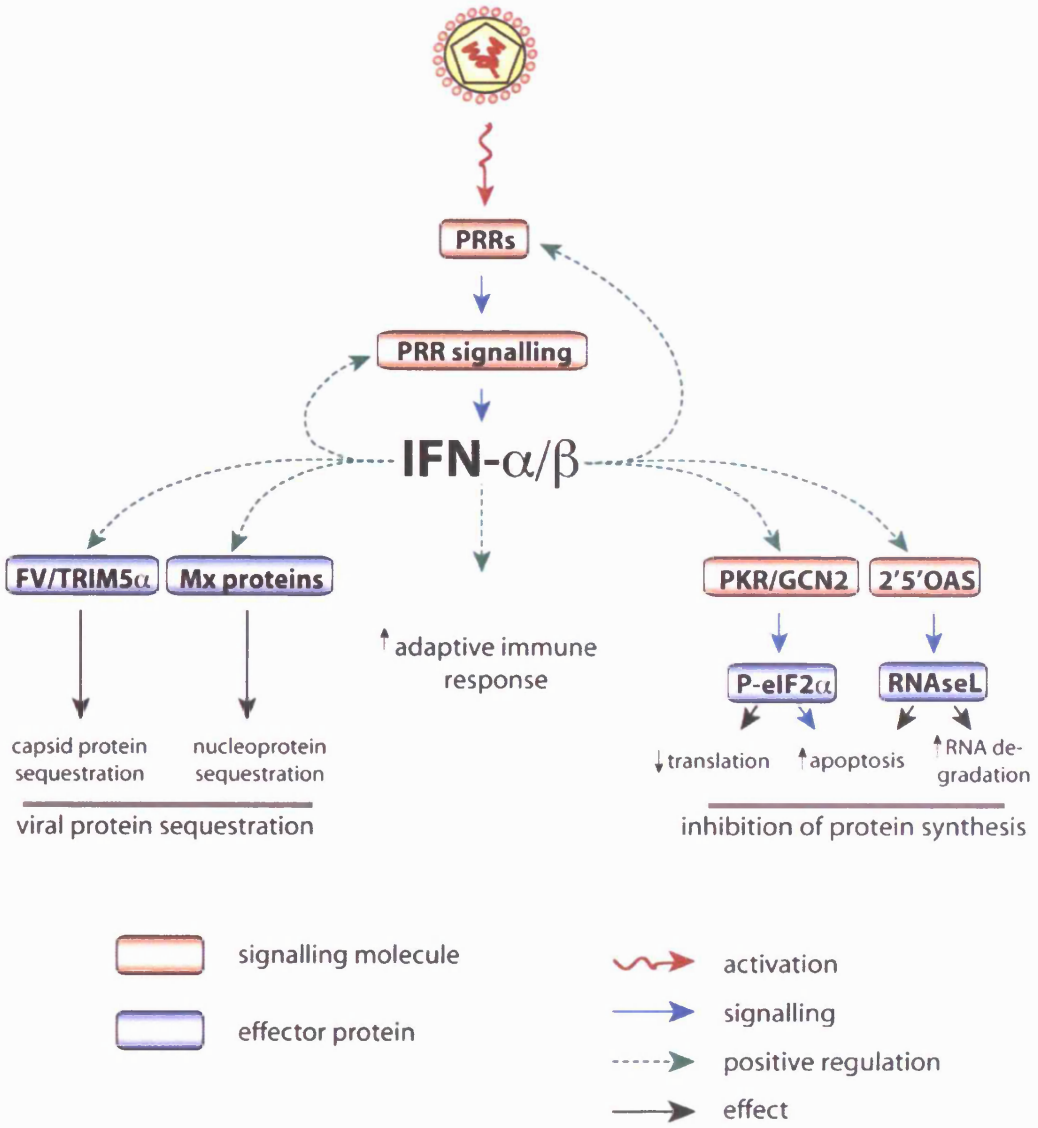


Figure 1.2.1: IFN-α/β increases expression of PRRs, signalling molecules and effector molecules

PRRs are activated by viral pathogens and initiate production of IFN-α/β. IFN-α/β increases expression of antiviral proteins like FV/TRIM5α and Mx proteins as well as the signalling molecules PKR and 2'5'OAS that modulate the activity of eIF2α and RNAse L, respectively. The latter proteins have the ability to regulate translation, apoptosis and RNA degradation, as indicated. Furthermore, IFN-α/β positively regulates expression of PRRs and signalling molecules thereby increasing sensitivity by providing a positive feedback loop. IFN-α/β contributes to shape adaptive immune responses.

Figure 1.3.4

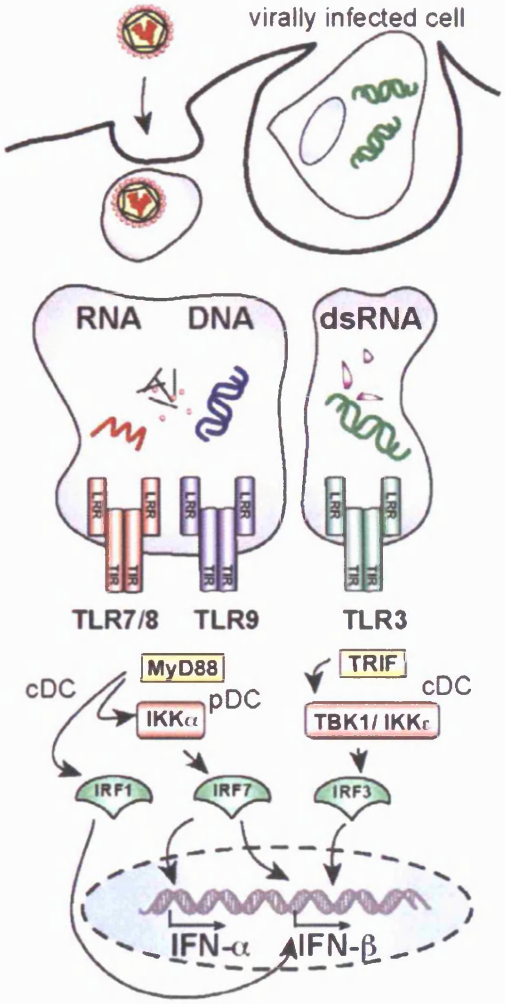


Figure 1.3.4: Endosomal TLRs recognise nucleic acid

Viral infection or engulfment for virally infected cells delivers the indicated nucleic acid into the endosomal compartment. In pDC TLR7, -8 and -9 recognise ssRNA and DNA, respectively, and activate a MyD88 and IKKα dependent signalling cascade. This leads to phosphorylation of IRF7 that translocates into the nucleus and stimulates expression of IFN-α. In cDC TLR9 activation results in signalling via IRF1 and this leads to IFN-β. TLR3 activation occurs in cDC and signalling goes via TRIF and involves TBK1 and IKKε which activate IRF3 to induce IFN-β. LRR: Lecuine rich repeat; TIR: Toll/IL1R domain

Figure 1.4.1

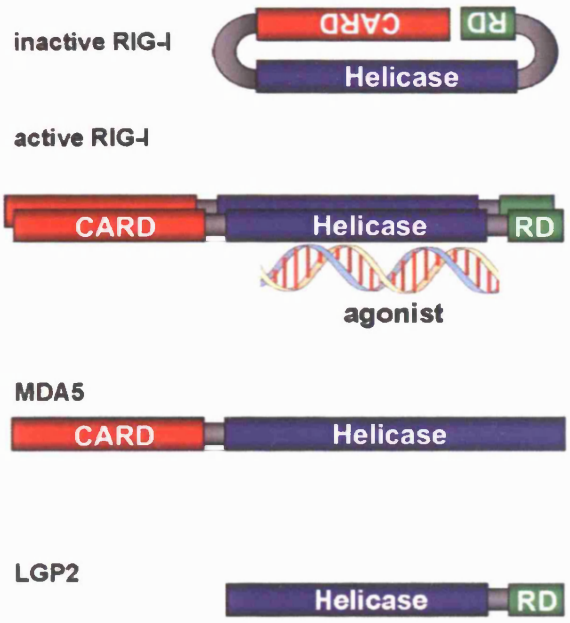


Figure 1.4.1: Schematic drawing of RIG-I Helicases

RIG-I consists of N-terminal tandem caspase activation and recruitment domains (CARD), a helicase domain and a repressor domain (RD). When inactive, the RD is believed to bind to the CARD and prevent downstream signalling. Binding of the RIG-I agonist results in a conformational change that allows homodimerisation and exposes the CARD. MDA5 also has tandem CARDs and a helicase domain but lacks the RD. LGP2 only consists of the helicase domain and a RD and lacks a CARD.

Figure 1.4.2

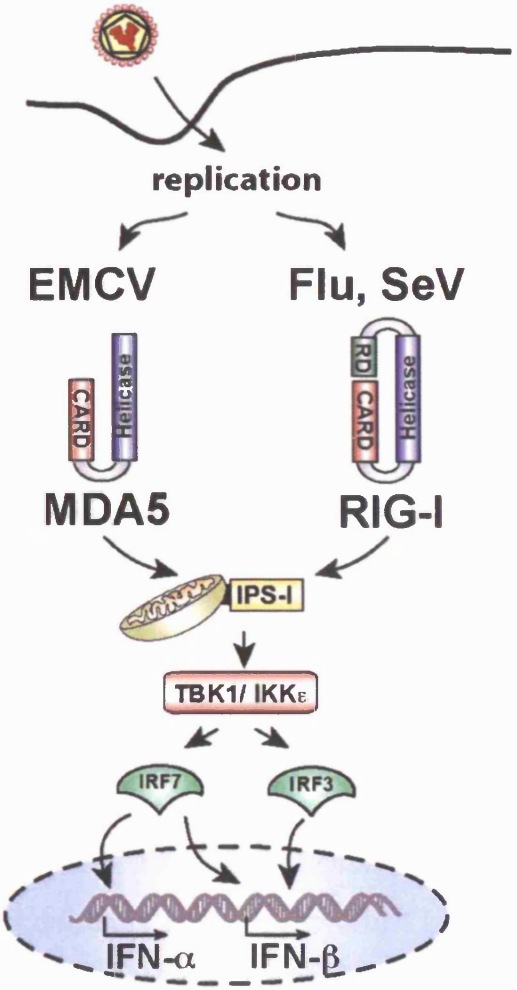


Figure 1.4.2: RIG-I and MDA5 have different virus specificity

MDA5 is activated by encephalomyocarditis virus (EMCV) whereas RIG-I is activated by viruses such as influenza (Flu) or Sendai virus (SeV). It is believed that virus replication is an essential step to activate MDA5 and RIG-I. Signalling downstream of both RNA helicases funnels through a common signalling molecule IPS-1 that activates TBK1 and IKKε. This results in phosphorylation and translocation of IRF3 and -7, which then stimulate the IFN-β and/or IFN-α promoters, respectively.

CHAPTER 2: Material and Methods

2.1. Reagents

2.1.1. Common Buffers

PBS-TC: (GIBCO-BRL, Gaithersburg, MD) for Tissue culture

PBS-EDTA-Trypsin: PBS-TC containing 2mM EDTA (Sigma, Poole, UK) and 0.2% (w/v) Trypsin (GIBCO-BRL)

RPMI 1640 medium: (GIBCO-BRL)

R10: RPMI 1640 medium supplemented with 10% (v/v) FCS, Penicillin 100U/ml, Streptomycin 100U/ml, L-Glutamine 0.3μg/ml (GIBCO-BRL), 50μM β-mercaptoethanol (GIBCO-BRL)

DMEM: (Dulbeccos Modified Eagle Medium): (GIBCO-BRL)

D10: DMEM (GIBCO-BRL) supplemented with 10% (v/v) FCS, Penicillin 100U/ml, Streptomycin 100U/ml, L-Glutamine 0.3μg/ml (GIBCO-BRL)

D5: DMEM (GIBCO-BRL) supplemented with 5% (v/v) FCS, Penicillin 100U/ml, Streptomycin 100U/ml, L-Glutamine 0.3μg/ml (GIBCO-BRL)

OptiMEM: (GIBCO-BRL)

VP-SFM: Virus production serum free medium (GIBCO-BRL) was supplemented with L-Glutamine 0.6μg/ml (GIBCO-BRL)

TC-grade agarose: (Sigma, Poole, UK) solved in TC-grade water (Sigma, Poole, UK) to give a final concentration of 4% (w/v), autoclaved

Red blood cell (RBC) lysis buffer: 155mM NH_4Cl (Sigma, Poole, UK), 10mM $KHCO_3$ (Sigma, Poole, UK), 0.1mM EDTA (Sigma, Poole, UK), MilliQ H_20, pH 7.0-7.2, sterile filtered

PBS (For analytical assays only): 8g NaCl, 0.25g KCl, 1.43g Na $2HPO_4$, 0.25g KH_2PO_4, (all from Sigma) dissolved in 1l H_2O, pH 7.2. Solution is prepared by Cancer Research UK and autoclaved before use

MACS-Buffer: PBS-TC containing 2mM EDTA (Sigma, Poole, UK) and 1% (v/v) FCS

FACS buffer: PBS, 5mM EDTA (Sigma, Poole, UK), 1% (v/v) FCS, 0.02% (w/v) NaN_3 (Sigma, Poole, UK)

PFA: 8% (w/v) PFA stock solution, pH7

ELISA buffer: PBS, 2.5% (v/v) FCS (Autogen Bioclear), 0.02% (w/v) NaN_3 (Sigma, Poole, UK)

ELISA coating buffer: 0.1M $NaHCO_3$ (Sigma, Poole, UK) in H_2O, pH 8.2

PBS-tween: 0.05% (v/v) Tween-20 (Sigma, Poole, UK) in PBS

ELISA wash: 0.05% (v/v) Tween-20 (Sigma, Poole, UK) in PBS

TAE buffer: 0.04M Tris-acetate (Sigma, Poole, UK), 0.002M EDTA (Sigma, Poole, UK)

ELISA block: PBS, 2.5% (v/v) FCS, 0.02% (w/v) NaN_3 (Sigma, Poole, UK)

ELISA coating buffer: 0.1M $NaHCO_3$ (Sigma, Poole, UK) in H_2O, pH 8.2

ELISA wash: 0.05% (v/v) Tween-20 (Sigma, Poole, UK) in PBS

TAE buffer: 0.04M Tris-Base (Sigma, Poole, UK), 2mM EDTA (Sigma, Poole, UK)

2x Laemmli buffer: 20% (v/v) Glycerine, 0.125M Tris-HCl pH 6.8, 2% (v/v) SDS, 0.02% (w/v) Bromphenol blue

2x western blot sample buffer: Laemmli buffer + 2% (v/v) β-mercaptoethanol

Western blot running buffer: 25mM Tris-Base, 250mM Glycine, 0.1% (w/v) SDS, pH 8.3

Western blot transfer buffer: 15,15g Tris-Base, 42,75g Glycine H_2O ad 4 litre, pH 8.3, add 20% (v/v) methanol were added before use

DNA extraction buffer: 100mM Tris-HCl, pH 8.5, 200mM NaCl, 5mM EDTA, 0.2% (w/v) SDS and Proteinase K 100µg/ml (Qiagen, Crawley, UK)

2.1.2. Tissue culture stimuli

CpG 1668 was synthesised by Sigma: TCCATGACGTTCCTGATGCT – all phosphorothioate linked and HPLC-purified. Loxoribine and R848 were purchased from InvivoGen (San Diego, CA). poly-I:C was from Amersham (Uppsala, Sweden). Spleen mRNA, Flu vRNA, and *in vitro* transcribed GFP RNA (transcribed by T7 or T3 polymerase, respectively) were a kind gift from Sandra Diebold. 7SK-as RNA (SP6 polymerase) was a kind gift of Choon-Ping Tan. Genomic RNA from VSV was

a kind gift of Andreas Bergthaler (University of Zurich, Switzerland). poly-U, total bovine RNA, bovine t-RNA and *E. coli* t-RNA were from Sigma. Genomic RNA of EMCV was isolated from concentrated virus particles. Briefly, 4ml of EMCV virus stock was ultracentrifuged through 1ml of a 20% (w/v) sucrose cushion in a MLS50 rotor (Becton Dickinson, Mountain View, CA) at 33.000 rpm for 2h and resuspended in 200µl PBS. vRNA was isolated using the Qiagen RNeasy RNA extraction kit according to the protocol provided by the manufacturer. siRNAs for reducing endogenous levels of human and murine RIG-I were bought from Qiagen, target sequences were ACGGATTAGCGACAAATTTAA for human RIG-I and CCGGACTTCGAACACGTTTAA for murine RIG-I.

2.1.3. Plasmids

name	features	source
pUC19	Plasmid from invitrogen	Invitrogen
pCDNA3	Cloning Vector, CMV-MCS-Neo	Invitrogen
pEGFP-C1	Cloning vector, eGFP under control of the CMV promoter	Clontech
pCAGGS	Cloning Vector, Chicken β-actin promoter upfront a multiple cloning site	Urs Schneider
pLL3.0	Lentiviral vector, siRNA expression from U6 promoter, eGFP from CMV	Luc van Paris
pLLCG	pLL3.0 without U6 promoter	self cloned
pSIN-OVA	Ovalbumin expressed from SFFV promoter in lentiviral vector	Mary Collins
pLentiLox 3.7	Lentiviral vector designed for siRNA knockdowns	Luc van Paris
pRSV-REV	REV expression vector	Luc van Paris
pRREg/pMDL	HIV-gag-pol expression vector	Luc van Paris
pVSV-G	VSV glycoprotein (Indiana strain) expression plasmid. CMV-intron-VSV-G	Invitrogen
pCAAGS-LCMV.GP (ARM)	LCMV glycoprotein (Armstrong strain) in pCAAGS	Daniel Pinschewer
pCAAGS-THOV.GP	Thogotovirus glycoprotein (Strain: SiAR129) in pCAAGS	Georg Kochs
pMLV-AMPHO	Amphotropic murine leukaemia virus envelope	Mary Collins

	protein in pFB	
pMSCVeGFP	Retroviral vector	Clontech
pLeGFP-N1	Retroviral vector expressing GFP from a CMV promoter	Clontech
pLNC-VSV-G	VSV-G replacing eGFP in pLeGFP-N1	self cloned
pCAAGS-PR8 NS1 SAM	NS1 from Influenza virus (strain PR8) in pCAAGS	Adolpho Garcia-Sastre
pCAAGS-mutNS1 (R38A, K41A)	NS1 with indicated mutations in the dsRNA binding domain in pCAAGS, no dsRNA binding	Adolpho Garcia-Sastre
peGFP-RIG-I	huRIG-I-eGFP fusion protein in peGFP-C1	Friedemann Weber
peGFP-Helicase	huRIG-I-helicase domain-eGFP fusion protein in peGFP-C1	Friedemann Weber
pCDNA-MDA5	MDA5 in pCDNA3	Friedemann Weber
pCDNA-HA-MDA5	HA-tagged hu MDA5 in pCDNA3	self cloned
p125-luc	Reporter plasmid: IFN-β promoter driving firefly luciferase	Friedemann Weber
pRL-TK	Renilla-luciferase driven by Thymidin-kinase promoter	Promega
pCMVΔR8.91	2nd Generation HIV-gag-pol packaging construct	Mary Collins

I am grateful to those who provided constructs: Luc van Parijs (MIT, Cambridge, MA, USA), Mary Collins (University College London, UK), Daniel Pinschewer (University of Zurich, Switzerland), Adolpho Garcia-Sastre (Mount Sinai, New York, USA) and Urs Schneider, Georg Kochs, Friedemann Weber (all University of Freiburg, Germany).

2.1.4. DNA oligos (Primers)

All primers listed below were purchased from Sigma.

primer name	sequence
5' VSV-G	GAAGTGCCTTTTGTACTTAG
3' VSV-G	GATCGGATGGAATGTGTTAT
5' eGFP(AP1)	GGCCACAAGTTCAGCGTGTC
3' eGFP(AP1)	TTGCCGTCCTCCTTGAAGTC

5' HIV1-gag	GCAGTTAATCCTGGCCTGTT
3' HIV1-gag	GTGGCTCCTTCTGATAATGC
5' HIV1-pol	TCAGAAGCAGGAGCCGATAG
3' HIV1-pol	TGCAGCCAATCTGAGTCAAC
5' integrated LV	GGAGCTAGAACGATTCGCAGTTA
3' integrated LV	GGTTGTAGCTGTCCCAGTATTTGTC
5' β-actin	GTTTGAGACCTTCAACACCCC
3' β-actin	GTGGCCATCTCCTGCTCGAAGTC
5' HA-huRIG-I	GACAATGTATCCTTATGATGTTCCTGATTATGCTACCACCGA GCAGCGACGCAGCCT
3' SI-huRIG-I	GACAGTCGACTCATTTGGACATTTCTGCTGGATCAAA
T7 seq primer	TAATACGACTCACTATAGGG
5' huRIG-I 1222-ds	CTGACTGCCTCGGTTGGTGT
5' huRIG-I 1839-ds	CCTCTGCTTCATCTTACAAG
5' HA-huMDA5	GACAATGTATCCTTATGATGTTCCTGATTATGCTTCGAATGG GTATTCCACAGACGAGAATTT
3' MDA5	GACACTAATCCTCATCACTAAATAAACAGCATTCTGAAT

2.1.5. Antibodies for western blot

Primary antibodies			
antibody (clone)	**isotype**	**source**	**dilution**
anti-VSV-G (P5D4)	msIgG1	Cancer Research UK	1:1000
anti-GFP (3E1)	msIgG1	Cancer Research UK	1:2000
anti-β-actin (AC-15)	ms	Sigma	1:1000
bt anti-HIVp24 (BC1071-BIOT)	ms	AALTO Bio Reagents LTD	1:1000
anti-NS1 (1A7)	msIgG2a	Jon Yewdell	IP
bt anti-NS1 (1A7)	msIgG2a	Cancer Research UK	1:1000
anti-Tubulin (TAT-1)	ms	Cancer Research UK	
HRP anti-HA (HA7)	ms	Sigma	1:5000
anti-Influenza A (H1N1) virus	gt	Europa Bioproducts Ltd.	1:1000
Secondary antibodies and reagents			
HRP anti-ms	gt	Santa Cruz Biotechnology	1:5000

HRP anti-rb	gt	Southern Biotech	1:5000
HRP-Streptavidin		Sigma	1:2500
anti-gt	ms	Jackson ImmunoResearch	1:1000

2.1.6. Antibodies and protein standards for ELISA

Step	antibody (clone)	Isotype	source	dilution
IFN-α ELISA				
Coating	anti-mIFN-α (clone F18)	rt IgG1	Hycult Biotechnologies	1:200
Detection ABs	anti-IFN-α	rb polyclonal	PBL	1:5000
	anti-rabbit biotin	ms mAB	Jackson ImmunoResearch	1:1000
IL6 ELISA				
Coating	anti-IL6 (MP5-20F3)	rt IgG1	BD Pharmingen	4µg/m
Detection AB	biotin anti-IL6 (MP5-32C11)	rt mAB	BD Pharmingen	1µg/ml
HIV p24 ELISA				
Coating	anti-HIV-1-p24 (D7320)	sheep polyclonal	AALTO BIO REAGENTS LTD	5µg/ml
Detection AB	biotin anti-HIVp24 (BC1071-BIOT)	ms mAB	AALTO BIO REAGENTS LTD	1:1000
anti-OVA serum antibodies				
Coating	Ovalbumin	protein	Calbiochem	5µg/ml
Detection AB*	biotin anti-msIgG	rt mAB	Jackson ImmunoResearch	1:1000
anti-FCS serum antibodies				
Coating	ELISA buffer	protein		2% (v/v) FCS
Detection AB*	biotin anti-msIgG	rt mAB	Jackson ImmunoResearch	1:1000

*** reagents diluted in 1% (w/v) fish skin gelatine (FSG) in PBS**

The following protein standards were used for ELISA

protein	Source	top concentration
recombinant mIFN-α	Hycult Biotechnologies	10.000 Units/ml
recombinant mIL6		5ng/ml
recombinant HIVp24	AALTO BIO REAGENTS LTD	100ng/ml

2.1.7. Antibodies and reagents used for Flow cytometry

antibody (clone)	isotype	source	dilution
anti-CD11c-PE (HL3)	Hamster IgG1	Pharmingen (Becton-Dickinson, Oxford, UK)	1:100
anti-CD11c-APC (HL3)	Hamster IgG1		
anti-CD11b-APC (M1/70)	rat IgG2b		
anti-B220 (CD45R)-TC	rat IgG2a		
anti-Ly6C (GR1)-FITC	rat IgM		
anti-CD8-TC	rat IgG2b		
anti-IFN-γ-PE	rat IgG1		
anti-CD45.1-PE	ms IgG2a		
anti-CD4-FITC (RM4-5)	rat IgG2a		
anti-VSV-G (P5D4)	mouse IgG1	Cancer Research UK	1:100
anti-mIFNα (RMMA-1)	rat polyclonal	PBL Biomedical Therapeutics	1:50
anti-mIFNα (F18)	rat IgG1	HyCult Biotechnology	1:10
unspecific	rat IgG	Jackson ImmunoResearch	
anti-ms-IgG-Alexa546	gt polyclonal	Molecular probes	1:200
YOYO-1	Nucleic acid stain	Molecular Probes	1:1000
Streptavidin-APC	Conjugate	Pharmingen	1:200
OVA/H2-K^b-PE	SIINFEKL-tetramer PE conjugated		

2.1.8. Antibodies used for virus neutralisation

antibody (clone)	isotype	source	dilution
anti-VSV-G (VA-7)	rt IgG	Andreas Bergthaler	1:25
unspecific	rt IgG	Jackson ImmunoResearch	1:25

2.1.9. Antibody used for confocal microscopy

antibody (clone)	isotype	source	dilution
anti-NS1-Cy3 (1A7)	ms IgG2a	Oliver Schulz	1:100

2.2. Cells and mice

2.2.1. Cell lines

293FT were from Invitrogen (Invitrogen, Paisley, UK), 293T and STAR-HV cells (Ikeda et al., 2003) were a kind gift from Mary Collins (University College London, UK). GP293 (retroviral packaging cells) were from Clontech (Clontech, Becton-Dickinson, Oxford, UK). 293-HV cells were generated through infection with VSV-G LV produced in STAR-HV cells and subsequent FACS sorting for GFP positive cells. NIH3T3 fibroblasts were kindly provided from Richard Treisman (Cancer Research UK, London, UK). All cell lines were grown in D10 medium, were maintained at 37°C, 5% CO_2 and split regularly to constantly grow in a logarithmic phase.

2.2.2. Mice

C57BL/6 were purchased from Charles River. *myd88*$^{-/-}$, *tlr9*$^{-/-}$, *tlr7*$^{-/-}$ mice (a kind gift from S. Akira, Osaka, Japan) *RAG2*$^{-/-}$ and B6.SJL CD45.1 mice were bred at the Biological resources facility of Cancer Research UK (Clare Hall, South Mimms, UK). All mice were used at an age of 6-10 weeks.

2.2.3. Isolation of splenocytes

Spleens were removed under aseptic conditions and collected in RPMI 1640 medium. Thereafter individual spleens were injected with 1ml of RPMI 1640 medium containing Liberase (1.67 WünschU/ml, Boehringer Mannheim) and DNAse I (0.2mg/ml, Boehringer Mannheim) and incubated at 37°C for 20-30 minutes. The digested spleens were passed through a 70µm cell strainer (Becton Dickinson, Oxford, UK), if necessary cells were released through extrusion with a syringe plunger followed by several washes with PBS-EDTA. Splenocytes were collected by spinning at 1500 rpm in a Beckman Allegra 6R centrifuge at room temperature for 5min and re-suspension in an appropriate buffer or growth medium. One spleen yields approx 10^8 cells

2.2.4. Bone Marrow isolation

Mice femurs and tibias were removed under sterile conditions and bone marrow was flushed out with R10 medium using a 23G needle syringe. The resulting cell suspension was strained through a 70µm cell sieve, subjected to red blood cell lysis and used at a concentration of $5*10^6$/ml if not indicated otherwise.

2.2.5. Red blood cell lysis (RBC-lysis)

Splenocytes or BM cells were pelleted, resuspended in 10ml RBC-lysis buffer and incubated for 1min. After adding 10ml R10 cells were again pelleted and resuspended in an appropriate volume of the medium used for further experiments (R10 if not indicated otherwise).

2.2.6. Production of GM-CSF DC

BM cells that underwent RBC-lysis were resuspended in 10ml R10, plated in a 10cm TC plate (Falcon, BectonDickinson, Oxford, UK) and incubated at 37°C for 30 minutes. Non-adherent cells were recovered into 30ml R10 supplemented with granulocyte and monocyte colony stimulating factor (GM-CSF) (dilution 1:20.000) (made by the Cancer Research UK protein purification service, batches were tested to be endotoxin free and titrated to give optimal growth conditions for BM-DCs) and equally distributed in one sixwell plate (Falcon, BectonDickinson, Oxford, UK). After 48hrs incubation at 37°C, 5% CO_2, 2.5ml of medium was removed from each well and replaced with fresh R10 supplemented with GM-CSF. Cells were incubated over night at 37°C, 5% CO_2. The next day 90% of the medium, containing nonadherent and loosely adherent cells, was removed and the remaining cells, consisting of an adherent macrophage-like population and small round DC progenitors, supplemented with 5ml of R10 containing GM-CSF. DC were either used at day 4 (evening) or at day 5.

2.2.7. Production of Flt-3L DC

BM cells that underwent RBC-lysis were resuspended in R10 to give a final concentration of $1.5*10^6$ cells/ml. This cell suspension was supplemented with Flt-3L (R&D, Minneapolis) at a final concentration of 0.5µg/ml and seeded in six wells

(5ml/ sixwell cavity). Cells were maintained at 37°C, 5% CO_2. 90% of the medium was changed at day 5 and day 8. The purity of the culture was tested at day 10: cultures contained about 30% of pDC (bearing surface markers $CD11c^{positive}$, $B220^{positive}$ and $CD11b^{low}$) and were used for further experiments.

2.2.8. MACS enrichment of $B220^{positive}$ cells

BM cells from RAG2 deficient mice were MACS separated into $B220^{positive}$ and $B220^{negative}$ fractions to enrich for pDC. Briefly, BM cells were labelled with anti-CD45R (B220) magnetic MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the protocol of the manufacturer (30µl beads per 10^7 cells, incubated 15min at room temperature, washed twice in 5ml MACS buffer) and enriched using an AutoMACS (Miltenyi Biotech, Bergisch Gladbach, Germany) (Possel program) according to the manufacturer's instructions. Enriched cells were resuspended in R10 medium and enrichment evaluated by FACS for CD11c, B220 and CD11b. Importantly, the B220 antibody used for FACS recognises a different epitope than the one used for MACS enrichment.

2.3. Viruses

2.3.1. Production of recombinant lenti- and retroviruses:

LVs were produced as follows: $6*10^6$ 293FT or 293T cells were seeded in a 10cm dish and, at the next day, transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK) as per manufacturer's instructions with pMDLg/pRRE (5µg), pRSV-REV (5µg), pLLCG or pSIN-OVA (10µg) and the plasmid encoding desired envelope protein (5µg; pVSV-G unless stated otherwise). For LVs lacking the envelope protein (LVΔenv) the Env plasmid was omitted. For LVs without viral RNA (LVΔvRNA) pLLCG was omitted. For control supernatant (SN), all plasmids were omitted.

When LV produced by retrovirus infection was compared with LV produced through transient transfection, SRAR-HV cells, that stably express GFP encoding LV particles without envelope proteins (Ikeda et al., 2003), were infected with VSV-G encoding retrovirus (see below) or transfected with pVSV-G (5µg).

16 to 24h after transfection or retroviral infection, cells were washed and fresh medium was added. Supernatant was collected at 48, 60 and 72 hours after transfection, filtered through a 0.45µm Millex-HV filter (Millipore, Carrigwohill, Ireland) and stored at -80°C.

Before use, supernatants were pooled and concentrated through a 20% (w/v) sucrose cushion using an ultracentrifuge (Becton Dickinson, Oxford, UK) (85.000 g, 1.5h in a SW28 rotor).

To produce retrovirus-encoding VSV-G three 10cm dishes containing $6x10^6$ GP293 cells were used. Each plate was transfected with pLNC-VSV-G (10µg) and pVSV-G (5µg). Supernatant was harvested, filtered through a 0.45µm Millex-HV filter (Millipore, Carrigwohill, Ireland), concentrated 10-fold by ultracentrifugation and used to infect one 10cm plate containing $6x10^6$ STAR-HV cells.

For LV fractionation, a 20 – 60% (w/v) sucrose gradient was used. Briefly, a gradient mixer was installed at a stirring plate (8000rpm/min) and the outlet tubing connected to a gradient maker. Thereafter the mixer was filled with 6.5ml 20% (v/v)

sucrose at the outlet side and 6.5ml 60% (v/v) sucrose at the non-outlet side, respectively. The gradient maker slowly (approx 1ml/min) poured the mixed sucrose into a 15ml ultracentrifugation tube (Ultraclear, Becton Dickinson, Oxford, UK). Supernatant containing $4x10^8$ infectious viruses was loaded on top of the gradient and centrifuged in a SW40 Ti rotor (Becton Dickinson, Oxford, UK) at 85.000 g, 4°C for 16 h. 1.5ml fractions were collected, diluted in PBS and centrifuged for another 1.5h (SW40 Ti, 4°C, 85.000 g). Pellets were resuspended in R10 and used for further analysis

2.3.2. Virus isolates

EMCV, SFV and phage dsRNA (BRL 5907) were a kind gift of Ian Kerr (Cancer Research UK, London, UK). Influenza virus (strain influenza A/PR/8/34) and delta NS1 influenza virus (FluΔNS1) were a gift from Thomas Muster (University of Vienna, Vienna, Austria). SeV was purchased from LGC Promochem/ATCC. Cells infected with VSV (New Jersey strain) were a kind gift of Yasu Takeuchi (University College London, UK).

2.3.3. Infections with viruses and blocking with neutralising antibody

Viruses were diluted to infect cells at the MOI indicated in the figure legends. With the exception for plaque assays on Vero cells the medium was not changed before or after infection as this commonly resulted in detachment of cells.

The neutralizing anti-VSV-G antibody (clone VA7) (Bachmann et al., 1997) was incubated with TVS preparations or CpG and used in a final titre of 1:32000 as assessed in a VSV inhibition assay. After 30min treatment, viruses were added to BM and accumulation IFN-α was tested after over night incubation.

2.3.4. Blocking infection of LV with weak bases

293FT cells were seeded at a density of $5*10^5$ cells/sixwell and pre-treated with 0mM, 10mM and 25mM ammonium chloride (NH_4Cl) for 1h. Cells were then washed with PBS and infected for 1h with the indicated viruses. Medium containing

ammonium chloride was again added for over night incubation. At 24h cells were analysed by flow cytometry.

2.3.5. Titration of lentiviruses

Lentivirus titre was determined by transduction of 293FT cells. Briefly, $5*10^5$ cells were seeded into a sixwell plate, left over night to adhere and then infected with dilutions of the desired virus. 48h later cells were analysed by FACS and the number of GFP positive cells determined. One infectious virus can transduce one 293T cell. For GFP expressing LV the virus titre was measured and is depicted as green fluorescent units/ml (gfu/ml). To determine the virus titre the following formula has been used:

$$\text{Virus titre/ml (gfu/ml)} = \frac{\text{\% GFP pos cells * number of cells infected}}{\text{100 * dilution of virus used for infection}}$$

Non-infectious virus (LVΔenv and LVΔvRNA) was quantified by ELISA for HIV p24 according to the protocol provided by the manufacturer (AALTO Bio Reagents LTD, Dublin, Ireland). Infectious virus of known gfu was used as a standard.

2.3.6. Titration of cytopathic viruses

Plaque assay

EMCV and SFV were titrated by plaque-assay on Vero cells as follows: Vero cells were seeded at a density of 2.5 to $5*10^5$ per sixwell cavity and infected for 1h at 37°C with 500µl of 10 fold dilutions of the virus stock. Thereafter the virus soup was removed and cells covered with a mixture of hot 2.5ml TC-grade agarose (Sigma, Poole, UK), 10ml pre-warmed D5 and 15ml pre-warmed DMEM medium. Agarose was allowed to polyermerise at room temperature for 15 minutes and plates were incubated until clearly visible plaques appeared (24h for EMCV and 48h for SFV). Plaques were counted and virus titre was determined as plaque-forming units/ml (pfu/ml) follows:

$$\text{Virus titre/ml (pfu/ml)} = \frac{\text{number of plaques * 2}}{\text{dilution of virus used for infection}}$$

TCID50

Influenza virus was titrated by TCID50. Vero cells were resuspended in VP-SFM and seeded at a density of 10^4 cells per cavity 96-well plate. 24h later the virus stock of interest was serially 10-fold diluted in VP-SFM containing 1µg/ml TC-grade trypsin. Addition of trypsin is necessary in tissue culture to cleave and thereby activate the hemagglutinin protein of Influenza virus. 100µl of virus dilutions were used to infect Vero cells. Infections were done in quadruplets if not indicated otherwise. 3-4 days later, when cytopathic effects were clearly visible, the cells were or were not stained with giemsa stain (Cancer Research UK) and the virus titre was determined using the following formula:

$$\text{Virus titre/ml (TCID50/ml)} = 10 * 10^{y} \qquad y = -\log(X_0) - 0.5 + \left(\frac{1}{n} * \sum X \geq 1\right)$$

n = number of wells infected per dilution
X_0 = last dilution causing cytopathic effect (cpe) in all wells
$X \geq 1$ = Sum of wells with cpe after X_0

2.4. Tissue culture techniques

2.4.1. Transfection

Lipofection was commonly used to deliver nucleic acids into cells. Fibroblasts were seeded the day before transfection to allow attachment. To prepare the transfection reaction, 5ml polystyrene tubes (Becton Dickinson, Oxford, UK) were used. The indicated amount of Lipofectamine 2000 (see table below) was incubated with OptiMEM at room temperature for 5 minutes and then pooled with nucleic acids pre-diluted in OptiMEM. The following table shows the amount of reagents and nucleic acids used:

Tissue culture vessel/ number of cells	OptiMEM + LF2000	OptiMEM + DNA	OptiMEM + RNA
96 well cavity 10^4- 2 x 10^5	50 + 0.5µl	not done	50 + 0.004 – 0.5µg
24 well cavity 5 x 10^4- 1 x 10^6	100 + 1µl	100 + 0.25 – 0.8µg	100 + 0.004 – 1µg
6 well cavity 5 x 10^5- 2 x 10^6	500 + 5µl	500 + 0.5 – 1µg	not done
10cm dish 6 x 10^6- 1 x 10^7	1500 + 30µl	1500 + 6 – 25µg	not done

The combined mixture was incubated for another 5 – 15 minutes and then added dropwise to cells. 16-72h later transfected cells were used for experiments.

2.4.2. Electroporation

Electroporation was used to introduce poly-I:C into BM cells.

BM cells were washed 2 times in 1x electroporation buffer (120mM KCl; 0.15mM $CaCl_2$; 10mM K_2HPO_4, pH 7.6; 25mM Hepes, pH 7.6; 2mM EGTA, pH 7.6; 5mM $MgCl_2$; pH adjusted with KOH). Cells were resuspended in 200µl 1x intracellular buffer containing 2mM ATP (stock solution 50mM ATP, pH 7.6, sterile filtered),

glutathione (final concentration 1.6mg/ml) and 10µg/ml poly-I:C. $2*10^7$ cells per sample were transferred into an electroporation curette (Equibio ECU-104) and pulsed with 300V / 150µF using a BioRad electroporator. Cells were transferred immediately into a 96-well plate containing growth medium. The final concentration of cells was 10^6 cells/well.

2.4.3. Reporter assays

A dual luciferase reporter assay was used to measure activity of the IFN-β promoter. The assay is based on expression of two enzymes that have different substrate specificity and can be measured separately. One enzyme (firefly luciferase) is under control of the promoter of interest (IFN-β promoter) whereas the other enzyme (renilla luciferase) is controlled by a constitutively active promoter (Thymidine kinase promoter). The ratio of both activities gives clues on the activation status of the promoter of interest.

Briefly, $2*10^5$ 293HEK cells or $5*10^4$ 3T3 cells were seeded per cavity 24-well plate. After over night incubation, cells were transfected as described above with 250ng of the reporter plasmid p125-luc (IFN-β promoter driving firefly luciferase) and 25ng the control plasmid pRL-TK (Thymidine kinase promoter driving renilla luciferase). Where necessary, siRNAs for RIG-I (final concentration 100nM) or 500ng of pCAAGS-NS1 and pCAAGS-mutNS1, respectively, or plasmids encoding dominant negative RIG-I or empty vector were co-transfected. Cells were incubated for 16-72h and were mock treated, infected with viruses or transfected with RNAs using Lipofectamine 2000. Luciferase activity was measured 14-16h later using the Dual-luciferase Reporter assay system (Promega, Southampton, UK). For that, cells were lysed in 100µl 1x Passive lysis buffer (Promega, Southampton, UK) and incubated at room temperature for 15min. 20µl of cell lysate was pipetted into a 96-well plate and analysed in an Envision 96-welll plate Luminometer (Promega, Southampton, UK). First, 50µl of firefly luciferase substrate was added to measure the activity of firefly luciferase (equivalent to IFN-β reporter activity), then renilla values were determined by adding 50µl stop and glow substrate. Results are shown as fold induction of the ratio between firefly activity and renilla activity as compared to mock-treated control samples

2.5. Molecular Biology

2.5.1. RNA extraction and reverse transcription

RNA extraction using the RNeasy mini kit (Qiagen, Crawley, UK) and an on-column DNAse-I (Qiagen, Crawley, UK) digestion was performed according to the instructions given by the manufacturer. Where necessary RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Briefly, 20µl extracted RNA was incubated with 10µM random hexamer primers (Invitrogen, Paisley, UK) at 65°C for 5 minutes. The mixture was put on ice for 2min and 18µl of a master-mix containing 2µl 10mM dNTP (Promega, Southampton, UK), 8µl 5x first strand buffer (part of SuperScript II kit, Invitrogen, Paisley, UK), 4µl of 0.1M DTT (part of SuperScript II kit, Invitrogen, Paisley, UK), 3.5µl of H_2O and 0.5µl SuperScript II was added to each tube. The mixture was incubated in a PCR machine at 42°C for 1h followed by a denaturation step at 70°C for 15 minutes.

2.5.2. Dephosphorylation of RNA

For some experiments purified RNAs were subjected to mock or 5'-dephosphorylation using calf intestinal phosphatase (CIP)(New England Biolabs, Ipswich, UK). 2-10µg of RNA was incubated in a total volume of 100µl of NEBuffer 3 (1x) in the absence (NoCIP treated) or presence (CIP treated) of 20 units of CIP. The incubation was carried out at 37°C for 3-6h. Thereafter the enzyme was removed by phenol/chloroform extraction followed by RNA precipitation with sodium acetate/ethanol or by using the RNeasy RNA extraction kit.

2.5.3. DNA extraction from cells and virus preparations

LV preparations (35µl) and cells pelleted by centrifugation (10^6 cells), respectively, were resuspended in 500µl DNA extraction buffer and incubated at 55°C in a shaking heating block for 2h (LV preparations) or 16h (cells). 500µl of Isopropanol was added and the mixture incubated for 30min on dry ice. DNA was pelleted by centrifugation for 30min at 13.000rpm, 4°C, in an eppendorf benchtop centrifuge and

washed once with 500µl 70% (v/v) EtOH. The resulting DNA pellet was air-dried and resuspended in 20µl (LV) or 100µl (cells) TE-buffer.

2.5.4. Polymerase chain reaction (PCR)

PCR reactions were performed using Taq polymerase (Cancer Research UK) or, for cloning purposes, PWO polymerase (Roche, Mannheim). 10x PCR buffer (PWO polymerase kit, Roche, Mannheim) and dNTPs (Promega, Southampton, UK) were used as follows:

	PCR	cloning-PCR
dNTPs (10mM)	0.75 µl	1.25 µl
FW-primer (10µM)	0.3µl	0.5µl
RV-primer (10µM)	0.3µl	0.5µl
Buffer 10x	3µl	5µl
Polymerase	Taq 0.2µl	PWO 0.5µl
Template	≈ 200ng	≈ 200ng
H_2O	ad 30µl	ad 50µl

DNA and cDNA were amplified using the following program:

Step nr	Description	Temperature	Duration
1	Denaturing	96°C	2min
2	Denaturing	96°C	30s
3	Annealing	55°C	30s
4	Extension	60°C	2min
5	Return to step 2, 29 times		
6	Cool	4°C	Forever

PCR reactions were analysed on a 1% (w/v) agarose gel.

2.5.5. Sequencing of plasmids

DNA sequencing was done at the Cancer Research UK sequencing facility. The sequencing reaction mix contained 8µl of BigDye Terminator mix v3.1 (provided by

Cancer Research UK), the sequencing primer (equivalent of 3.2pmol), 150-300ng of plasmid and H_2O ad 20µl.

Following program was used for the PCR reaction:

Step nr	Description	Temperature	Duration
1	Denaturing	96°C	1min
2	Denaturing	96°C	30s
3	Annealing	50°C	15s
4	Extension	60°C	4min
5	Return to step 2, 24 times		
6	Cool	4°C	Forever

DNA was precipitated by adding 2µl 125mM EDTA, 2µl 3M NaAC and 50µl EtOH (100%) and spinning at 2500 rpm in an eppendorf benchtop centrifuge for 20min. After washing once with 100µl 70% (v/v) EtOH the pellet was dried and handed over to the sequencing facility.

2.5.6. Cloning of plasmids

To generate a lentiviral vector coding for eGFP under the control of a CMV promoter (pLLCG), the XbaI – XhoI fragment of pLentiLox 3.7, containing the U6 promoter, was excised and circularised using the quick ligation kit (Qiagen, Crawley, UK).

pLNC-VSV-G, a retroviral vector expressing VSV-G, was generated by replacing the eGFP gene in pLeGFP-N1 with VSV-G. Briefly, pLeGFP-N1 was cut with ClaI and HindIII. The backbone fragment was circularised after blunting the overhangs using Klenow fill-in. The resulting plasmid was cut by Bgl-II, dephosphorylated and ligated to the VSV-G encoding BamHI fragment of pVSV-G.

HA-MDA5 and HA-RIG-I were cloned from cDNA of human 293T cells (HA-MDA5) or peGFP-RIG-I (HA-RIG-I) and ligated into pCDNA3 using TOPO-cloning.

2.5.7. *Restriction Enzyme digests*

For cloning purposes or to test the integrity of plasmids, restriction digests were performed. Restriction enzymes and buffers were purchased from New England Biolabs (NEB, Ipswich, UK) and used according to the manufacturer's instructions. Briefly, 0.5-3µg of plasmid was added to a mix containing 2-10 Units of enzyme in the appropriate buffer. The restriction reaction commonly had a final volume of 20µl (0.5µg DNA) for test digestions or 80µl (3µg DNA) for cloning purposes and was incubated at 37°C for 3h.

2.5.8. *Gel Electrophoresis*

TAE-Buffer was supplemented with 1% (w/v) agarose and melted by heating in a microwave oven. 1µg/ml ethidium bromide (Sigma, Poole, UK) was added and the solution was poured into a gel tray equipped with an appropriate comb. After polymerisation of the gel, the tray was transferred into a running chamber (BioRad, Hemel Hempstead, UK) containing TAE buffer and DNA, supplemented with DNA loading Buffer (Sigma, Poole, UK) to a final 1x concentration, was loaded into the slots. Gels were commonly run with a current of 100-120 for 1h.

When staining agarose gels with acridine orange 1% (w/v) agarose gels (without ethidium bromide) were run as described above. Staining of nucleic acid was accomplished using 30µg/ml Acridine orange in a final volume of 150ml 1% (w/v) $NaHCO_3$ for 15 min. Thereafter the gel was de-stained by rinsing in hot tap water for 2h. Nucleic acid was visualised on a UV transilluminator and a picture taken using a Dimage Xt digital camera (Minolta, Tokyo, Japan). The blue channel, showing the UV bulbs of the transilluminator, was removed electronically using Adobe Photoshop.

2.5.9. *Extraction of DNA from agarose-gels*

After size-separation of the DNA by agarose-gel electrophoresis, the DNA fragment of the appropriate size was excised using a sterile scalpel. DNA was purified from the gel slice using the Qiagen gel extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. DNA was eluted from the column in 20µl TE buffer.

2.5.10. Klenow fill-in

Overhangs after restriction digests were filled up by using the large subunit of DNA polymerase-I (Klenow) (NEB, Ipswich, UK). For that, DNA fragments were resuspended in a buffer containing a final concentration of 1x NEBuffer 2 and 33µM dNTPs (Promega, Southampton, UK). 5 units of DNA polymerase-I were added and the mixture incubated for 15min at 25°C, followed by a denaturation step at 72°C for 20min.

2.5.11. Dephosphorylation of DNA

In order to remove phosphates on the 5' terminus, 1µg DNA was diluted in a total volume of 18µl of 1x shrimp alkaline phosphatase buffer and incubated with 2µl of shrimp alkaline phosphatase (NEB, Ipswich, UK) for 1h at 37°C. To inactivate the enzyme the mixture was heated to 65°C for 15min and the DNA was directly used for further cloning applications.

2.5.12. Ligation of DNA fragments

DNA fragments were ligated using the QuiaQuick ligation kit (NEB, Ipswich, UK) according to the protocol of the manufacturer.

2.5.13. TOPO-cloning

TOPO-cloning was performed using the pcDNA3.1/V5-His©TOPO®TA cloning kit (Invitrogen, Paisley, UK) according to instructions provided by the manufacturer. Briefly, PCR reactions or blunted DNA fragments were separated by size on an agarose gel and the DNA fragment corresponding to the size of the gene of interest was excised from the agarose gel with a sterile scalpel. DNA was released from the gel slice using the Gel extraction kit (Qiagen, Crawley, UK) according to the instructions of the manufacturer. Appropriate amounts of 10 x PCR Buffer, dATP (to a final concentration of 0.2nM) and 0.5µl Taq polymerase (Cancer Research UK) were added and the mixture incubated at 72°C for 15min. 2µl of the resulting PCR-fragment containing adenine overhangs was added to 3µl of the T/A cloning buffer, incubated with 0.5µl of the T/A cloning vector for 5min and transformed into competent bacteria.

2.5.14. Transformation of competent E. Coli

1-10µl of plasmid DNA solution was added to 20-50µl of ice-cold DH5α (Top10 one shot) competent cells (Invitrogen, Paisley, UK) in a 1.5ml eppendorf tube and incubated on ice for 30-60min. The bacteria were heat-shocked by transferring the tube into a 42°C heating block for 60s and then put on ice for an additional 2min. 200µl SOC medium (Invitrogen, Paisley, UK) was added and the suspension incubated in a heating block at 37°C for 30-60 minutes under constant shaking (500 rpm). Bacteria were plated on LB-agar plates containing an appropriate antibiotic for selection (Ampicillin or Kanamycin, both 50µg/ml). Plates were incubated over night at 37°C.

2.5.15. Plasmid isolation from bacteria

Single resistant clones were isolated from LB-agar plates using sterile pipette-tips and incubated in 2ml LB containing appropriate antibiotics (Ampicillin or Kanamycin at 50µg/ml) over night at 37°C on a shaker. The cells were pelleted by spinning 5min at 13000rpm in an eppendorf benchtop centrifuge and plasmids were isolated from the resulting bacteria pellet using the Qiagen plasmid mini-kit (Qiagen, Crawley, UK), according to the protocol provided by the manufacturer.

For large-scale plasmid isolation 500µl of the over night culture was added to 150ml of LB containing appropriate antibiotics (Ampicillin or Kanamycin at 50µg/ml) and incubated over night at 37°C on a shaker. Over night cultures were centrifuged at 3500rpm for 30min (Allegra 6R centrifuge, Beckman-Coulter), supernatants were discarded and plasmids were isolated using the PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, Paisley, UK) following the instructions given by the manufacturer.

2.6. Protein biochemistry

2.6.1. Western Blot

A western blot is a method to detect a specific protein in a given sample. It uses gel electrophoresis to separate proteins by size. The proteins are then transferred to a membrane, where they can be detected using antibodies specific to the target protein. For western blotting, cells were lysed in 1x passive lysis buffer (Promega, Southampton, UK) for a minimum of 10 minutes on ice. Lysates were spun in an eppendorf benchtop centrifuge at full speed for 5 min.

Concentrated LV preparations, cell lysates or immunoprecipitated proteins were mixed 1:1 with western blot sample buffer and denatured at 94°C for 5min. In the meantime a 10% Tris-glycine pre-cast mini-gel (Novex, Invitrogen, Paisley, UK) was assembled in a NOVEX running chamber filled with western blot running buffer. 20µl per sample were loaded in each gel slot and proteins separated at a currency of 125 volts for 1-1.5h. Proteins were blotted onto an Immobilon P PVDF membrane (Millipore, Carrigwohill, Ireland) using a wet transfer method. For that, the gel was transferred into a gel holder cassette (BioRad, Hemel Hempstead, UK) and fitted with a methanol activated Immobilon P PVDF membrane and whatman filter paper (Millipore, Carrigwohill, Ireland) according to the protocol provided by BioRad. The cassette was then assembled in a Mini Trans-Blot cell (BioRad, Hemel Hempstead, UK) filled with western blot transfer buffer and a Bio-Ice cooling unit (BioRad, Hemel Hempstead, UK). Proteins were blotted for 2-3h at a maximum of 0.35 ampere.

The membrane was blocked in 5% (w/v) BSA or 5% (w/v) dried skimmed milk powder in PBS-tween for 0.5 to 16h at 4°C.

Primary antibodies were diluted in 1% (w/v) dried skimmed milk powder in PBS-tween and incubated with the membrane for 1-16h on a shaker. The membrane was washed 3-5 times in PBS-tween (5 minutes each wash). Then the secondary reagent, diluted in 1% (w/v) dried skimmed milk powder in PBS-tween, was added for another hour. 3-5 thorough washes in PBS-tween removed unbound antibodies and

the streptavidin-conjugate, respectively. HRP was detected using the Supersignal WestPico Chemiluminsecent Substrate (Pierce, Perbio, Rockford, IL). The membrane was wrapped in cling film and used to expose X-ray films (Amersham, Uppsala, Sweden) for 5s to over night. X-ray films were developed in an X-ray film developer.

2.6.2. Immunoprecipitation

For immunoprecipitation, 1-6*10^6 293T cells were lysed in PBS containing 1% (v/v) Triton, complete® protease inhibitor cocktail (Roche, Mannheim, Germany), orthovanadate 1mM, NaF 5mM and sodium orthophosphate 1mM (all Sigma). Lysates were spun in an eppendorf benchtop centrifuge at full speed for 5min and the supernatant incubated with GammaBind streptavidin sepharose (Amersham, Uppsala, Sweden) (50µl beads per sample) at 4°C for 1 hour to precipitate unspecifically binding proteins. Meanwhile, GammaBind streptavidin sepharose (50µl beads per sample) was coated with antiNS1 antibody (10µg/sample) for 1h at room temperature on an inverting wheel. Lysates were spun at low speed (1000 rpm) for 5min and incubated with NS1-loaded GammaBind sepharose on an inverting wheel for 3 hours at 4°C. Samples were washed five times in ice cold lysis buffer diluted 1:5 in PBS. Proteins attached to the beads were extracted using 20µl sample buffer and denaturation at 94°C and samples were subjected to western blotting.

2.6.3. Biotinylation of NS1 antibodies

Purified NS1 AB was incubated with biotin (final concentration: 1mg/ml, final volume 500µl) for 3min, thereafter injected into a pierce dialysis chamber (molecular cut off: 30.000 Dalton) and dialysed against 3 litres of PBS for 2 days at 4°C. During this period PBS was exchanged twice.

2.6.4. Enzyme-Linked Immuno Sorbent Assay (ELISA)

ELISA is a method to quantify the concentration of a protein of interest in solution. All ELISAs were carried out in MAXISORP 96-well immunoplates (Nunc, Roskilde, Denmark) and all incubation steps were done in a humidified chamber. Capture antibodies or capture proteins were diluted in ELISA coating buffer and

50µl added to each well. Plates were covered with flexi-lids (Falcon, BectonDickinson, Oxford, UK) and incubated over night at 4°C. Coated plates were then washed 3 times with ELISA wash buffer and blocked at 4°C for 1-16h by addition of 200µl ELISA buffer (for cytokine ELISAs) or 200µl 1% (w/v) fish skin gelatine (FSG, for detection of serum antibodies). This step is essential to reduce unspecific binding of proteins during the procedure. To detect FCS-specific antibodies, ELISA buffer was used for coating. Coated ELISA plates were either used directly or stored at minus 80°C until use. The ELISA buffer was discarded and 50µl of serial dilutions of a protein standard and experimental samples (cell supernatants or diluted mouse serum) were added per well. Plates were covered with flexi-lids and incubated for 2h at RT on a shaker or over night at 4°C. After 4 washes with ELISA wash buffer, 50µl per well of the detection antibody (diluent: ELISA-buffer for cytokines and proteins, 1% (w/v) fish skin gelatine in PBS for serum-antibodies) was added to the ELISA plate. This step (4 washes, 1h incubation with antibody) was repeated for all staining steps during the ELISA procedure.

For detection of biotinlyated antibodies, streptavidin-alkaline phosphatase conjugate (ExtrAvidin-AP, Sigma) was used at a dilution of 1:5000 in ELISA buffer (detection of cytokines and HIVp24) or 1% (w/v) FSG in PBS (detection of serum-antibodies). After streptavidin-alkaline phosphatase incubation for 1h, the plates were washed 6 times with ELISA wash buffer and 100µl of ELISA substrate (Sigma-fast p-Nitrophenyl Phosphate, Sigma) was added per well. A SpectraMax 190 ELISA reader (Molecular Devices, Wokingham, UK) was used to read the absorbance at 405nm after appropriate developing time (10min to over night). To analyse the data, SoftMax® Pro software (Molecular Devices, Wokingham, UK) and Excel (Microsoft) were used. Graphs show the average of measurements ± standard deviation of triplicate samples. Only data within the range of the experimental standard curve were used. The IFN-α/β ELISA used in this thesis has a detection limit of 30U IFN-α/ml.

2.7. Flow cytometry and FACS

Flow cytometry is a powerful technique for examining and sorting microscopic particles suspended in a stream of fluid. It allows analysis of single cells on multiple physical parameters (defined by size and granularity) and chemical parameters (defined by fluorescent markers). Fluorescent activated cell sorting (FACS) is a specialised type of flow cytometry that allows isolation of homogenous cells from a heterogeneous population.

2.7.1. Surface staining of DC

To prepare cell suspensions for flow cytometry, cells were washed twice with pre-cooled FACS buffer. The antibody or antibody-combination of interest was diluted in 50µl/sample FACS buffer and used to stain the cells on ice for 30min. This procedure (2 washes and incubation on ice for 30min) was repeated when secondary reagents were used. After the last staining step cells were washed in FACS buffer and data acquired using a FACS Calibur cytometer (Becton Dickinson, Mountain View, CA). Data was analysed using FlowJo software (Treestar, San Carlos, CA).

2.7.2. Intracellular stain for IFN-α in BM

BM cells were left untreated or treated with LV preparations at an MOI of 0.2. After 3h incubation at 37°C, 10% CO_2, Brefeldin A (Sigma, Poole, UK) was added at a final concentration of 5µg/ml and cells incubated for another 3h. Thereafter cells were harvested, washed once in PBS and fixed in 4% (w/v) PFA for 15min. After fixation, the cells were washed twice in PBS and left in ELISA buffer over night at 4°C. All following steps were performed using FACS buffer containing 0.1% (w/v) saponin (Sigma, Poole, UK). Cells were washed once and resuspended in the primary antibody solution containing a mixture of rat anti-IFN-α antibodies (clone F18 (1:10) and RMMA-1 (1:50)). After 1h incubation on ice, the primary antibodies were washed away thoroughly (2 washes), the secondary antibody (biotinylated ms anti-rat-IgG 1:100) added and incubated for another hour. The last staining step consisted of CD11c, Ly6C, B220 antibodies and Streptavidin-PE conjugate. After 1h

staining on ice, the cells were washed twice in FACS buffer and analysed in a FACS Calibur cytometer.

2.7.3. Intracellular stain for IFN-γ in restimulated CD4 T-cells

Splenocytes were restimulated over night with or without 10% (v/v) FCS and Brefeldin A (5µg/ml) was added for another three hours. This was followed by two washes in PBS and a fixation step with 4% (w/v) PFA for 15min. After three more washes with PBS, cells were resuspended in ELISA buffer for over night incubation at 4°C. Cells were then washed with FACS buffer containing 0.1% (w/v) saponin and incubated for 1h with a FITC conjugated CD4 and a PE conjugated IFN-γ antibody (each 1:100 diluted). Cells were washed twice in FACS buffer and data acquired with a FACS Calibur cytometer.

2.7.4. Flow cytometric analysis of TVS

TVS could be detected as population with distinct forward- and sideward scatter blot properties. To analyse TVS, forward- and sideward scatter axes had to be set to a logarithmic scale and data needed to be collected with low acquisition speed.

For staining TVS with antibodies and subsequent FACS, TVS preparations were stained with anti-VSV-G (clone P5D4) or a control ms IgG1 (each 10µg/ml) and then with an Alexa546 conjugated anti-mouse-IgG antibody (1:200). Each staining step (30 min, 4°C, dark) was followed by a wash with 5ml PBS in a MLS50 rotor in a benchtop ultracentrifuge (Becton Dickinson, Oxford, UK), 33.000 rpm, 4°C, 30 min. Pellets were resuspended in PBS and analysed using a FACS calibur cytometer.

To stain nucleic acid for flow cytometric analysis LV and TVS preparations were fixed in 4% (w/v) PFA and stained with YOYO-1 (dilution 1:1000) for 5min to visualise nucleic acid. YOYO-1 is a dimeric cyanine nucleic acid stain that intercalates into the backbone of nucleic acid and only then gains fluorescent activity and emits light at 509nm when excited at 491nm (Molecular probes, Poort Gebouw, The Netherlands) (Rye et al., 1992). Intercalated YOYO-1 can be detected in FL1 of the FACS calibur cytometer.

2.7.5. FACS sorting of cells

To generate a homogenous population of cells expressing the lentiviral genome (293-HV cells), 293T cells were infected with LV produced in STAR-HV cells and GFP positive cells sorted 3 days later. Briefly, infected cells were trypsinised, washed twice in PBS-TC containing 2% (v/v) FCS and filtered through a 0,45µm cell strainer. Cells were then sorted for GFP expression using a FACS Aria (Becton Dickinson, Mountain View, CA) by the Cancer Research UK FACS facility.

2.8. Confocal and electron microscopy imaging

2.8.1. Confocal microscopy

HEK293 cells were seeded on 13mm diameter coverslips at a density of $4x10^5$ cells/sixwell plate and transfected with GFP-RIG-I the next day. 24h later cells were infected with wild type influenza virus. After another 16h cells were fixed in 4% (w/v) PFA, permeabilised with 0.1% (v/v) Triton X-100 and stained with a 1:100 diluted Cy3-conjugated anti-NS1 antibody (clone 1A7, kindly provided by Oliver Schulz). After three washes in PBS, cover slips were mounted with Fluoromount (Southern Biotech, Birmingham, AL, USA) and images were taken using a laser scanning confocal microscope (LSM 510, Zeiss, Jena, Germany).

2.8.2. Electron microscopy

Electron microscopy was carried out with a lot of help from Steven Gschmeissner, Cancer Research UK Electron microscopy facility.

Cells were fixed in 4% (v/v) glutaraldehyde for 1h, before being processed for routine araldite processing and sectioning by the Electron microscopy unit of Cancer Research UK. Sections were placed on 200 mesh nickel grids and post stained in lead citrate and uranyl acetate. For negative staining 20µl concentrated supernatants were fixed with 20µl 8% (v/v) glutaraldehyde. 10µl of this mixture was spread on glow discharged carbon/formvar 400 mesh nickel grids and allowed to settle in a moist chamber for a few minutes. Grids were washed twice in distilled water and stained with 1 drop aqueous 1% uranyl acetate. Specimens were examined using a JEOL 1010 (JEOL Ltd., Tokyo, Japan) electron microscope.

2.9. *In vivo* applications

2.9.1. Immunisation

Immunisation with LV

Mice were immunised with concentrated OVA-expressing lentivirus or egg white equivalent to 250µg ovalbumin supplemented with PBS, CpG (25µg) or poly-I:C (50µg). A total volume of 200µl was injected into each mouse.

Immunisation with TVS

Mice were immunised intraperitoneally with egg white equivalent to 250µg ovalbumin, in PBS alone, mixed with 320µl VSV-G-TVS, mixed with CpG (25µg) or mixed with poly-I:C (50µg).

2.9.2. Staining for tetramer positive T-cells

A tetramer assay is used to detect the presence of antigen specific T-cells. A tetramer consisting of 4 MHC molecules with its bound peptide will only bind the T-cells that bear the T-cell receptor specific for the particular tetramer.
1 week after immunisation, blood was analysed for the presence of OVA/H2-K^b tetramer positive T-cells. For that, 100-300µl blood was obtained from the tail vein and collected into a tube containing heparin, which prevents coagulation. Whole blood was then stained for 30min with PE conjugated SIINFEKL-tetramer (10µl per sample) and a TC conjugated CD8 antibody. Thereafter, cells were washed in FACS buffer and data was acquired in a FACS Calibur cytometer.

2.9.3. In vivo killing assay

Activation of adaptive immune responses can be monitored by assessing the ability of cytotoxic T-lymphocytes (CTL) to specifically eliminate target cells that present a peptide of the immunogen on their class I MHC. In case of TVS and LV immunisation, 10-12 days after immunisation an *in vivo* target killing assay was performed to test the induction of OVA specific immune responses. Briefly,

splenocytes from mice carrying a congenic marker (C57BL/6SJL-CD45.1) were isolated and incubated with different amounts (0, 20 and 200nM) of SIINFEKL peptide (Cancer Research UK peptide synthesis service) in RPMI 1640 medium at 37°C for 1h. Cells were then washed twice in PBS and then resuspended in 2ml PBS supplemented with different amounts of Carboxyflourescein (CFSE) (2.5μM CFSE, unloaded cells; 0.25μM CFSE, 200nM peptide loaded cells and 0.025μM CFSE, 20nM peptide) and incubated for 10min in a water bath at 37°C. Aliquots were washed three times in PBS, pooled and passed through a 40μm strainer (Becton Dickinson, Oxford, UK). 200μl cell suspension (containing approximately 10^7 cells) was injected intravenously into each mouse. 24h later the mice were sacrificed, splenocytes and blood collected.

For the *in vivo* killing assay $1/20^{th}$ of every spleen was used to stain with a Tri-colour conjugated anti-CD8 AB (1:100) and incubated for 30min on ice. Cells were washed in FACS buffer and data acquired with a FACS Calibur cytometer.

2.9.4. In vitro re-stimulation of T-cells

For CD4 T-cell responses, isolated splenocytes were incubated over night in RPMI 1640 medium in the presence or absence of 10% (v/v) FCS. Brefeldin A was added for the last 3 hours and cells stained for intracellular IFN-γ as described above.

2.9.5. Serum isolation and antibody responses

Whole blood isolated from 10-12 days immunised mice was incubated at room temperature for 2 hours, which resulted in blood coagulation. Blood was then spun at max speed in an eppendorf benchtop centrifuge for 2min and serum that accumulated in the top layer was carefully removed. ELISA for serum antibodies was performed as described above.

CHAPTER 3: Lentiviral vector preparations activate dendritic cells via Toll-like receptor 9

3.1. Introduction

3.1.1. Virus infection routes

Viruses are intracellular parasites and rely on living cells in order to replicate. Each virus only has a single chance to infect a cell, and, consequently, the virus particle has to enter the living cell at the first contact. Whether or not this infection is successful relies on proteins present on the virus surface. In fact, there are two basic mechanisms that guarantee a virus to infect the right target:

Some viruses bear surface proteins that have to co-engage multiple receptors on the host cell in order to be activated. Upon receptor binding, fusogenic domains of these surface proteins are exposed, leading to penetration of the host plasma membrane and delivery of the virus core into the cytoplasm. Examples of viruses using this route are members of the retro- and paramyxovirus family (Smith and Helenius, 2004; Stein et al., 1987). The best-understood and most prominent example for this kind of infection is the human immunodeficiency virus -1 (HIV-1). HIV-1 has a surface protein named gp120 that co-engages CD4 and the co-receptor CCR5 and CXCR4, respectively. Only binding of receptor and co-receptor leads to release of a fusion protein gp41 that inserts into the plasma membrane of the cell and mediates delivery of the virus capsid into the cellular cytoplasm (Colman and Lawrence, 2003). However, most viruses infect cells by a different mechanism, i.e. through the endocytic pathway. Endosomal acidification activates virus surface proteins and leads to delivery of the virus core into the cytoplasm (Colman and Lawrence, 2003). This mechanism guarantees infection of a metabolically active cell. The pH–dependent fusogenic properties of the virus surface proteins can easily be tested *in vitro* by using weak bases that inhibit acidification of the endosome and thereby virus delivery into the cytoplasm (Smith and Helenius, 2004).

3.1.2. *TLRs sense nucleic acid in the endosome*

TLR3, -7, -8 and -9 are activated by viral nucleic acid and therefore came into focus of virologists and immunologists (Akira et al., 2006; Bowie, 2007). These TLRs are present in endosomal compartments of immune cells and have the ability to recognise nucleic acid delivered into this cellular compartment.

Single stranded RNA and DNA viruses that infect cells via the endosome trigger TLR7 and -9, respectively. This leads to IFN-α production in plasmacytoid DC (pDC). It is believed that incoming viruses activate endosomal TLRs. However, how the virus entry route affects activation of TLR7 and -9 is not entirely understood (Fig 3.1.1). Direct comparison between viruses can be complicated as some viruses may differ in their stimulatory activity, regardless of their entry route and others may actively suppress TLR activation or signalling. The HIV-1 surface protein gp120, for instance, can selectively inhibit TLR9 dependent responses through an unknown mechanism (Martinelli et al., 2007). Hepatitis C virus NS3/4A protease cleaves TRIF (Li et al., 2005) and therefore potentially interferes with TLR3 dependent responses. Finally, the porcine circovirus can inhibit activation of TLR9 (Vincent et al., 2007). Therefore, more standardised models are required to understand the relationship between virus entry and TLR activation. One way to proceed is to compare very similar virus particles that only differ in their entry routes.

3.1.3. *Lentiviral vectors as tools for innate immunity*

Lentiviral vectors (LVs) are promising agents for genetic therapy of human diseases (Cockrell and Kafri, 2003; Galimi and Verma, 2002; Wiznerowicz and Trono, 2005). They have several advantages over other virus-based vectors, which include integration of the transgene into the genome and the lack of pre-existing immunity against the viral vector.

Remarkably, little is known about innate immune responses to recombinant LVs. Recombinant LVs are replication-incompetent and intracellular sensing mechanisms by means of dsRNA helicases are therefore unlikely to contribute to their immunogenic potential. The most likely stimuli for inducing innate and adaptive immunity to LVs are the nucleic acids carried by the virions, including the ssRNA viral genome or short DNA transcripts that are produced after maturation of the

virion (Trono, 1992). It has been reported that HIV-1 can stimulate IFN-α production in human pDC most probably by triggering TLR7 (Beignon et al., 2005; Fonteneau et al., 2004; Schmidt et al., 2005; Yonezawa et al., 2003).

Recombinant lentiviruses are produced by transient transfection of producer cells with a plasmid coding for the viral RNA genome (bearing the transgene), as well as additional plasmids that provide *in trans* the necessary components for virus assembly and budding (Gag, Pol, Rev and Env proteins) (Fig 3.1.3) (Wiznerowicz and Trono, 2005). This transient transfection method is the basis for simple manipulation of LVs: The envelope protein, being responsible for the infection route, can be replaced with other surface proteins and viral entry can thereby be altered easily. Furthermore, this technique provides a standardised system, as the rest of the lentiviral particle is not affected by exchanging the envelope protein.

3.1.4. Aim of this Chapter

I wanted to investigate whether LV can induce an innate immune response and how the virus entry route influences this. On the one hand, studying the interaction between LVs and cells of the innate immune system might suggest strategies to minimise LV immunogenicity and maximise therapeutic use in gene delivery. On the other hand, further insight could tell us how to increase immune responses against HIV-1.

This study was designed to gain knowledge of the importance of the virus entry route in regard of TLR activation. I hypothesized that the entry route of virus particles contributes to the activation of the innate immune system: LV entering the cell via the endosome may give a stronger innate immune response than particles entering the cell via fusion at the plasma membrane (Fig 3.1.4). I further hypothesized that incubation of virus particles with antibodies or complement components could enhance innate sensing of LVs as opsonisation increases their uptake via the endosomal pathway. If this were the case it would emphasize the role for antibodies and the complement system as 'adjuvant' of innate immunity.

3.2. Results

3.2.1. LV preparations simulate IFN-α from BM-pDC

In order to establish whether LV induce innate immune responses I examined whether exposure of LV to leucocytes induces IFN-α, one of the earliest innate responses to invading viruses. For that purpose I incubated total murine bone marrow (BM) cells isolated from C57Bl/6 mice with graded doses of LV preparations and measured IFN-α in the supernatant after over night culture. CpG-containing DNA oligonucleotides that stimulate TLR9 in pDC and lead to production of high amounts of IFN-α were used as positive control. As shown in Fig 3.2.1A, standard LV preparations are very potent IFN-α inducers in BM. LV preparations were comparable or superior to CpG used at an optimal concentration (Fig 3.2.1B). Concentration of the LV-containing supernatant also increased its ability to induce IFN-α and resulted in a bell-shaped dose response courve (Fig 3.2.1A), a feature that is commonly seen for TLR agonists or agonists of cytoplasmic PRRs. I cannot exclude the possibility, however, that high concentrations of LV induce cell death and that this results in loss of IFN-α induction. In order to investigate which cells were responsible, I stimulated BM cells with LV and stained for intracellular IFN-α. Although all nucleated cells can produce IFN-α/β only a small subset of cells produced IFN-α in these experiments (Fig 3.2.1C). The cells responsible for this were $CD11c^{low}$, $B220^{positive}$ and $Ly6c^{positive}$, a phenotype indicative of pDC (Liu, 2005) (Fig 3.2.1C). To confirm this I enriched pDC from BM of RAG2-deficient mice on the basis of B220 expression by magnetic activated cell sorting (MACS) (Fig 3.2.1D). The only cell type expressing B220 in BM from *rag2*$^{-/-}$ mice are pDC (Shinkai et al., 1992). Only cultures containing B220-positive cells produced significant levels IFN-α in response to LV preparations and CpG (Fig 3.2.1E). Similarly, LV elicited IFN-α from BM cultured in the presence of Fms-like tyrosine kinase 3 ligand (Flt3L), which is a standard method to generate pDC *in vitro* (Fig 3.2.1F).

I concluded that LV preparations stimulate pDC to produce IFN-α.

3.2.2. *LV that infect cells via the endosome do not necessarily induce IFN-α*

Standard LV preparations bear the glycoprotein of vesicular stomatitis virus (VSV-G) on the surface, a protein that mediates uptake via endocytosis. This uptake mechanism may deliver the virus genome into endosomes to trigger a TLR-dependent response. To investigate whether endocytic uptake is necessary for IFN-α production, I generated virus particles carrying four different surface proteins that mediate infection via different routes. *In vitro*, virus infection through the endocytic route can be blocked by weak bases (Smith and Helenius, 2004). Treatment of 293FT cells with ammonium chloride (NH_4CL) did not affect GFP expression after infection with LV bearing the amphotropic murine leukaemia virus envelope protein (MLV-A) (Fig 3.2.2A), consistent with the notion that MLV-A mediates pH-independent fusion at the plasma membrane (Hernandez et al., 1996; McClure et al., 1990). In contrast, the same treatment reduced GFP expression in cells infected with LV equipped with VSV-G (Fig 3.2.2A), as expected (McClure et al., 1990; Puri et al., 1988). Infection of LV pseudotyped with the hitherto uncharacterised Thogotovirus glycoprotein (THOV-G) could also be blocked by treatment with weak bases (Fig 3.2.2A), indicative for endosomal uptake. Lymphochoriomeningitis virus glycoprotein (LCMV-G) is reported to deliver the virus particle into an endosomal compartment (Borrow and Oldstone, 1994).

To assess their innate stimulatory activity, pseudotyped viruses were normalised for virus titre on 293T cells and added to BM at a MOI of 1. Surprisingly, only preparations containing VSV-G pseudotyped virus generated detectable IFN-α in BM after over night treatment (Fig 3.2.2B). Thus, only LV preparations containing VSV-G stimulate IFN-α production from BM pDC. This was surprising as VSV-G was not unique in mediating endocytic uptake (Fig 3.2.2A, and (Borrow and Oldstone, 1994)). However, this result may be explained by the fact that VSV-G has a remarkable host range and for that reason VSV-G pseudotyped LV could be the only virus allowing successful infection of pDC. Testing this hypothesis was not possible as GFP expression was not detectable in the short-term assays necessary when using primary pDC.

3.2.3. IFN-α induction is independent of viral RNA but depends on VSV-G

I hypothesised that the virus genome present in virus particles acted as the agonist in the observed IFN-α response. However, virus particles lacking virus RNA (LVΔvRNA) induced high amounts of IFN-α (Fig 3.2.3A). As expected, omitting the VSV-G (LVΔenv) plasmid or transfecting the viral RNA only (vRNA) did not result in preparations with the capacity to induce IFN-α (Fig 3.2.3A). Unexpectedly, however, transfection of VSV-G and Rev alone (VSV-G) was sufficient to render the cell supernatant stimulatory (Fig 3.2.3A).

I next investigated whether functional VSV-G is necessary for IFN-α induction. Inactivation of VSV-G LV preparations by treatment with acidic medium or heat diminished the IFN-α response in BM (Fig 3.2.3B). Furthermore, pre-incubation of VSV-G containing preparations with a neutralising anti-VSV antibody abrogated IFN-α induction but the same treatment had no effect when using the control stimulus CpG (Fig 3.2.3C).

I concluded that VSV-LV preparations induce IFN-α in the absence of viral RNA. Transient transfection of VSV-G, renders the supernatant stimulatory and this depends on functionally active VSV-G.

3.2.4. VSV-G transfection leads to accumulation of VSV-G-bearing tubulo-vesicular structures in the extracellular milieu

The standard production of LV involves an ultracentrifugation step in order to concentrate virus particles. This concentration step also increased IFN-α inducing activity of LV containing supernatants (Fig 3.2.1A), which suggests the presence of a particulate stimulus. I therefore examined the transfected cells and cell supernatants by electron microscopy for the presence of particles that are associated with VSV-G expression. 293T cells were transfected with three combinations of plasmids, supernatants and cells harvested 48h later and processed for imaging in an electron microscope. Cells transfected with the four plasmids used for virus production (LV) showed abundant tubulo-vesicular structures (TVS) in the

extracellular space (Fig 3.2.4A, iv, black arrow). These structures could also be found in LV preparations (Fig 3.2.4A, i) and greatly outnumbered the actual virus particles (Fig 3.2.4A, iv, white arrow). Transfection of plasmids coding for vRNA, Gag-Pol and Rev (LVΔenv) was not sufficient to trigger formation of TVS but led to generation of virus particles (Fig 3.2.4A, iii and vi, white arrows). In contrast, TVS could be found when cells were transfected with the VSV-G plasmid alone (VSV-G) (Fig 3.2.4A, ii and v, black arrows).

TVS were also detectable by flow cytometry of cell supernatants as a population with distinct scatter properties (Fig 3.2.4B), which stained positive with an antibody against VSV-G (Fig 3.2.4C) indicating that this protein is associated with TVS. I confirmed this by western blot analysis (Fig 3.2.4D). Notably, LV and VSV-G but not control preparations of LVΔenv contained VSV-G and other proteins, like GFP and β-actin, present in cells originally used for transfection (Fig 3.2.4D). However, as expected, the LV capsid protein (HIV-p24), which is the main structural component of lentiviral particles, was present in LV and LVΔenv preparations (Fig 3.2.4D).

Thus, VSV-G pseudotyped LV preparations, as well as supernatants from VSV-G transfected cells, contain large amounts of TVS that are associated with VSV-G and other proteins present in cells used for virus production.

3.2.5. Separation of TVS from LV suggests minor innate immune response to virus particles

I speculated whether the virus particles and TVS synergised in IFN-α induction from LV preparations. Membrane vesicles have a lower density than lentiviruses (1.08 vs. 1.16-1.18g/ml) (Goff, 2001). Therefore I fractionated LV preparations by continuous sucrose gradient ultracentrifugation and tested each fraction for interferon inducing activity on BM cells and the presence of virus by infecting 293T cells. Further, I analysed the presence of VSV-G and HIV-p24 by western blot. IFN-α induction was found in distinct fractions, mainly fractions 5 and 6 (Fig 3.2.5). These fractions contained virus and the majority of the VSV-G protein indicating the presence of TVS. However, fraction 8 did not contain detectable VSV-G and did not induce

detectable IFN-α, yet contained comparable amounts of infectious particles and HIV-p24 capsid protein (Fig 3.2.5). These results suggest that the actual virus particle has little interferon inducing activity and defines TVS as the major IFN-α stimulus in LV preparations. The VSV-G protein itself, however, was probably not responsible for IFN-α induction as the protein was detectable in fraction 3 that did not activate IFN-α production (Fig 3.2.5). Only minute amounts of VSV-G may be necessary to confer infectivity to a lentiviral particle (fraction 8).

I concluded that TVS rather than virions or the VSV-G protein constitute the main stimulus for IFN production upon treatment of BM with LV preparations.

3.2.6. TLR9 is the main receptor responsible for IFN-α production after LV treatment of BM

In order to investigate which PRR is involved in IFN-α induction from LV I used BM cells from MyD88-deficient mice that are devoid of TLR7 and TLR9 signalling. Electroporation of poly-I:C, which is recognised by the intracellular pattern recognition receptor MDA5 (Kato et al., 2006), triggered similar amounts of IFN-α in wild-type and *myd88*$^{-/-}$ BM. In contrast, the response to LV was completely lost, arguing for involvement of TLR signalling in the recognition process of LV preparations in BM (Fig 3.2.6A). As LV carry a RNA genome I investigated whether TLR7 would be the major receptor involved. Surprisingly, treatment of BM from wild-type and from *tlr7*$^{-/-}$ mice showed no differences in terms of IFN-α induction when exposed to LV preparations whereas the response to the TLR7-dependent control stimulus R848 was decreased in *tlr7*$^{-/-}$ BM (Fig 3.2.6B). In contrast, TLR9-deficient BM cells had a more than 100-fold reduced ability to produce IFN-α in response to LV preparations or the TLR9-dependent stimulus CpG whereas the response to Loxoribine, a TLR7 stimulus, was equal in both cell types (Fig 3.2.6C). Similarly, IFN-α triggered by supernatants from VSV-G transfected cells was also dependent on TLR9 (Fig 3.2.6C).

From these data I concluded that TLR9 is the main pattern recognition receptor mediating IFN-α production from BM cells treated with preparations containing LV and TVS.

3.2.7. Plasmid DNA is present in TVS

The TLR9 requirement for IFN-α induction suggested the presence of DNA. To investigate this I stained cell supernatants with YOYO-I, a dye that intercalates into nucleic acid and thereby gains fluorescence activity that can be detected by flow cytometry. LV and TVS but not control preparations contained YOYO-I positive populations bearing the scatter properties of TVS (Fig 3.2.7A).

RT-PCR and PCR analysis of nucleic acids extracted from LV and TVS preparations confirmed the presence of RNA and DNA, corresponding to each of the plasmids that had been transfected into the producer cells (Fig 3.2.7B, C). Furthermore, I could detect DNA for β-actin, presumably derived from the genome of the producer cells (Fig 3.2.7B). In contrast, preparations containing LV but no TVS (LVΔenv) did not generate amplicons for plasmid or β-actin DNA by PCR analysis (Fig 3.2.7B, C). However, RNA coding for the virus genome (GFP) and β-actin could be amplified, consistent with the fact that lentiviral particles can package viral and cellular RNA (Muriaux et al., 2001).

To confirm the presence of intact plasmids, I transformed bacteria with DNA extracted from LV preparations. DNA corresponding to 1ml of LV preparation generated five times as many antibiotic resistant bacterial colonies than 10pg of the control plasmid pUC19 (Fig 3.2.7D). In contrast, no plasmids could be recovered from preparations of LVΔenv (Fig 3.2.7D). To exclude a possible contamination I isolated DNAs from individual bacteria colonies and compared their restriction pattern with that of the plasmids. The results indicate that each of the plasmids used for virus generation could be recovered from the transformants (Fig 3.2.7E, F).

I concluded that DNA of plasmid and cellular origin is present in LV and TVS preparations and this DNA may constitute the main IFN-α trigger in BM.

3.2.8. Transfer of plasmid DNA and/or cellular contents within TVS

The presence of DNA within TVS suggests that TVS might have the potential to transfer intact plasmids into target cells. To test this, I transfected 293T cells with a GFP expression plasmid together with or without a VSV-G plasmid and 48h later

transferred the supernatant to fresh 293T cells (Fig 3.2.8A). Cells exposed to supernatant of GFP transfected cells remained GFP negative (Fig 3.2.8B), whereas a portion of cells exposed to supernatants from GFP and VSV-G transfected cells contained GFP (Fig 3.2.8B) suggesting either carry over of intact GFP and/or nucleic acid resulting in new generation of GFP.

3.2.9. Generation of LV in cells exposed to TVS

Transfer of plasmids used for generation of LV could potentially convert target cells into virus producers. Indeed, supernatant of 293T cells exposed to high doses of LV contained small amounts of infectious LV at 120h after infection and after seven washes with PBS (Fig 3.2.9A). As I could not entirely exclude the possibility that remaining input virus is detected in this assay, I resorted to an alternative strategy to convincingly show that new virus was generated. A preparation of VSV-G pseudotyped LV lacking the viral genome (LVΔvRNA) was used to infect 293T cells containing a stably integrated lentiviral genome encoding GFP but none of the genes encoding viral structural proteins (293HV cells) (see scheme in Fig 3.2.9B). Only TVS-mediated transfer of nucleic acid expressing viral structural proteins can result in generation of new virus that contains a GFP genome (Fig 3.2.9B). Indeed, small amounts of indicator 293T cells could be transduced with a GFP expressing virus present in 293HV supernatant that was exposed to LVΔvRNA preparations (Fig 3.2.9C). FACS sorting and expansion of GFP positive cells for six weeks resulted in a population containing more than 30% GFP positive cells (Fig 3.2.9D). Amplification of a DNA fragment within the LTR that is only present in infected cells confirmed that GFP-positive cells were infected with a lentivirus (Fig 3.2.9E).
Taken together, these experiments indicate that VSV-G pseudotyped LV preparations generated by transient transfection contain TVS that carry DNA, which may provide the major stimulus for triggering TLR9. Furthermore, TVS can act as gene delivery vehicles in a VSV-G dependent manner, resulting in transfection of cells with residual plasmid DNA that potentially lead to the generation of new virus particles.

3.2.10. Avoiding transient transfection of producer cells decreases the innate stimulatory potential of LV preparations

Plasmid derived DNA is present in LV preparations and may be the major stimulus for innate immune responses. To test whether the stimulatory potential of LV can be decreased by nuclease digestion I exposed LV or a control plasmid (pMSCV-eGFP) to benzonase. The virus titre was not affected by benzonase treatment (data not shown), consistent with the notion that virus genomes are protected from nuclease activity by the membranous envelope and capsid proteins. Similarly, benzonase treatment of LV did not change the ability to induce IFN-α in BM, whereas treatment of a control plasmid abrogated IFN induction (Fig 3.2.10A). This suggests that immunostimulatory nucleic acid within LV preparations is not accessible to nucleases, probably due to localisation within a membranous compartment. Virus particles present in the same preparations could be responsible for IFN-α production in BM exposed to benzonase-treated LV. However, this is unlikely as an previous experiment (see section 3.2.5) suggested minor innate immune stimulation from virus particles.

To evaluate whether plasmids associated with LV are the actual source of IFN-α I resorted to an alternative strategy to generate LV. I made use of STAR-HV cells, which contain a HIV-based packaging vector encoding GFP and stably express HIV Gag, Pol and Rev proteins, needing only an exogenously-provided Env gene in order to secrete infectious LVs encoding GFP (Ikeda et al., 2003). I introduced VSV-G Env into these cells by transduction with a retrovirus and compared this to the plasmid transfection method used previously. STAR-HV cells transduced with VSV-G encoding retrovirus are not exposed to plasmids and only produce limiting amounts of VSV-G (Fig 3.2.10B) probably resulting in less TVS. For both reasons, LV preparations generated by this method should display a markedly diminished ability to activate pDC. VSV-G gene introduction by either plasmid transfection or retroviral transduction allowed LV production by STAR-HV cells as determined by titration on 293T cells (data not shown). The titre of LV produced through retroviral infection was significantly reduced when compared to LV produced through transient transfection. The preparations were normalised for viral titre and used to

stimulate BM cells. LV preparations produced by regular plasmid transfection induced IFN-α, as expected (Fig 3.2.10B). In contrast, LV preparations generated by retroviral transduction did not elicit IFN-α from BM cells (Fig 3.2.10B).
Therefore, I concluded that the interferon inducing ability of LV preparations clearly depends on the method of virus generation and not on the viral particles themselves.

3.2.11. LV can induce adaptive immune responses

Adaptive immune responses are initiated through delivery of an antigen together with an innate immune stimulus. An adaptive immune response against a viral pathogen is characterised by induction of cytotoxic $CD8^+$ T-cells that can recognise and kill cells displaying peptide-bound MHC-I and by antibody production that requires help from $CD4^+$ T-cells. Activated $CD8^+$ and $CD4^+$ T-cells can be re-stimulated *in vitro* to produce IFN-γ. LV have been described to give rise to adaptive immune responses against the encoded antigen. However, it is unclear what the innate stimulus promoting this immune response results from. I wanted to test whether successful vaccination with LV requires TLR signalling, which could suggest a role for TVS in this process. To investigate this, I immunised mice lacking the signalling molecule MyD88 with an ovalbumin (OVA) expressing LV or egg-white (as an adjuvant-free source of OVA protein) together with PBS, CpG or poly-I:C. 10 days after immunisation mice were injected with splenocytes pulsed with OVA or not and *in vivo* killing tested 48h later. In my hands, CpG that should have served as control for a TLR9 dependent adjuvant response did not act as potent adjuvant. *In vivo* killing of OVA loaded splenocytes was marginally detectable in wt mice and reduced in *myd88*$^{-/-}$ mice (Fig 3.2.11C, D). As expected, killing and expansion of tetramer positive $CD8^+$ T-cells was comparable in wt and *myd88*$^{-/-}$ mice when poly-I:C was used as adjuvant (Fig 3.2.11). Unexpectedly, when LV was used for immunisation, *myd88*$^{-/-}$ mice mounted an efficient adaptive immune response against OVA. Expansion of tetramer positive $CD8^+$ T-cells and killing of OVA loaded target cells was comparable in wt and *myd88*$^{-/-}$ mice immunised with OVA expressing LV (Fig 3.2.11C, D).

This suggests that activation of the adaptive immune system in response to LV immunisation does not rely on MyD88 signalling. However, MyD88-dependent responses may be present but masked by alternative innate stimuli.

3.2.12. TVS act as adjuvant in vivo

Given that LV preparations can act as immunogens but their innate stimulatory properties *in vitro* appear highly dependent on the presence of TVS contaminants, I wondered to what extent TVS modulate adaptive immune responses *in vivo*. I therefore intraperitoneally immunised wt and TLR9-deficient mice with egg white together with TVS, CpG or poly-I:C and tested for $CD8^+$ T-cell responses by monitoring expansion of OVA-specific tetramer positive T-cells and *in vivo* killing of OVA-pulsed splenocytes. *In vivo* killing of OVA-loaded splenocytes and expansion of tetramer positive $CD8^+$ T-cells was reduced in *tlr9*$^{-/-}$ mice immunised with CpG (Fig 3.2.12A-D) whereas poly-I:C immunisation resulted in a TLR9 independent immune response (Fig 3.2.12A-D). Control mice immunised with egg white alone did only show background levels of tetramer stain and no killing of OVA-pulsed splenocytes (Fig 3.2.12A-D). Surprisingly, however, when TVS were used as adjuvant, expansion of tetramer positive $CD8^+$ T-cells and *in vivo* killing of OVA-loaded splenocytes was similar in wt and *tlr9*$^{-/-}$ mice (Fig 3.2.12A-D).

In vitro re-stimulation of splenocytes with OVA did not result in any detectable IFN-γ production by T-cells (data not shown). However, FCS re-stimulation of splenocytes from OVA-TVS immunised mice triggered $CD4^+$ T-cells to produce IFN-γ (Fig 3.2.12E). Furthermore, I could detect antibodies against OVA and FCS (Fig 3.2.12F) suggesting that immunisation elicits both, a B- and T-cell response against TVS-associated proteins.

In conclusion, TVS clearly have adjuvant activity *in vivo*. However, this effect is TLR9-independent, suggesting the presence of additional innate stimuli in TVS preparations. These additional stimuli make TLR9 activation *in vivo* redundant for activating adaptive immune responses.

3.2.13. Supernatant of VSV infected cells does not contain TVS

Finally, I considered the possibility that TVS-like structures are produced during infection with VSV. VSV particles have a diameter of about 70nm, a length of approximately 170nm and bear an electron-dense core protein. As shown in Figure 3.2.4A, TVS, in contrast, only show a diameter of 35-45nm, are up to 2μm long and do not show electron-dense structures of particular order. Therefore, it is feasible to discriminate TVS from virus particles by electron microscopy. Yasu Takeuchi (UCL, Windeyer Institute of Virology) infected 293T cells with VSV (strain: New Jersey) at an MOI of 10 and we harvested the cells 24 hours after infection to assess the presence of TVS in the electron microscope. The majority of cells showed vast amounts of virus that was in the process of budding off the plasma membrane. An example is shown in Fig 3.2.13A. Although particles were present in great abundance, especially in the intercellular space (Fig 3.2.13B, white arrows), I could not detect structures that were reminiscent of TVS. In fact, most particles were viruses as judged by diameter, length and electron density (examples given in Fig 3.2.13C, D).

This suggests that the virus coordinates expression of its structural proteins. This regulation might partly have evolved to avoid formation of TVS that potentially could alert the immune system.

3.3. Discussion

Gene transfer for therapeutic purposes should, if possible, be immunologically silent or provoke immunological tolerance. Paradoxically, lentiviral vectors have also been used as tools for immunisation and shown to elicit powerful cytotoxic T-cell (CTL) responses against transgene-encoded proteins (Dullaers et al., 2004; Esslinger et al., 2003; Firat et al., 2002; He et al., 2005; Iglesias et al., 2007; Palmowski et al., 2004; Rowe et al., 2006). Similarly, HIV-1, from which most recombinant LVs are derived, elicits potent cell and antibody-mediated responses in humans (McMichael and Phillips, 1997). This indicates that lentiviruses and recombinant LV are intrinsically immunogenic, suggesting that they have the capacity to activate innate viral sensing pathways, which subsequently couple to adaptive immunity.

Recombinant LVs are used extensively to transduce non-dividing cells and are an attractive vehicle for gene delivery. LVs are less immunogenic than other viral vectors (Bessis et al., 2004; Chen et al., 2003), but induce an immune response *in vivo*, which limits their application for gene therapy (Kafri, 2001). This immunogenicity has been linked to their ability to transduce dendritic cells (Esslinger et al., 2003) although it is elusive why this should be sufficient as antigen targeting to DC in the absence of innate stimuli induces tolerance rather than immunity (Iwasaki and Medzhitov, 2004; Steinman and Hemmi, 2006). Therefore, it seems likely that the ability of LVs to prime immune responses reflects their ability to deliver both antigen and innate stimuli for DC activation. However, the ability of recombinant LVs to stimulate conventional DC is unclear and their capacity to stimulate pDC is ill defined despite the importance of this cell type in inducing anti-viral immune responses.

In this chapter I show that standard VSV-G-pseudotyped LVs prepared by transient transfection methods activate pDC, which results in high levels of IFN-α production in BM (Fig 3.2.1). Most of this activity appears to be contained in tubulo-vesicular structures (TVS) that outnumber the virus particles in standard LV preparations (Fig 3.2.4). TVS carry DNA (Fig 3.2.7) and activate TLR9 (Fig 3.2.6), a receptor implicated in innate immune responses against DNA viruses. Purified TVS can act

as potent adjuvant for co-administered antigens *in vivo* (Fig 3.2.12). TLR9, that is required for production of IFN-α *in vitro*, is not required for initiating adaptive immune responses against TVS (Fig 3.2.12) and LV (Fig 3.2.11). This could be attributable to alternative stimuli present in the supernatant of VSV-G transfected cells. Indeed, it has been proposed recently that the VSV-G protein has the ability to activate TLR4 (Georgel et al., 2007). However, additional stimuli might well be present in TVS preparations as TLR7/9 and TLR4 responses are reduced in *myd88*$^{-/-}$ mice (Akira et al., 2006; Kawai and Akira, 2007), yet wt and *myd88*$^{-/-}$ mice mount a similarly strong immune response when immunised with TVS (Fig 3.2.11).

TVS may be similar to microvesicles found within HIV preparations, which appear to be the actual source of many cellular proteins previously thought to be associated with the virus envelope (Bess et al., 1997; Gluschankof et al., 1997; Trubey et al., 2003). Like those microvesicles, TVS carry proteins of producer cell origin, including proteins encoded by plasmids (Fig 3.2.4D) and proteins derived from the culture medium (FCS components; Fig 3.2.12C, D). Notably, TVS may mediate transfer of several of these proteins (e.g. GFP) into target cells (Fig 3.2.8), likely explaining the phenomenon of "pseudo-transduction" observed with LV preparations (Nash and Lever, 2004). The actual origin of TVS and microvesicles is unclear although the presence of cell-derived vesicular structures within supernatants of cultured cells is a long-established phenomenon (Dalton, 1975). TVS are unlikely to be apoptotic bodies because preliminary experiments suggested that deliberate induction of apoptosis in cells transfected with MLV-A, THOV-G or LCMV-G Env genes does not generate supernatants capable to stimulate cytokine production from BM cells (data not shown). Similarly, TVS are unlikely to be exosomes, which have a different EM appearance (Fevrier and Raposo, 2004). I favour the possibility that TVS are distinct structures, which are actively induced by VSV-G overexpression. This is supported by the fact that VSV-G is known to promote budding of vesicular stomatitis virus (Brown and Lyles, 2003b). Furthermore, an alphavirus replicon encoding for VSV-G but not for viral capsid proteins spontaneously generates infectious virus-like particles (Rolls et al., 1994). VSV-G is not the only glycoprotein with budding activity. In fact, virus surface proteins expressed by Ebola-, Respiratory syncytial virus and Rift valley fever virus are important for

efficient budding of virus particles (Jasenosky and Kawaoka, 2004; Techaarpornkul et al., 2001)(and Friedemann Weber, personal communication). Bearing that in mind could be of importance when using these proteins to generate recombinant viruses for means of gene therapy or immunisation (Kobinger et al., 2001; Swenson et al., 2005) as TVS-like structures could be present in these virus preparations.

VSV-G is expressed in cells that are infected with VSV. However, this abundant presence does not result in TVS accumulation, as seen when transfecting the VSV-G plasmid (Fig 3.2.13). TVS could potentially alert the immune system and the virus might therefore carefully balance the expression of VSV-G. The matrix (M) protein is mainly responsible for budding of VSV (Mebatsion et al., 1999). VSV-G accumulates in foci on the plasma membrane and facilitates the budding process of preformed virus particles that are localising underneath the cell surface. It is believed that the virus ensures efficient coating of the virus particles with VSV-G by this mechanism (Brown and Lyles, 2003a, b). It may be that this budding mechanism minimises formation of TVS-like structures.

Functional VSV-G is involved in TVS formation (Fig 3.2.4) and necessary for induction of IFN-α from pDC (Fig 3.2.3). This, together with the fact that VSV-G was unique in promoting innate stimulation, prompted me to speculate that the VSV-G itself may promote IFN-α production. Fractionation experiments, resulting in VSV-G containing fractions that do not activate an antiviral response, argue against this hypothesis: Fraction 3 and 4 in Fig 3.2.5 contain immunodetectable VSV-G protein but elicit no or little IFN-α. Furthermore, the TLR9 dependency for IFN-α induction (Fig 3.2.6C) suggests that TVS-associated DNA rather than the VSV-G protein is the actual stimulus.

The VSV-G protein most likely promotes delivery of TVS into endosomal compartments of pDC where DNA coming from the producer cell is sensed by TLR9. LVs pseudotyped with other glycoproteins like THOV-G, LCMV-G and MLV-A do not act as strong activators of BM (Fig 3.2.2), underlining the importance of the VSV-G protein for the described phenomenon. LCMV-G and THOV-G promote virus entry via the endosomal route (Borrow and Oldstone, 1994) (Fig 3.2.2A) but may not promote formation of DNA-containing TVS.

It is surprising that murine pDC do not produce IFN-α in response to LV particles (Fig 3.2.2), which is in contrast to human pDC that produce IFN-α and mature in a TLR-dependent manner when exposed to HIV-I (Beignon et al., 2005; Francis and Meltzer, 1993). It may reflect intrinsic differences between murine and human pDC in response to lentivirus particles. For instance, murine pDC could be different in handling endosomal cargo, or, alternatively, HIV could be recognised by TLR8 that is thought to be non-functional in mice (Heil et al., 2004; Jurk et al., 2002). A simple explanation could be insufficient uptake of virus particles by pDC although this is unlikely as VSV-G allows uptake of TVS, which results in a TLR9 response. However, in comparison to other stimuli like influenza, VSV or HSV-2, which trigger IFN-α responses via TLR7 and TLR9, respectively, HIV-I is only poorly stimulatory in human pDC (Beignon et al., 2005; Diebold et al., 2004; Fonteneau et al., 2004; Lund et al., 2004; Schmidt et al., 2005; Yonezawa et al., 2003).

A provocative possibility could be that replication-competent HIV-I is only poorly recognised upon uptake of virus particles, but that lentiviral particles are delivered into an endocytic compartment later during the virus life cycle. HIV is believed to leave the cell through a process called inward budding. This process involves delivery of virus particles into the endosome followed by exocytosis (Kramer et al., 2005). One could speculate that, newly generated HIV particles contribute to TLR7 or -8 mediated interferon responses in human pDC. This hypothesis can easily be tested as reverse transcriptase inhibitors like AZT should not influence TLR activation if the virus is solely activated through incoming viruses. If virus replication and budding is necessary for efficient TLR activation AZT treatment should reduce TLR activation. If inward budding was involved in lentivirus sensing, expression of the HIV gag protein in pDC should result in a TLR7-dependent IFN-α response. Alternatively, BM from transgenic mice bearing receptors for successful entry of HIV (Schule et al., 2006) could be infected with wt HIV and this should give a TLR7-dependent IFN response. Further, if the virus is recognised during entry, TLR activation should be possible in cells lacking a functional ESCRT system, which is critically involved in budding of HIV particles (Stuchell et al., 2004). Sensing of viruses budding into the endosome would add another aspect to

TLR recognition and would underline the notion that the TLR system may also have evolved to as a means to ensure self-control in pDC (Lee et al., 2007).

In vitro, human pDC produce little IFN-α when exposed to HIV-I but this may be different *in vivo*, as the virus could be opsonised and thereby would be better endocytosed by pDC (Palmer et al., 2000). In that regard, it has been shown that antibodies present in the serum of HIV infected patients can enhance IFN-α production if added together with HIV-1 to peripheral blood mononuclear cells (Green et al., 2002), an effect likely explained by increased Fc-receptor mediated internalisation of HIV particles into pDC endosomes and recognition via TLR7.

I found that LV preparations can induce efficient CTL priming in MyD88-deficient mice (Fig 3.2.11). Thus, *in vivo*, LVs contain immunostimulatory properties that cannot be attributed to TLR4 or TLR7/9 stimulation as those receptors signal - at least in part - via MyD88. A source of this activity could be DNA that is delivered into the cytoplasm of cells and then would activate cytoplasmic receptors for DNA, like the recently identified DAI (Takaoka et al., 2007) and others, that signal in a MyD88 independent way. How does DNA that is present in LVs enter the cell? Reagents such as DOTAP or PEI are routinely used to transfect cells but have also been exploited experimentally to deliver nucleic acids to endosomes for recognition by TLR7, -8 and -9 (Diebold et al., 2004; Heil et al., 2004). Similarly, just like they deliver DNA for innate sensing in endosomes, VSV-G-coated TVS also act as transfecting particles and deliver DNA directly into cells (Fig 3.2.8 and Fig 3.2.9). The ability of VSV-G to act as a transfection agent when added to plasmid DNA or to retroviruses lacking envelope proteins has been noted previously (Abe et al., 1998; Okimoto et al., 2001) and, given that cytosolic delivery of DNA activates anti-viral responses (Ishii et al., 2006; Stetson and Medzhitov, 2006a), this ability could account for TLR-independent innate stimulatory activity of TVS *in vivo*.

The notion that DNA can be present in LV preparations is not new (Sastry et al., 2004). DNA delivery raises the possibility that TVS within LV preparations might transfer plasmids used for virus production into target cells. Indeed, I could show that cells exposed to VSV-G-pseudotyped LV preparations can be converted into virus producing cells (Fig 3.2.9). Recombinant LVs have been tested extensively for potential clinical use (Fuller and Anson, 2004) and it is unlikely that plasmid transfer

could ever constitute a risk in gene therapy protocols, especially as I detect only low titres of newly generated infectious virus (Fig 3.2.9). Nevertheless, it may be of value to assess LV preparations for the presence of TVS as this may further enhance their safety profile.

My results on the innate stimulatory potential of LV preparations have important practical implications for the use of recombinant LVs in gene therapy. There are many examples in which innate responses to vectors have adverse effects (Bessis et al., 2004; Chen et al., 2003). For adenovirus vectors it has been noticed that induction of cytokines limits high expression of delivered genes (Bessis et al., 2004). Similarly, interferons reduce gene expression from retroviral vectors, which might contribute to the relatively poor performance of such vectors *in vivo* (Ghazizadeh et al., 1997). In mice, intracranial delivery of VSV-G-pseudotyped LVs induces local inflammation and a systemic immune response which can limit transduction efficiency (Baekelandt et al., 2003). Therefore, gene therapy approaches, as well as *in vitro* transduction of immune cells, would likely benefit from having LVs with low innate stimulatory potential. In this regard, my results suggest that pseudotyping with THOV-G, LCMV-G or MLV-A may be preferable to the more widely used VSV-G. This needs to be weighed against the fact that THOV-G, LCMV-G or MLV-A also result in production of LV preparations containing lower virus titres than those obtained by VSV-G-pseudotyping (references (Cockrell and Kafri, 2003; Verhoeyen and Cosset, 2004) and data not shown). If VSV-G cannot be avoided, my results suggest that either careful purification of virus particles or generation of virus in stable producer cell lines by retroviral transduction (Fig 3.2.10B) or inducible VSV-G expression (Ory et al., 1996) might help to decrease their immunogenic potential. However, I was unable to purify large enough quantities of virus by gradient fractionation and yet would have to generate a stable LV producer cell line expressing a suitable antigen to set up *in vivo* experiments to address this. In this line, it has been reported by others that sucrose gradient fractionation decreases LV immunogenicity (Baekelandt et al., 2003). More work will be needed to see how different virus generation and purification methods impact on contamination by TVS and on immune responses.

3.4. Figures

Figure 3.1.3

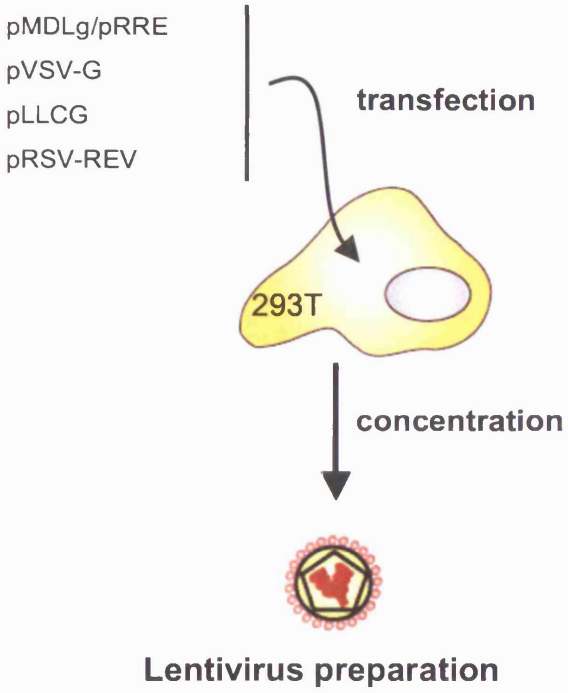


Figure 3.1.3: Generation of recombinant lentiviruses

Transfection of 293T cells with four plasmids coding for the lentiviral capsid protein (pMDLg/pRRE), an envelope protein (pVSV-G), the lentiviral genome (pLLCG) and a helper plasmid (pRSV-REV) results in production of replication incompetent lentiviral particles that are released into the cell supernatant. The standard procedure of LV production involves ultracentrifugation as a means to increase the virus titre.

Figure 3.1.4

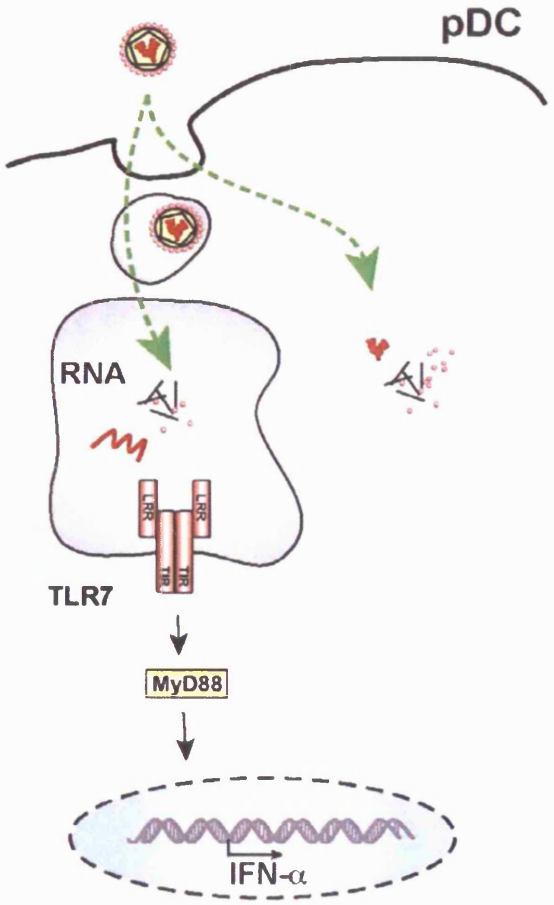


Figure 3.1.4: What is the influence of the virus infection route on TLR activation?

Recombinant lentiviruses (LV) can infect the cell via two routes (green arrows). Left arrow: Virus entry through the endosome should theoretically deliver ssRNA that activates TLR7, resulting in MyD88 dependent downstream signalling. In pDC this would result in expression of IFN-α. Right arrow: Changing the envelope protein of the virus should mediate fusion of the virus particle at the plasma membrane. Therefore such a virus is not delivered into the endocytic compartment.

Figure 3.2.1

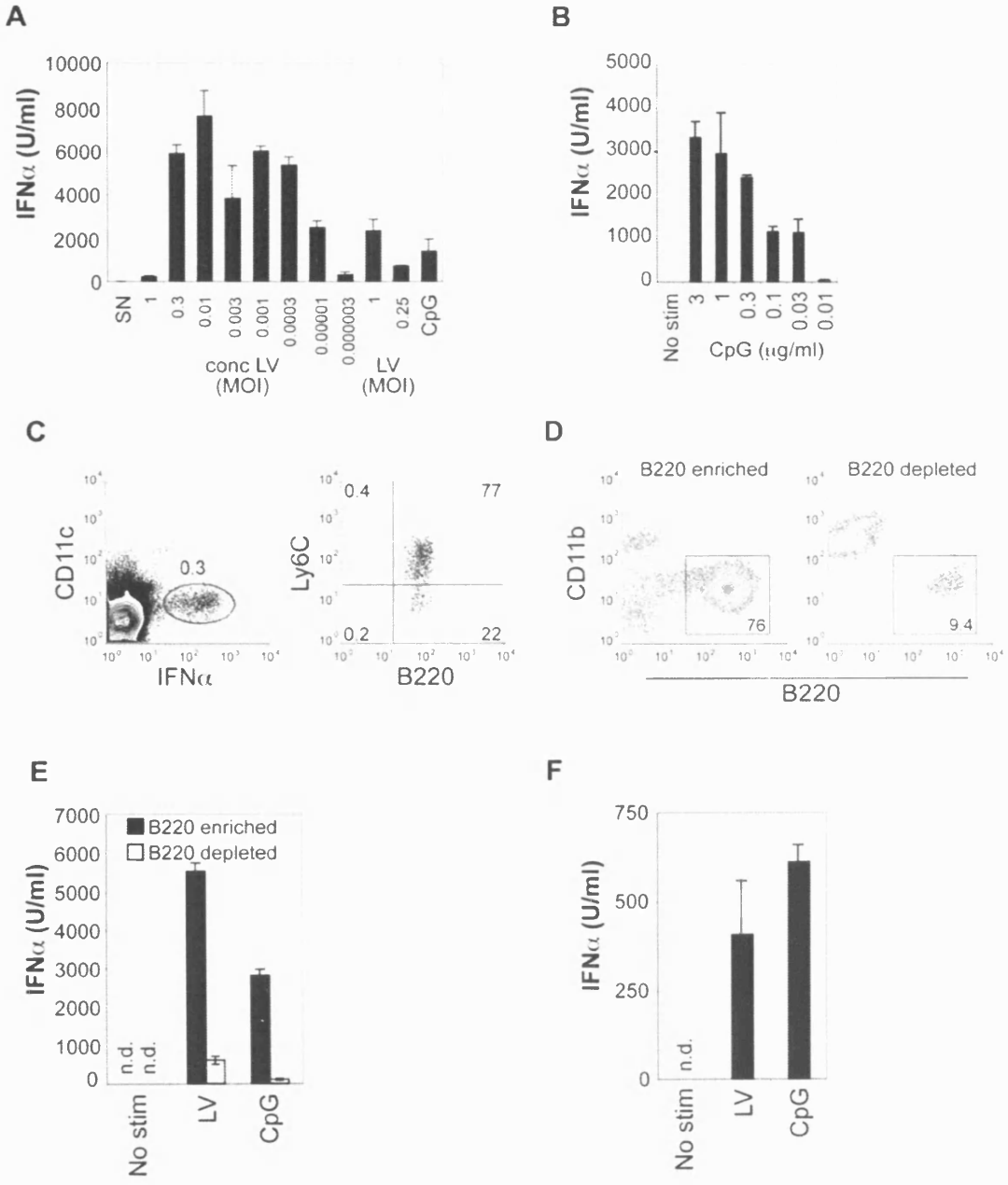

A
IFNα (U/ml)
10000
8000
6000
4000
2000
0
SN
1
0.3
0.01
0.003
0.001
0.0003
0.00001
0.000003
1
0.25
CpG
conc LV (MOI)
LV (MOI)
B
IFNα (U/ml)
5000
4000
3000
2000
1000
0
No stim
3
1
0.3
0.1
0.03
0.01
CpG (μg/ml)
C
CD11c
0.3
IFNα
Ly6C
0.4
77
0.2
22
B220
D
B220 enriched
B220 depleted
CD11b
76
9.4
B220
E
IFNα (U/ml)
7000
6000
5000
4000
3000
2000
1000
0
B220 enriched
B220 depleted
n.d.
n.d.
No stim
LV
CpG
F
IFNα (U/ml)
750
500
250
0
n.d.
No stim
LV
CpG

Figure 3.2.1: LV preparations induce IFNα production by pDC

(A) $1*10^6$ BM cells were stimulated with VSV-G-pseudotyped LVs at the indicated MOI or with CpG (0.5µg/ml; positive control) or with 100-fold concentrated cell supernatant of untransfected cells (SN; negative control). Shown is the concentration of IFN-α in the supernatant as measured by ELISA 16h after treatment. (B) BM was treated with graded doses of CpG and IFN-α measured by ELISA after over night stimulation. (C) Intracellular staining for IFN-α in BM cells stimulated with LVs at a MOI of 0.2 for 6h. Left panel: IFN-α vs. CD11c. Right panel: Ly6C and B220 expression on IFN-α positive cells gated as indicated in the left panel. Numbers represent the percentages of cells in each gate. (D, E) IFN-α secretion from B220 MACS-enriched and B220 MACS-depleted BM cells isolated from *rag2*$^{-/-}$ mice. (D) Panels show FACS blots of cells from B220 enriched and B220 depleted fractions. Numbers represent percentage of cells in the indicated gate. (E) Accumulation of IFN-α in supernatant of cells from (D) treated over night with VSV-G-pseudotyped LVs (MOI 0.2) or CpG (0.5µg/ml). (F) $2*10^5$ Flt-3L derived BM-DC (approx 30% pDC) were treated with LV (MOI 1) and IFN-α measured after over night incubation. One representative experiment of two (B), three (C, D, E, F) or more (A) experiments is shown.

Figure 3.2.2

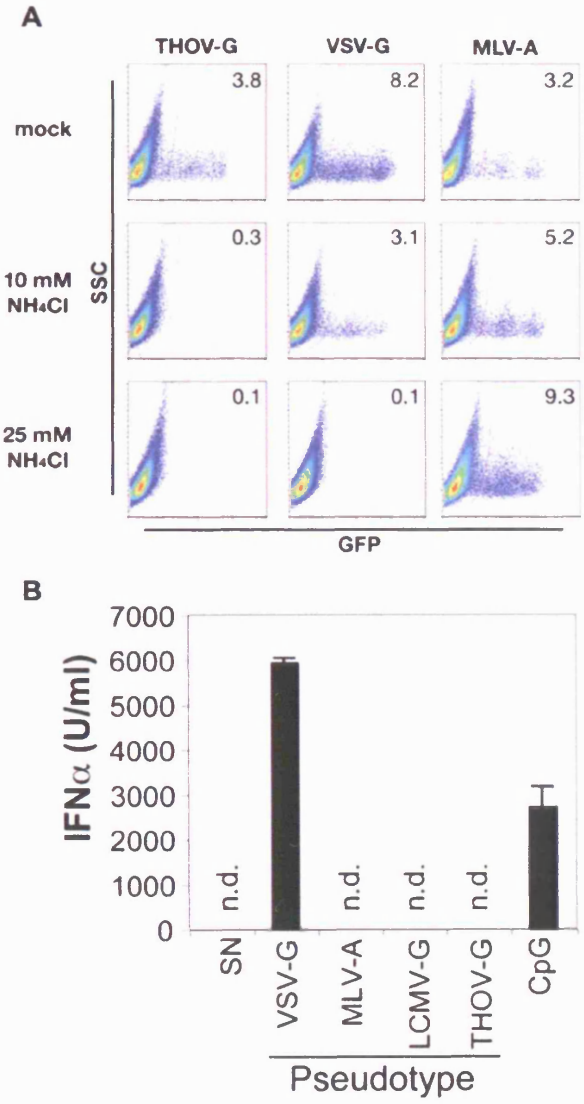


Figure 3.2.2: LV entering the endosome do not necessarily stimulate IFN-α

(A) 293FT cells were treated with medium containing the indicated amount of ammonium chloride (NH_4Cl) for 1h. Cells were then washed with PBS and infected for 1h with the indicated viruses. Medium containing ammonium chloride was again added for over night incubation. At 24h cells were analysed by flow cytometry. FACS blots show cells gated on sideward and forward scatter. SSC: sideward scatter. Numbers indicate percentage of cells in the indicated gate. (B) BM cells were stimulated with preparations containing LV bearing the indicated glycoproteins (MOI 1); IFN-α was measured by ELISA after over night culture. n.d., not detected. One of three (A) or more (B) experiments with similar results is shown.

Figure 3.2.3

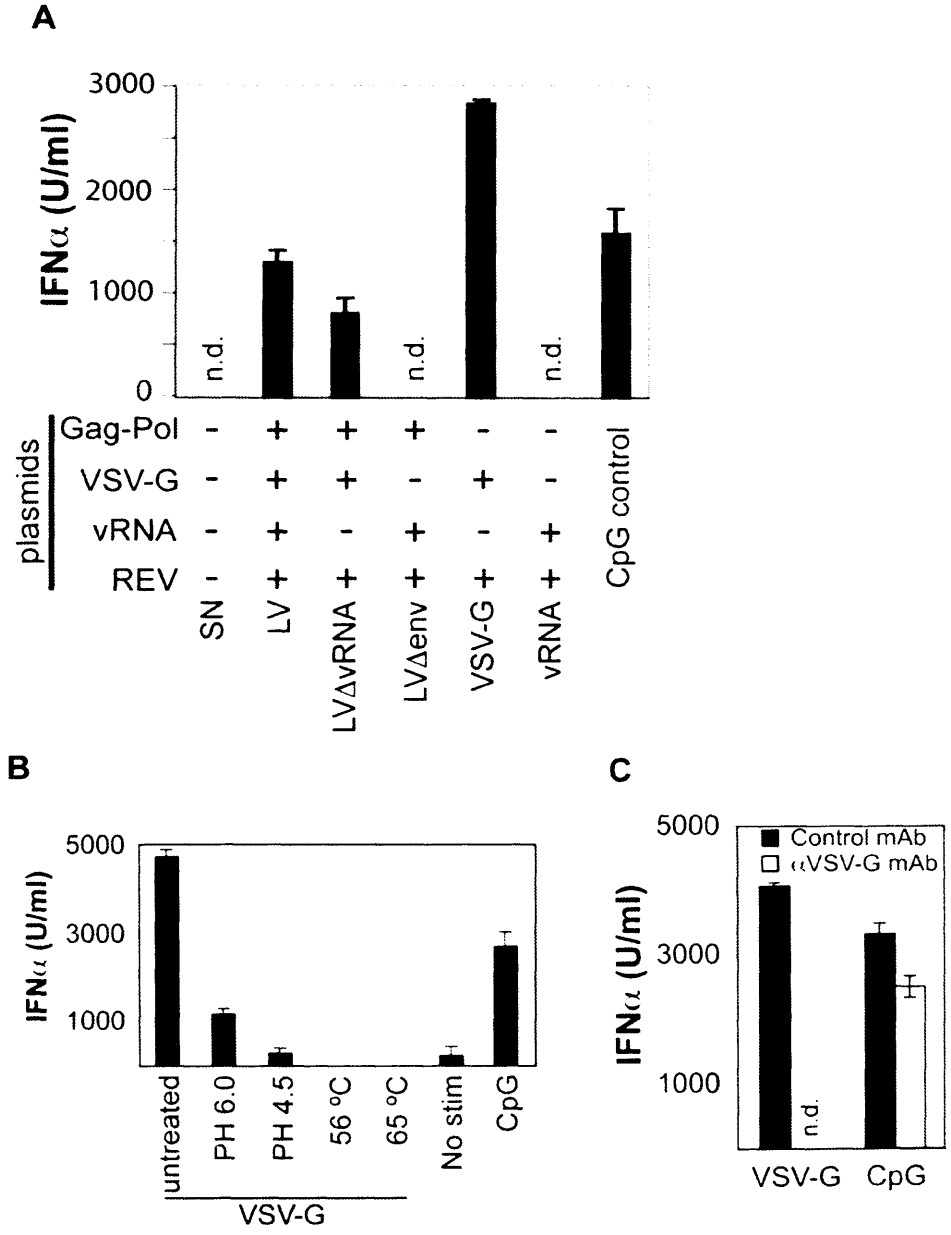

A
IFNα (U/ml)
3000
2000
1000
0
n.d.
n.d.
n.d.
plasmids
Gag-Pol - + + + - -
VSV-G - + + - + -
vRNA - + - + - +
REV - + + + + +
SN
LV
LVΔvRNA
LVΔenv
VSV-G
vRNA
CpG control
B
IFNα (U/ml)
5000
3000
1000
untreated
PH 6.0
PH 4.5
56 °C
65 °C
No stim
CpG
VSV-G
C
IFNα (U/ml)
5000
3000
1000
Control mAb
αVSV-G mAb
n.d.
VSV-G
CpG

Figure 3.2.3: VSV-G expression is required for the IFN-α response to LVs

(A) Treatment of BM with concentrated supernatants from 293FT cells transfected with the indicated combinations of plasmids to generate preparations of VSV-G-pseudotyped LV (LV), LV lacking viral RNA (LVΔvRNA) or LV lacking the envelope protein (LVΔenv). Concentrated supernatants (dilution 1:20) from untransfected cells (SN) or from cells transfected with plasmids encoding REV + VSV-G (VSV-G) or REV + viral RNA (vRNA) were tested as controls. Where applicable, stimuli were normalised for HIV p24 content (data not shown). (B) Concentrated supernatant from VSV-G transfected cells (VSV-G) was pretreated as indicated for 30min and used to stimulate BM. (C) VSV-G supernatant (VSV-G) and CpG (0.5μg/ml) were pre-incubated with a neutralizing anti-VSV-G or unspecific msIgG (control mAb) before adding to BM cells. (A-C) IFNα in supernatants was measured by ELISA 16h after stimulation. n.d., not detected. One representative experiment of three (B, C) or more (A) is shown.

Figure 3.2.4

A

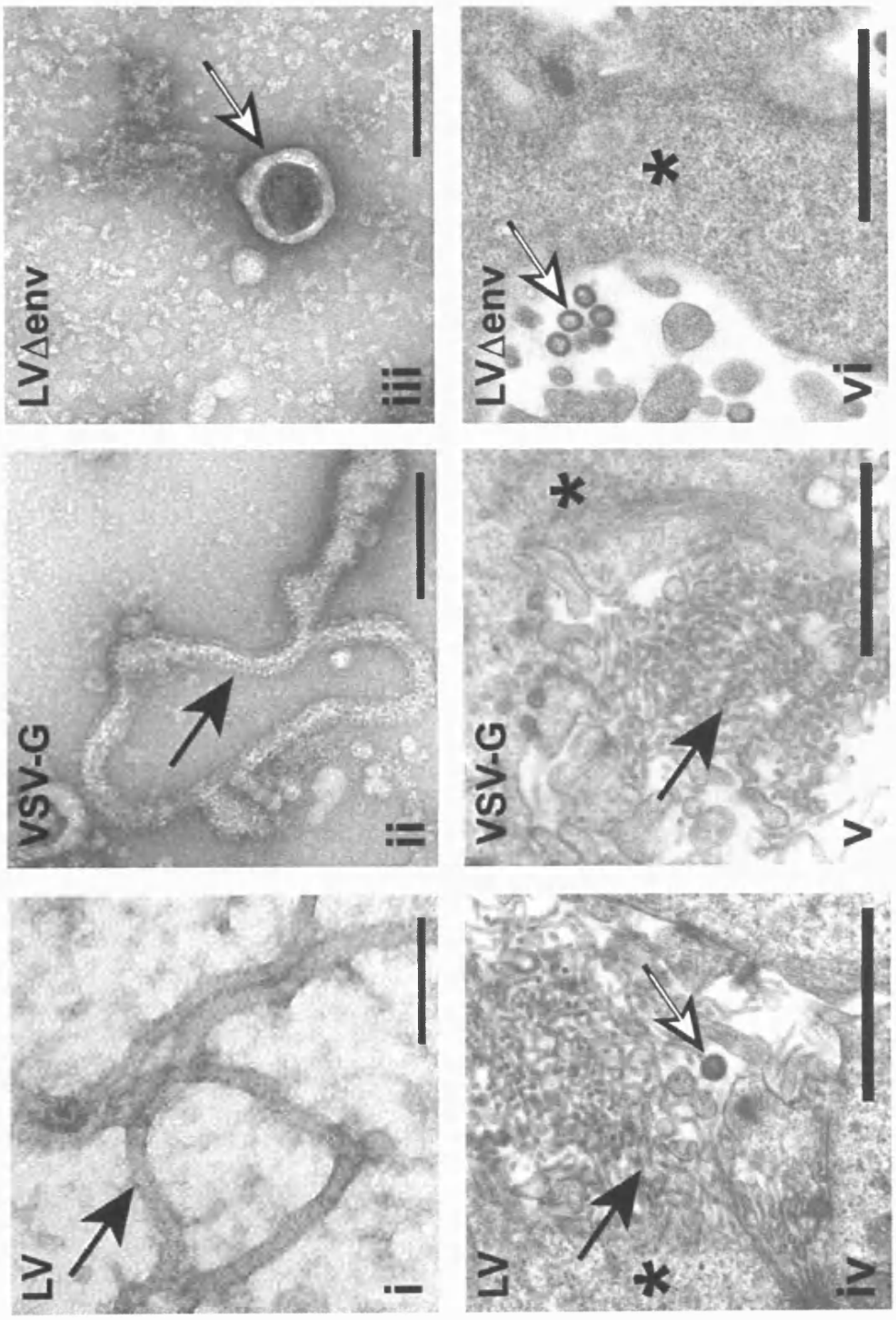


Figure 3.2.4

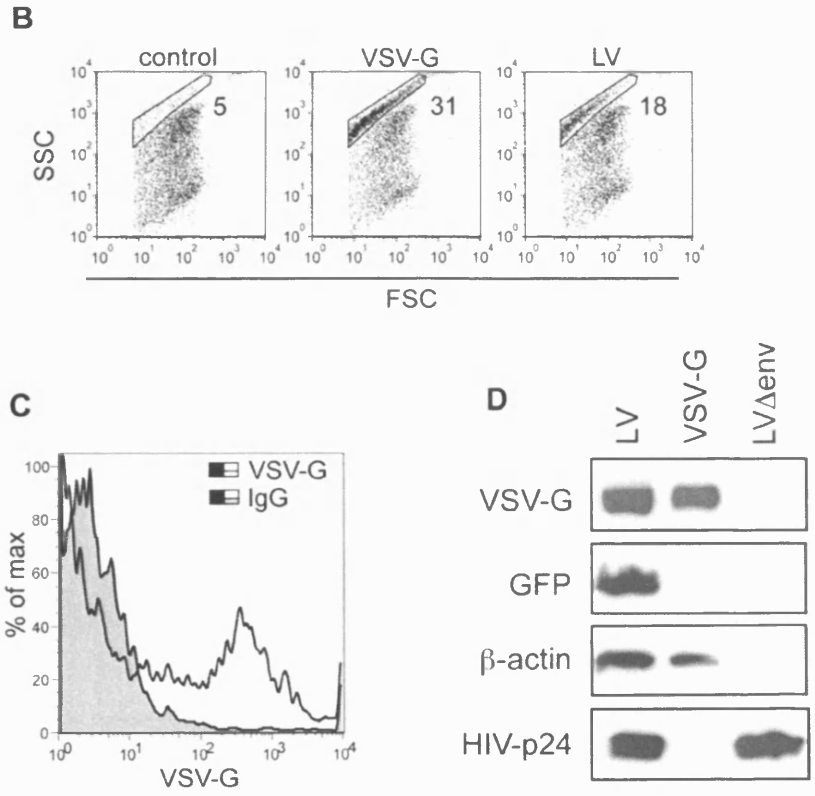


Figure 3.2.4: VSV-G transfection induces formation of tubulo-vesicular structures (TVS) that carry proteins of cellular origin

(A) Electron micrographs of supernatants (upper panels) and transfected cells (lower panels) producing VSV-G-pseudotyped LV, VSV-G only or LV without envelope protein (LVΔenv). Bars represent 200nm (upper panels) and 1μm (lower panels) respectively. Black arrows indicate TVS, white arrows show virus particles. * indicates the cell cytoplasm. (B) FACS blots of concentrated supernatant of cells that were left untransfected (control) or transfected to produce LV and VSV-G. Numbers show percentage of particles in indicated gates. (C) Histogram of concentrated supernatant from VSV-G transfected cells stained with a VSV-G antibody or an isotype control. The panel shows particles of the indicated gate in (B). (D) Western blots of 20μl concentrated supernatant of the indicated preparations were stained with the indicated antibodies. Experiments were repeated two (C) or three (A, B, D) times.

Figure 3.2.5

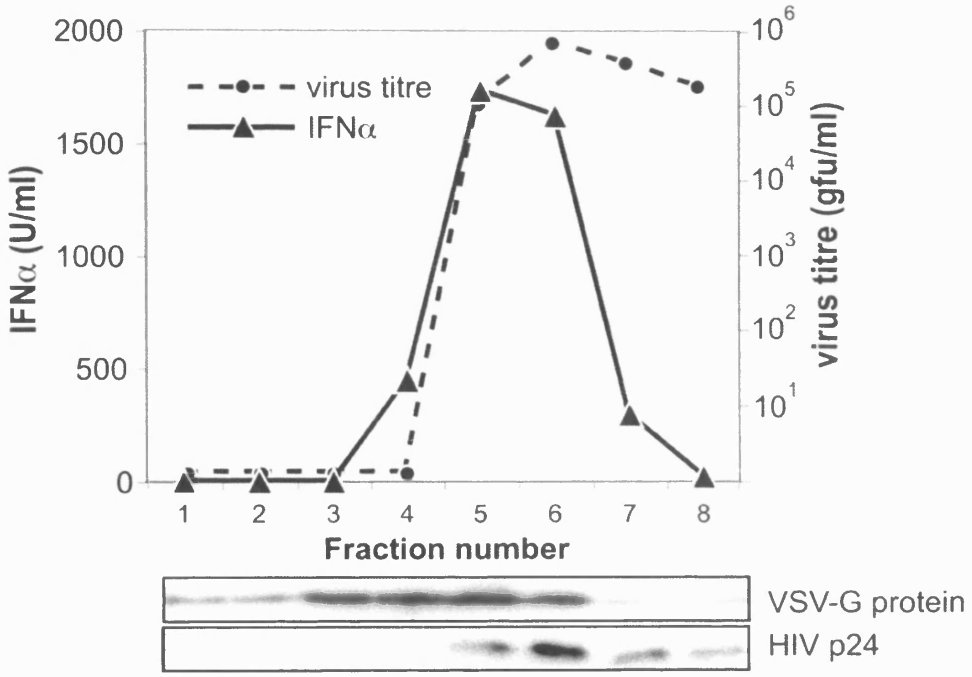


Figure 3.2.5: TVS constitute the main pDC stimulus in VSV-G-containing LV preparations

VSV-G-LV preparations were fractionated on a continuous sucrose gradient. Each fraction was assessed for IFN-α induction in BM cells (dilution 1:20, solid line) and for virus titre in 293T cells (dashed line). Western blots (20µl of each fraction) show the relative amount of VSV-G and the viral capsid protein (HIV p24) in each fraction. Fraction 8 represents the bottom of the gradient (dense fraction).

Figure 3.2.6

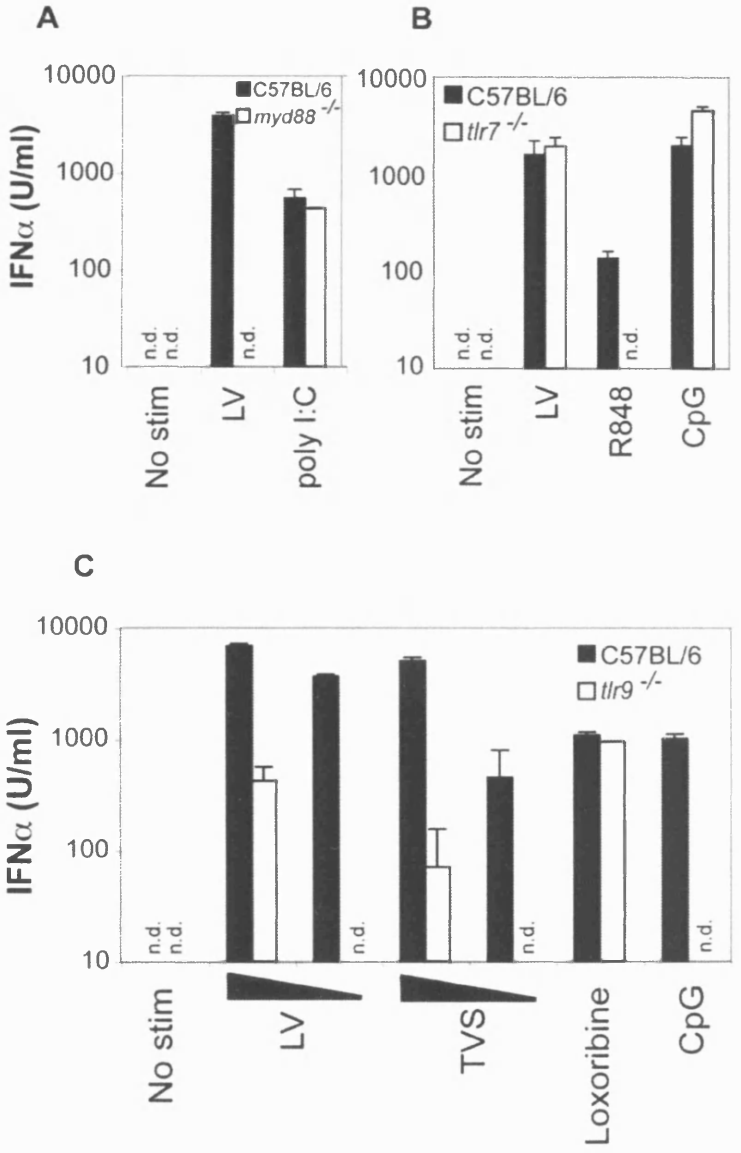


Figure 3.2.6: IFN-α induction by LV preparations and TVS is dependent on TLR9

(A) BM cells from C57BL/6 or *myd88*$^{-/-}$ mice were cultured in medium alone (No stim), with LV (MOI 0.1) or were electroporated with poly-I:C (0.5μg). IFN-α was measured by ELISA after over night incubation. (B) IFN-α from *tlr7*$^{-/-}$ or wild type BM cells after stimulation with LV (MOI 0.1), R848 (1μg/ml) or CpG (0.5μg/ml). (C) BM cells from wild type or *tlr9*$^{-/-}$ mice were stimulated with LV (MOI 0.1 and 0.01), TVS (dilution 1:20 and 1:200), Loxoribine (20mM) or CpG (0.5μg/ml). IFN-α was measured after over night culture. n.d., not detected. All experiments were repeated more than three times, representative experiments are shown.

Figure 3.2.7

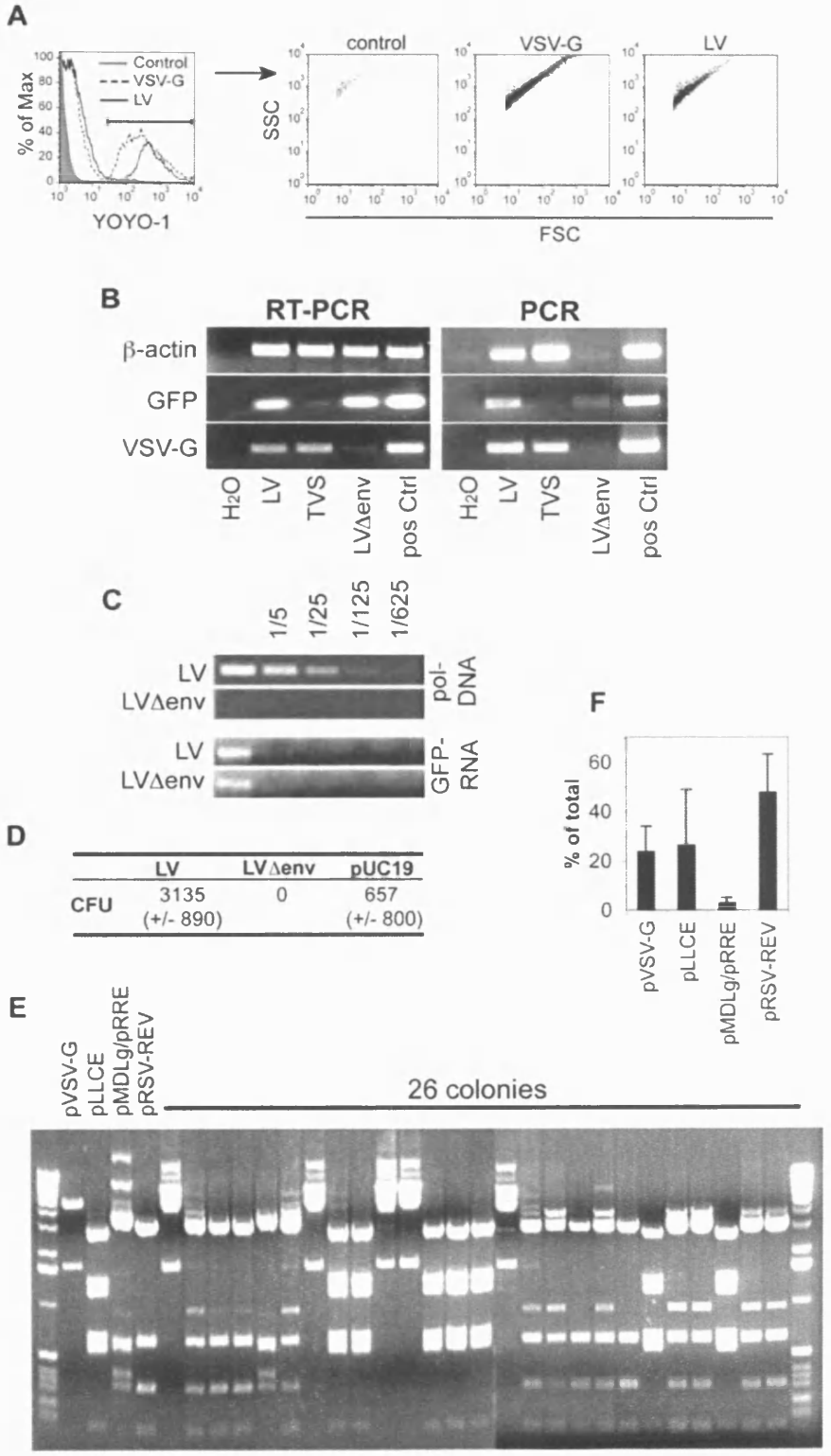

A
Control
VSV-G
LV
% of Max
YOYO-1
control
VSV-G
LV
SSC
FSC
B
RT-PCR
PCR
β-actin
GFP
VSV-G
H2O
LV
TVS
LVΔenv
pos Ctrl
C
1/5
1/25
1/125
1/625
LV
LVΔenv
pol-DNA
GFP-RNA
D
LV
LVΔenv
pUC19
CFU
3135 (+/- 890)
0
657 (+/- 800)
E
pVSV-G
pLLCE
pMDLg/pRRE
pRSV-REV
26 colonies
F
% of total
pVSV-G
pLLCE
pMDLg/pRRE
pRSV-REV

Figure 3.2.7: TVS and LV preparations contain plasmid DNA

(A) Paraformaldehyde fixed preparations of LV, TVS and control transfected cells (pLLCG) were stained with the nucleic acid stain YOYO-1 (dilution 1:1000); histograms show YOYO-1 staining of ungated particles. Dot blots show particles gated on YOYO-1 as indicated in the histogram. (B, C) RT-PCR and PCR for the indicated genes on RNA and DNA isolated from preparations of LV, LV lacking the envelope protein (LVΔenv), or TVS (concentrated supernatant from VSV-G transfected cells). Expression plasmids for VSV-G and GFP or cDNA from cells served as positive controls (pos Ctrl) (B). In (C) the indicated serial dilutions of template were used for PCR and RT-PCR. Starting concentration: $1/10^{th}$ of nucleic acid isolated from 35µl concentrated supernatant. (D-F) DNA extracted from LV preparations was used to transform bacteria. (D) The table shows the number of ampicillin-resistant colonies after transforming bacteria with DNA isolated from 1ml LV or 1ml LVΔenv. 10ng pUC19 plasmid was used as control. The average of three experiments (± SD) is shown. (E) Ampicillin-resistant individual colonies were picked, the plasmid DNA extracted and analysed by restriction double-digest with EcoRI and HindIII for 3h. Lanes show the restriction pattern of a random sample of 26 colonies and the pattern of the plasmids that were used for generation of LV as a control. The first and last lanes show molecular weight markers. (F) Frequency of bacterial colonies containing each plasmid (average ± SD from 3 independent experiments analysing a total of more than 150 colonies).

Figure 3.2.8

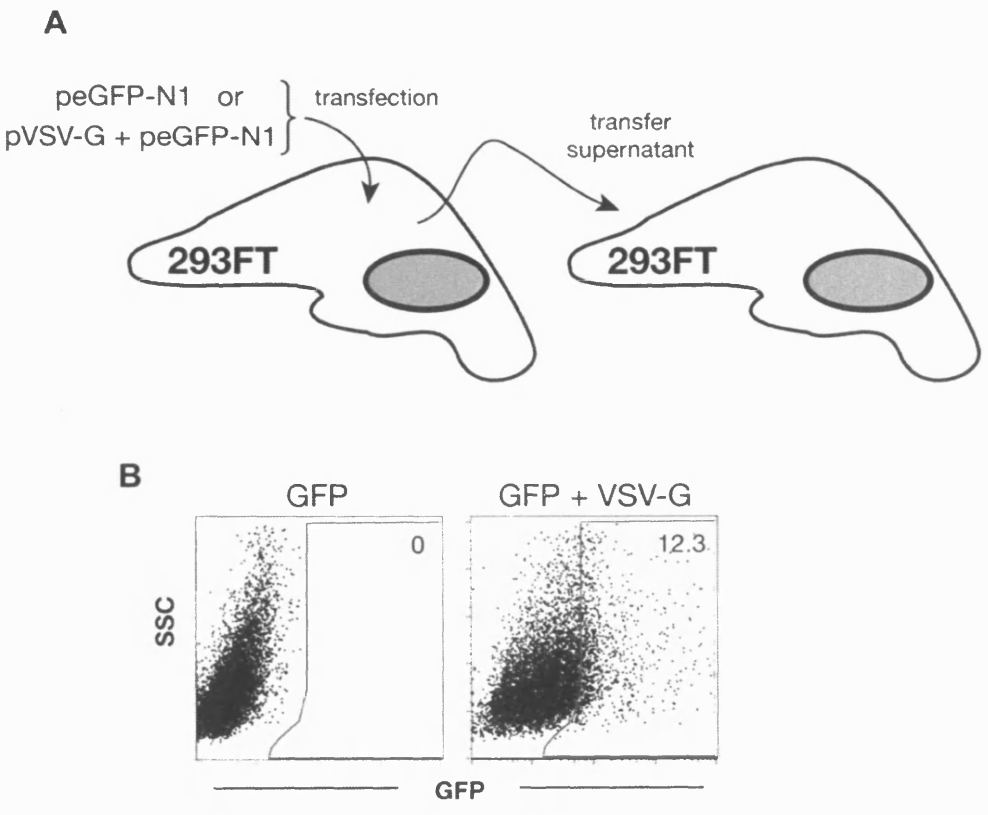


Figure 3.2.8: TVS transfer protein and/or nucleic acid

(A) Schematic drawing of the experiment. 293FT cells were transfected with plasmids coding for GFP (peGFP-N1) and VSV-G (pVSV-G) or the GFP expression plasmid only. 48h later cell supernatant was filtered and transferred to indicator cells. (B) FACS blots of indicator cells 48h after exposure to supernatant of transfected cells. The numbers indicate percentage of cells in the indicated gates. SSC: sideward scatter.

Figure 3.2.9

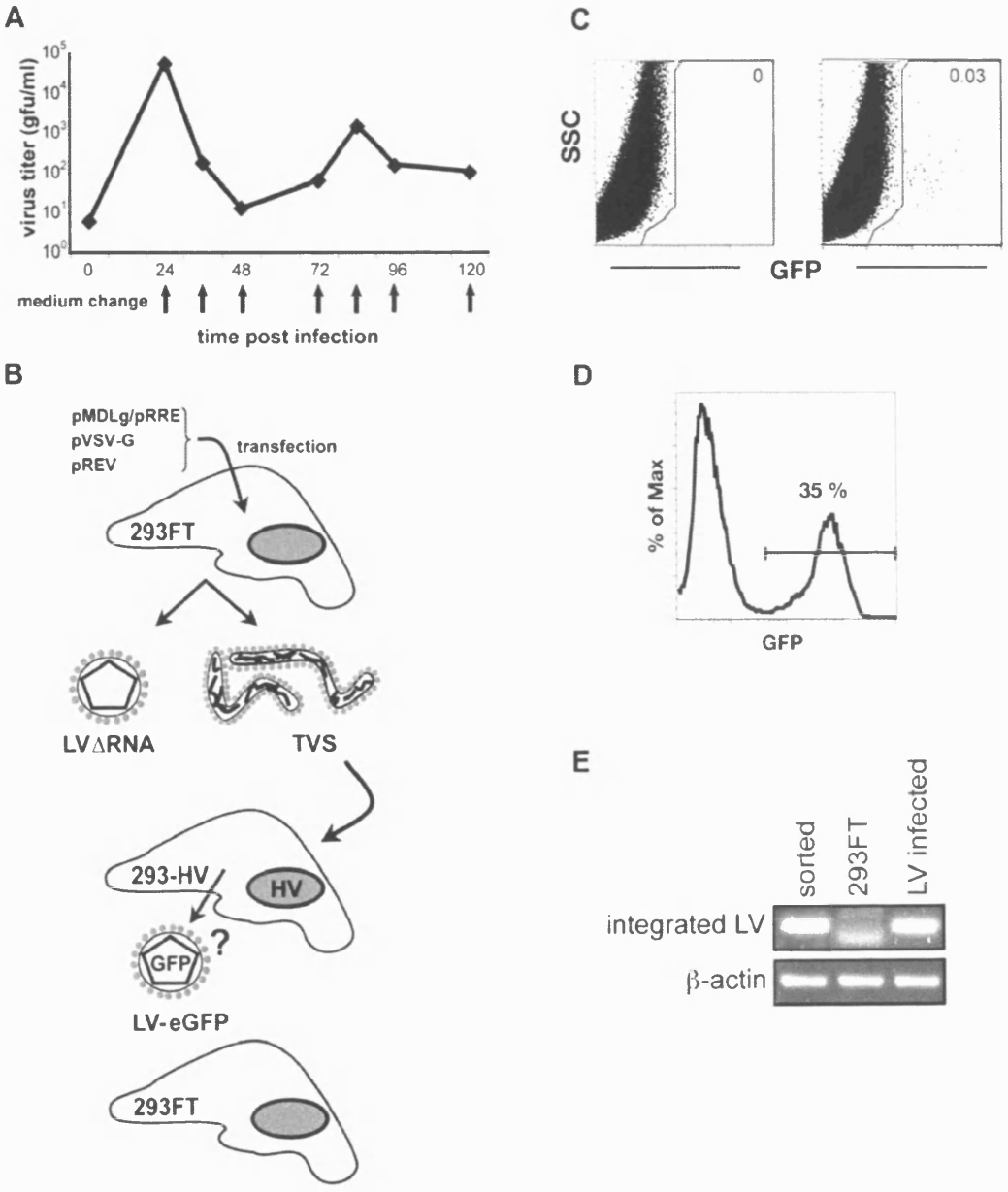

A
virus titer (gfu/ml)
10^5
10^4
10^3
10^2
10^1
10^0
0 24 48 72 96 120
medium change
time post infection
C
SSC
0
0.03
GFP
B
pMDLg/pRRE
pVSV-G
pREV
transfection
293FT
LVΔRNA
TVS
293-HV
HV
GFP
?
LV-eGFP
293FT
D
% of Max
35 %
GFP
E
sorted
293FT
LV infected
integrated LV
β-actin

Figure 3.2.9: LV preparations can transfer plasmid DNA to target cells leading to production of new LV particles

(A) 293T cells were infected with LV containing $2*10^8$ gfu LV. The medium was changed at the indicated time points (arrows) and tested for the presence of GFP-transducing virus. (B) Schematic outline of the experiment shown in (C): cells were transfected with 3 plasmids coding for the lentiviral capsid protein (pMDLg/pRRE), VSV-G (pVSV-G) and REV (pREV). Concentrated supernatant from these cells, containing both TVS and LV lacking viral genome (LVΔvRNA), was transferred onto 293HV cells that carry an integrated lentiviral genome coding for GFP. Newly generated eGFP expressing virus was assayed by monitoring GFP expression in 293FT indicator cells. (C) Supernatant of 293HV cells only (293HV) or 293HV cells exposed to LVΔRNA (293HV LVΔRNA) was added to 293T cells and GFP expression was assayed 72h later by flow cytometry. (D, E) GFP positive cells from (C) were sorted, expanded for 6 weeks and analysed by flow cytometry (D) or used to isolate genomic DNA to test the presence of integrated virus and β-actin by PCR (E). Untransduced and LV-infected 293FT cells were used as negative and positive controls, respectively (E). All experiments were repeated three times, one representative experiment is shown.

Figure 3.2.10

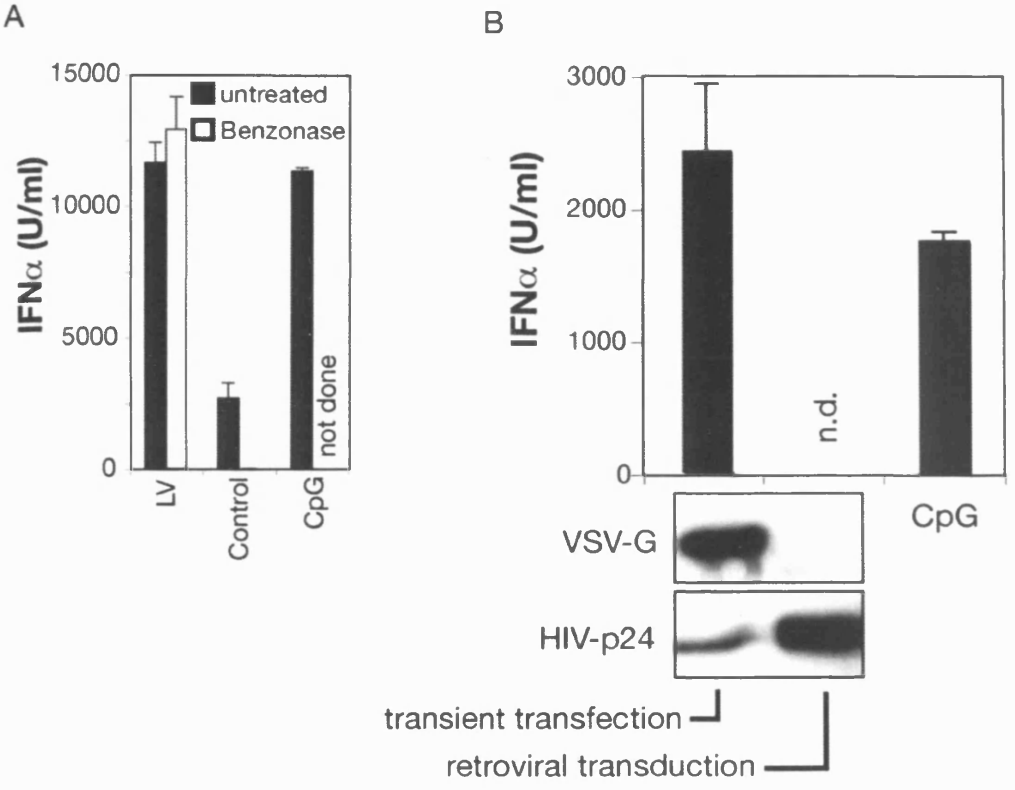


Figure 3.2.10: Avoiding transient transfection reduces IFN-α stimulatory activity of LV

(A) IFN-α production in BM after stimulation for 16h with LV, a control plasmid (pMSCFeGFP, control) or CpG (0.5µg/ml). Where indicated, LV and the control plasmid were pretreated with benzonase for 2h at 37°C (B) VSV-G was introduced into STAR-HV cells by transient transfection or by retroviral transduction. Supernatant containing VSV-G pseudotyped LV (MOI 0.1) was added to BM cells or used for western blot analysis. IFN-α was measured by ELISA 16 hours after treatment. CpG (0.5µg/ml) was used as a positive control. n.d., not detected. One of three experiments with similar results is shown.

Figure 3.2.11

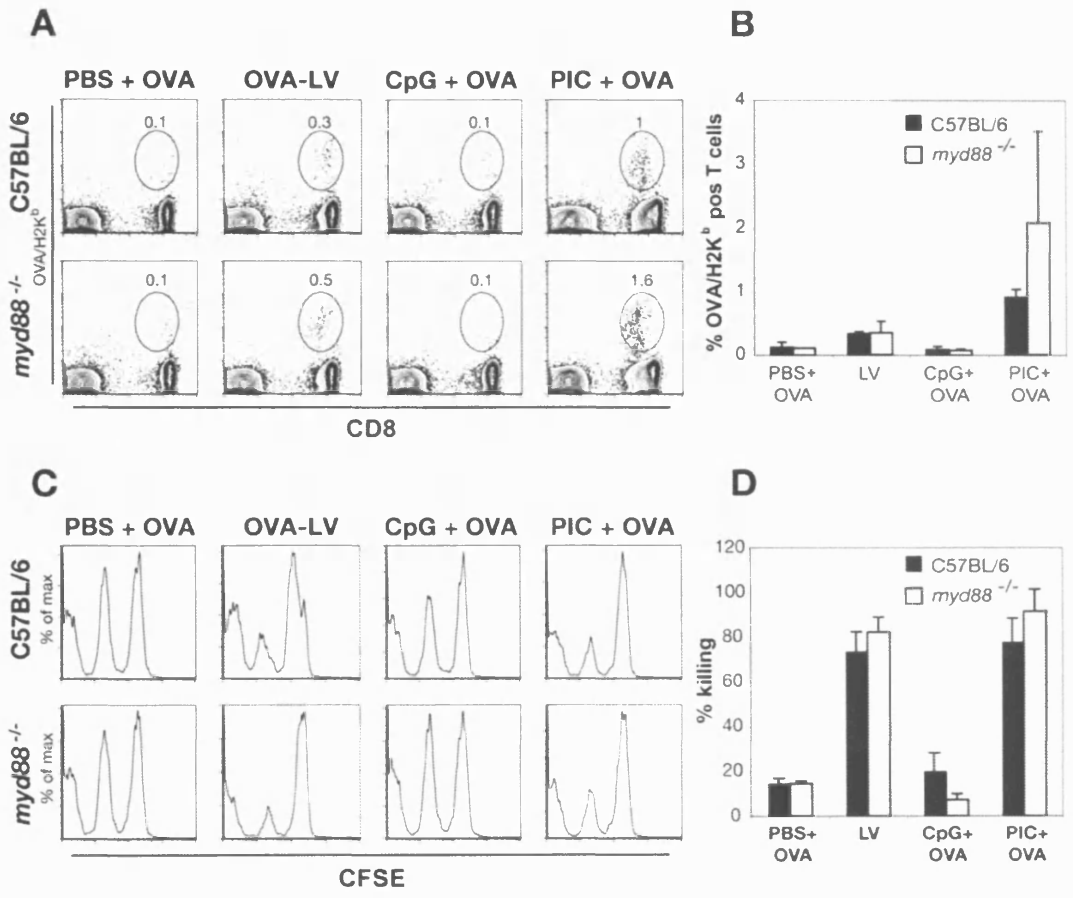


Figure 3.2.11: LV induce adaptive immune responses in the absence of MyD88

C57BL/6 and *myd88*$^{-/-}$ mice were immunised intravenously with OVA expressing LV (n = 4) or egg white in PBS (n = 2), CpG (n = 2) or poly-I:C (PIC; n = 2). (A) Contour plots show Thy1.2^{+} cells in blood of representative mice one week after immunisation. Numbers indicate the percentage of OVA/H-2K^{b} tetramer positive cells, as gated. The graph in (B) shows the average ± SD frequency of OVA/H2-K^{b} tetramer positive Thy1.2^{+} cells for all mice. (C, D) 10 days after immunization mice were challenged with congenic CD45.1 splenocytes loaded with 20nM (CFSE low), 200nM (CFSE intermediate) or 0nM (CFSE high) of OVA peptide (SIINFEKL). (C) Histograms show the CFSE profile of target cells (gated on CD45.1) from representative mice 48h after injection. (D) The graph shows the amount of specific killing of 200nM OVA peptide loaded splenocytes as compared to splenocytes without OVA peptide. The average of all mice ± SD is shown.

Figure 3.2.12

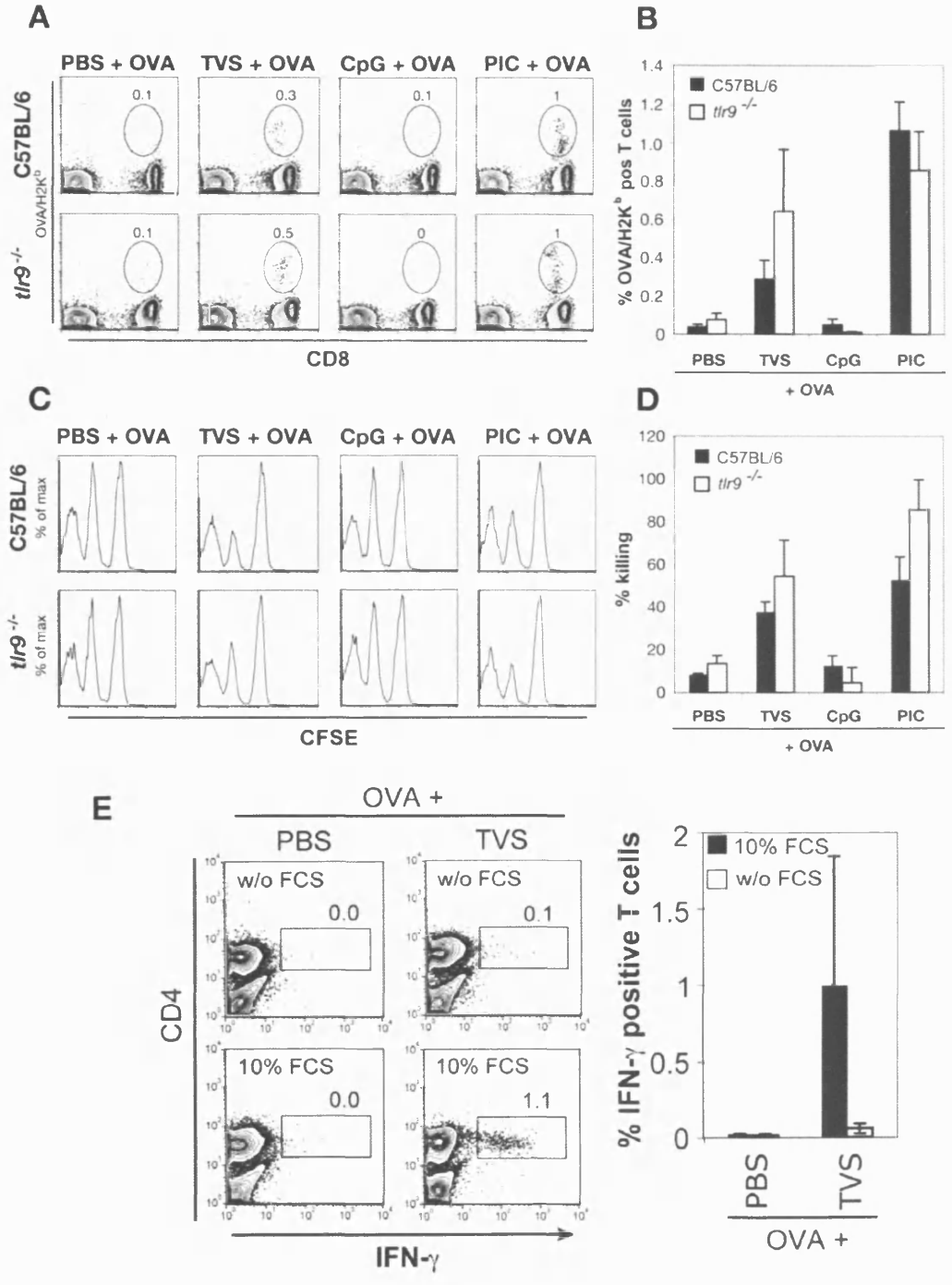

A
PBS + OVA
TVS + OVA
CpG + OVA
PIC + OVA
C57BL/6
tlr9 -/-
OVA/H2K^b
0.1
0.3
0.1
1
0.1
0.5
0
1
CD8
B
C57BL/6
tlr9 -/-
% OVA/H2K^b pos T cells
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
PBS
TVS
CpG
PIC
+ OVA
C
PBS + OVA
TVS + OVA
CpG + OVA
PIC + OVA
C57BL/6
% of max
tlr9 -/-
% of max
CFSE
D
C57BL/6
tlr9 -/-
% killing
120
100
80
60
40
20
0
PBS
TVS
CpG
PIC
+ OVA
E
OVA +
PBS
TVS
w/o FCS
0.0
w/o FCS
0.1
10% FCS
0.0
10% FCS
1.1
CD4
IFN-γ
10% FCS
w/o FCS
% IFN-γ positive T cells
2
1.5
1
0.5
0
PBS
TVS
OVA +

Figure 3.2.12

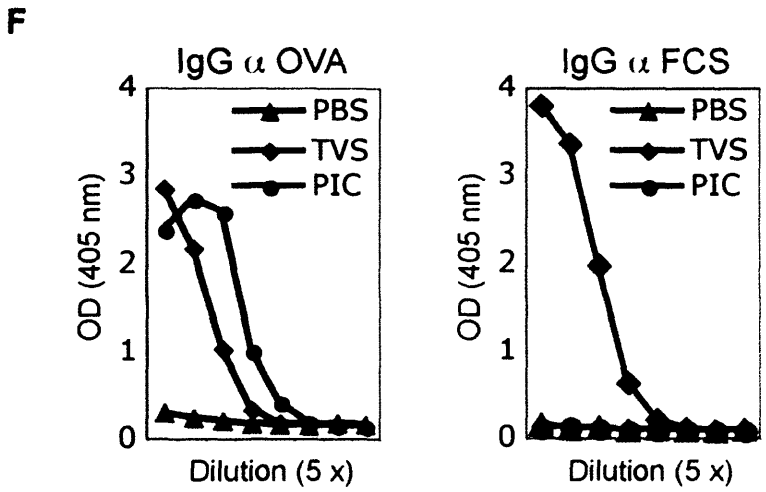


Figure 3.2.12: TVS act as adjuvant for adaptive immune responses

C57BL/6 and *tlr9*$^{-/-}$ mice were immunised intraperitoneally with egg white in PBS (n = 2) or with added TVS (n = 4), CpG (n = 2) or poly-I:C (PIC; n = 2). (A) Contour plots show Thy1.2^{+} cells in blood of representative mice one week after immunisation. The numbers represent the percentage of OVA/H-2K^{b} tetramer positive cells, as gated. The graph in (B) shows the average ± SD frequency of OVA/H2-K^{b} tetramer positive Thy1.2^{+} cells for all mice. (C, D) 10 days after immunization mice were challenged with congenic CD45.1 splenocytes loaded with 20nM (CFSE low), 200nM (CFSE intermediate) or 0nM (CFSE high) of OVA peptide (SIINFEKL). (C) Histograms show the CFSE profile of target cells (gated on CD45.1) from representative mice 48h after injection. (D) The graph shows the amount of specific killing of 200nM SIINFEKL-loaded splenocytes as compared to splenocytes not loaded with OVA. Shown is the average of all mice ± SD. (E) 12 days after immunization splenocytes of wt mice were isolated, cultured over night in the absence or presence of FCS and stained for intracellular IFN-γ. Contour plots show cells gated on Thy1.2. The numbers indicate frequency of IFN-γ positive Thy1.2^{+} CD4^{+}cells, as gated (average ± SD of all mice). (F) 12 days after immunization serum of wt mice was tested for the presence of specific antibodies against OVA and FCS. Data are displayed as titration curves from individual representative mice.

Figure 3.2.13

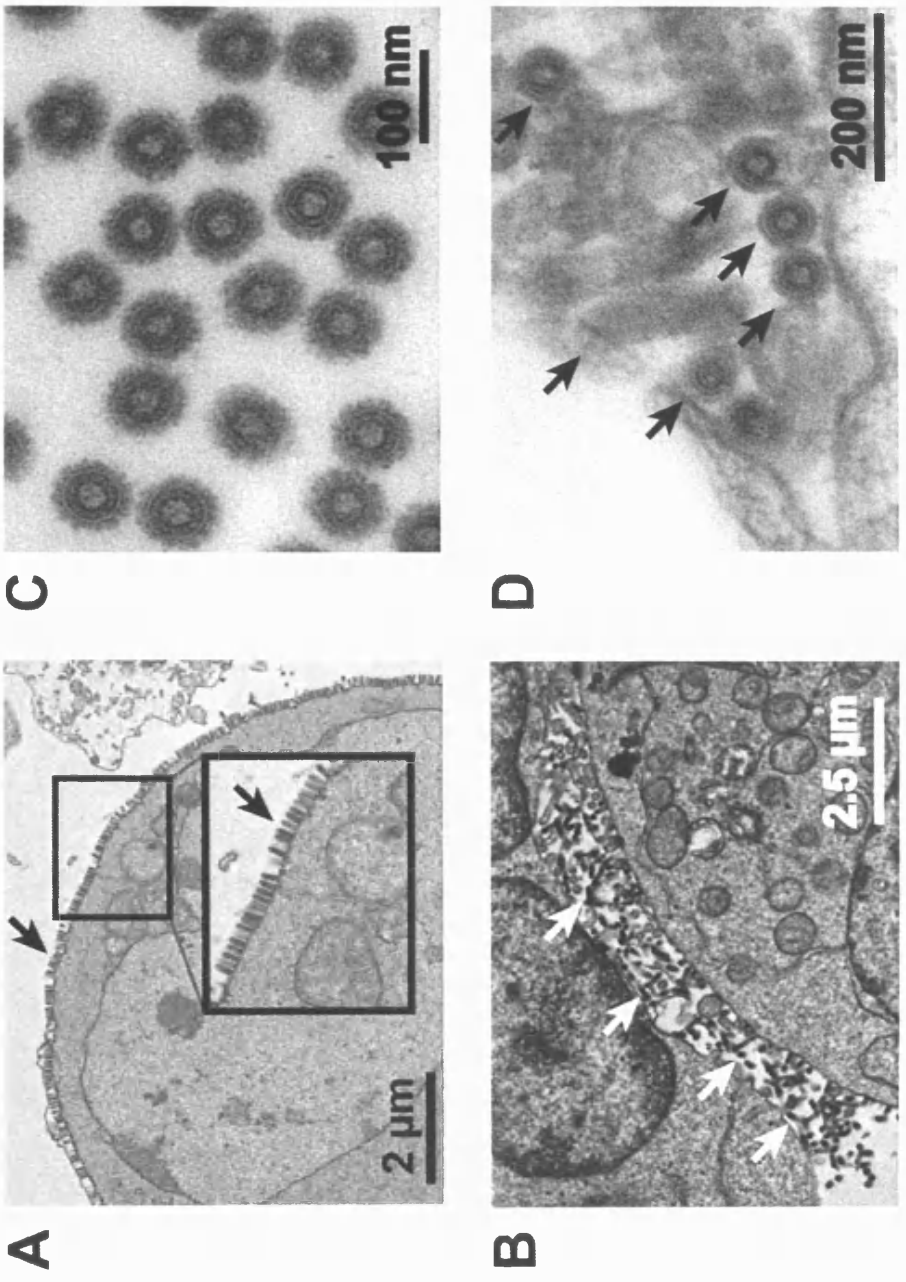


Figure 3.2.13: VSV does not generate detectable TVS

Representative electron micrographs of 293T cells infected with VSV for 24h. (A, B) low magnification. (A) budding of virus off the plasma membrane. Insert shows a further 2-fold magnification. (B) virus accumulates in intrercellular gaps. (C, D) Most particles have size and electron density characteristic for VSV. No TVS could be detected in VSV infected cells. Bars represent the indicated length. Arrows in (A), (B) and (D) point to virus particles.

Figure 3.3.1

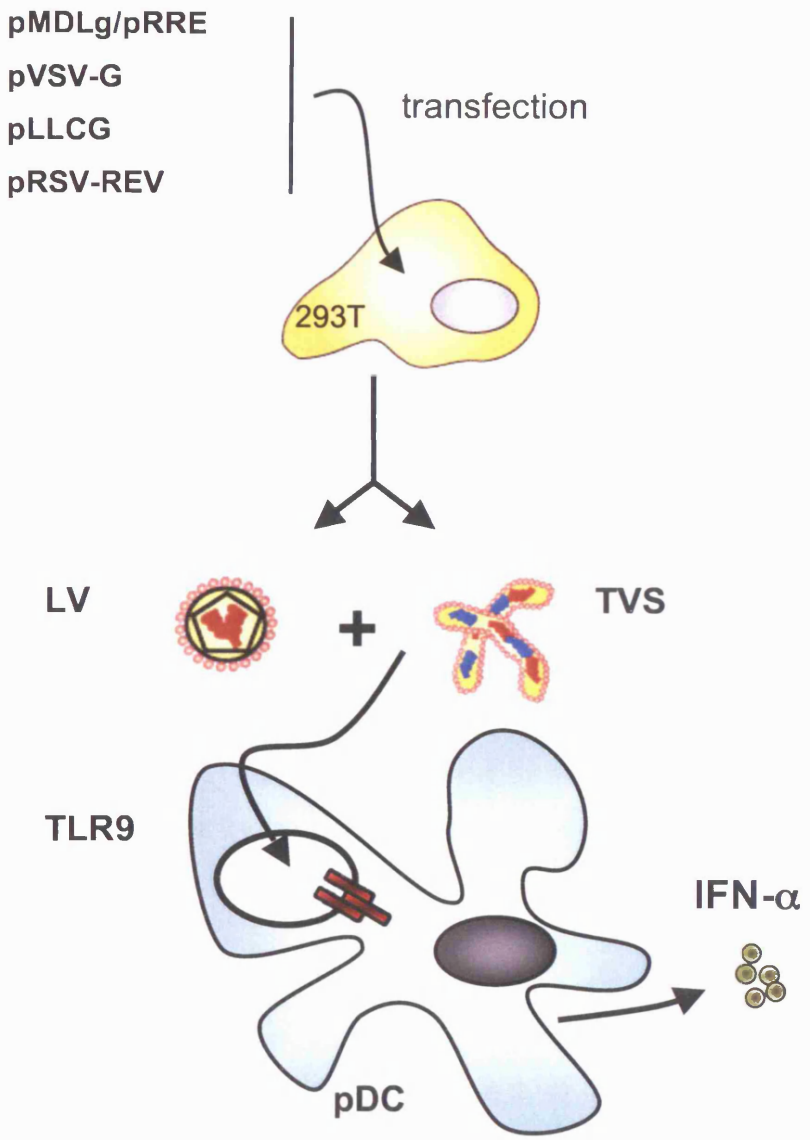


Figure 3.3.1: Model of IFN-α induction by LV preparations

293T cell transfection with plasmids commonly used for LV production results in generation of virus particles (LV) and tubulo-vesicular structures (TVS). The latter carry VSV-G on their surface and are delivered into the endosome of pDC. Plasmids (blue) present in TVS can activate TLR9 and this results in production of IFN-α.

CHAPTER 4: RIG-I is activated by viral RNA bearing 5' triphosphates

4.1. Introduction

dsRNA is believed to be the main virus associated pathogen associated molecular pattern recognised within infected cells (Akira et al., 2006). Intracellular delivery of dsRNA by lipofection or electroporation rapidly stimulates high amounts of IFN-α/β and other cytokines that are critical for inducing antiviral immunity (Diebold et al., 2003). Recently, RIG-I and MDA5 have been defined as critical pattern-recognition receptors (PRRs) for RNA viruses (Gitlin et al., 2006; Kato et al., 2005; Kato et al., 2006; Yoneyama et al., 2005; Yoneyama et al., 2004). Both proteins are capable of binding to poly-I:C but display distinct specificities for certain viruses. RIG-I senses viruses like NDV, VSV, Flu and SeV whereas MDA5 is activated by the picornaviruses EMCV and Theiler's virus (Gitlin et al., 2006; Kato et al., 2006). Likewise, RIG-I is necessary for inducing IFN-α/β in response to *in vitro* transcribed dsRNA whereas MDA5 is indispensable for recognition of poly-I:C (Gitlin et al., 2006; Kato et al., 2006). The reason for this discrepancy between both RNA-helicases was not known.

$CD8^+$ DC, a DC subset with special abilities to phagocytose apoptotic material, produce IFN-β and IL6 when co-cultured with cells that were infected with EMCV or SFV or pulsed with poly-I:C (Schulz et al., 2005). TLR3 present in endosomes of $CD8^+$ DC recognises dsRNA, which is thought to be commonly generated during virus replication. However, when Oliver Schulz in my laboratory co-cultured $CD8^+$ DC with Flu infected Vero-cells no IL6 production could be seen (Pichlmair et al., 2006). We hypothesised that this could be due to the influenza virus interferon antagonist NS1 as this protein is supposed to exert its function by sequestering dsRNA. However, cells infected with a mutant virus, lacking a functional NS1 protein (FluΔNS1) were still not able to activate $CD8^+$ DC although all Vero cells

were expressing Influenza virus nucleoprotein suggesting that the cells were homogenously infected (Pichlmair et al., 2006).

Possible explanations for the lack of TLR3 activation could be that dsRNA is not accessible in Flu infected Vero-cells or that replication of the Flu genome does not result in accumulation of dsRNA. To test the latter hypothesis, Oliver infected Vero cells and measured the content of intracellular dsRNA by ELISA (Pichlmair et al., 2006) and intracellular FACS (Fig 4.1.1A). As expected, EMCV and SFV infected Vero cells contained substantial amounts of dsRNA (Fig 4.1.1A and (Pichlmair et al., 2006)). Surprisingly, however, Flu and FluΔNS1 infected cells did not accumulate detectable amounts of dsRNA (Fig 4.1.1A and (Pichlmair et al., 2006)), despite the fact that they were uniformly infected (Fig 4.1.1B). This result is in line with a recent report from Friedemann Weber, stating that negative strand RNA viruses like Influenza or SeV do not generate detectable amounts of dsRNA (Weber et al., 2006). The lack of dsRNA production was not dependent on the infected cell line as this phenomenon was conserved in monkey fibroblasts (Vero), human fibroblasts (HEK293) murine fibroblasts (NIH3T3), and murine BM-DC (personal communication Oliver Schulz). We concluded that influenza virus infection appears to generate no or only marginal amounts of dsRNA during its infectious life cycle.

As dsRNA was considered to be the main stimulus for intracellular PRRs and we could not detect dsRNA in influenza virus infected cells, I set out to test whether ssRNA can elicit an IFN-α/β response.

4.2. Results

4.2.1. *Influenza virus lacking a functional NS1 protein is a potent IFN inducer*

dsRNA is reported to be critically involved in IFN-α/β production by the cytoplasmic PRRs RIG-I and MDA5 (Kato et al., 2006). As there was no or little detectable dsRNA in cells infected with FluΔNS1 I tested whether this virus induces IFN-α/β. I co-transfected HEK293 cells with a reporter plasmid that carried the firefly-luciferase gene under control of the IFN-β promoter and a plasmid coding for renilla-luciferase under the control of a constitutive promoter, which served as a transfection control. 24h later the cells were infected with FluΔNS1, SeV, EMCV and SFV at a comparable MOI. As reported previously (Garcia-Sastre et al., 1998), FluΔNS1 and SeV strongly activated the IFN-β promoter, whereas EMCV and SFV were less strong inducers of the IFN-β promoter (Fig 4.2.1). Oliver Schulz obtained similar results when he stimulated BM-DC: compared to EMCV and SFV, FluΔNS1 was always superior in inducing IFN-α from BM-DC (Pichlmair et al., 2006). The superiority of FluΔNS1 and SeV over EMCV and SFV could be explained by the fact that the latter viruses are wild-type strains that are very often bad inducers of IFN-α/β. However, I concluded that IFN-α/β production does not require accumulation of detectable amounts of dsRNA.

4.2.2. *The NS1 protein inhibits IFN-α/β production from some but not from all viruses*

As discussed in the introduction, the NS1 protein suppresses IFN-α/β production in virally infected cells and is important for efficient influenza virus replication in interferon competent systems (Diebold et al., 2003; Garcia-Sastre et al., 1998). The most accepted belief is that the RNA binding site of the NS1 protein sequesters dsRNA and thereby prevents induction of IFN-α/β (Diebold et al., 2003; Garcia-Sastre et al., 1998). As no dsRNA could be detected in Flu infected cells (Fig 4.1.1 and (Weber et al., 2006)), I analysed how the NS1 protein might function.

I asked whether NS1 has the ability to sequester dsRNA produced during an infection with EMCV and SFV and thereby suppresses IFN induction elicited by those viruses. As shown previously by others, transfection of a NS1 (derived from influenza strain A/PR8/34) expressing plasmid into HEK293 cells actively suppressed IFN-β luciferase production in FluΔNS1 and SeV infected cells (Hayman et al., 2007; Kochs et al., 2007; Talon et al., 2000), whereas an empty control plasmid was not able to do so (Fig 4.2.2). Surprisingly, however, NS1 showed remarkable specificity, as it appeared not to influence IFN-β promoter activation from EMCV or SFV (Fig 4.2.2). Notably, the former two viruses produce minimal amounts of dsRNA but strongly activated the IFN-β promoter whereas EMCV and SFV generate high levels of dsRNA but lower levels of IFN-β luciferase (Fig 4.1.1, Fig 4.2.1 and (Pichlmair et al., 2006; Weber et al., 2006)). Oliver Schulz obtained similar data by showing that a recombinant SFV expressing either the NS1 protein or an irrelevant protein induce similar amounts of IFN-α in BM-DC (Pichlmair et al., 2006).

Collectively, these data indicate that neither IFN-α/β induction nor the inhibitory effect of NS1 correlate with levels of dsRNA.

4.2.3. Flu NS1 co-localises and co-precipitates with RIG-I

Interestingly, recent studies have demonstrated that both SeV and FluΔNS1 are recognised via RIG-I whereas EMCV elicits responses via MDA5. I therefore investigated whether the virus-specific effects of NS1 correlated with its ability to interact with RIG-I. Consistent with this possibility, the NS1 protein co-immunoprecipitated with an eGFP tagged version of RIG-I when both proteins were expressed in 293T cells (Fig 4.2.3A). Similarly, NS1 and eGFP-RIG-I co-immunoprecipitated when influenza virus infection was used to introduce the NS1 protein into eGFP-RIG-I expressing 293T cells (Fig 4.2.3A). In addition, immunofluorescence staining revealed that in influenza virus infected 293T cells the cytoplasmic fraction of NS1 co-localised with transfected GFP-RIG-I (Fig 4.2.3B).

As NS1 was not able to block activation of the IFN-β promoter when a MDA5 dependent virus (EMCV) was used for stimulation, I investigated the possibility that NS1 specifically interacts with RIG-I but not with MDA5. Therefore I transfected

293T cells with HA tagged versions of RIG-I and MDA5 and subsequently infected those cells with Flu. As expected, HA-RIG-I could be co-immunoprecipitated with anti-NS1 antibody after influenza virus infection (Fig 4.2.3C). In contrast, MDA5 did not co-precipitate with NS1 (Fig 4.2.3C).
These results suggest that NS1 specifically targets RIG-I during Flu infection.

4.2.4. The ssRNA genome of Flu induces IFN-α and IL6

I considered how infection with Influenza virus might lead to RIG-I activation in the absence of detectable dsRNA and how NS1 interferes with this process. TLR-mediated virus recognition process evolved to recognise virus genomes delivered into endosomes and I speculated that the cytoplasmic recognition system might have evolved ways to sense cytoplasmic presence of virus genomes. Furthermore, RIG-I binds RNA, and I considered the possibility that RIG-I might recognise the influenza single-stranded RNA genome directly. Consistent with this hypothesis, BM-DC transfected with genomic RNA isolated from influenza virus particles (Flu vRNA) produced high amounts of IFN-α and IL6, comparable or superior to those elicited by poly-I:C (Fig 4.2.4A). Flu vRNA was unique in terms of IFN-α induction as other transfected RNAs like spleen mRNA, total bovine RNA, bovine t-RNA or E. coli tRNA did not elicit IFN-α above background (Fig 4.2.4B).
Cytoplasmic virus recognition is believed to critically depend on virus replication. However, genomes of negative strand RNA viruses are not infective and transfection cannot result in replication or production of progeny virus (Lamb and Krug, 2001). Consistent with this notion, 293HEK cells transfected with Flu vRNA did not produce Flu specific proteins (Fig 4.2.4C) as tested by western blot. Further, supernatant of the same cells did not contain cytopathic activity on Vero cells, suggesting the absence of a cytolytic virus like influenza (Fig 4.2.4D). Dominant interfering particles of the ssRNA virus VSV can contain dsRNA, which is believed to act as potent IFN-α/β inducer (Marcus and Sekellick, 1977). To exclude a possible dsRNA contamination of Flu vRNA I used acridine orange staining of Flu vRNA separated on an agarose gel, a method that has been used extensively in the past to easily discriminate between single- and double stranded nucleic acid (Lauretti et al., 2003). Acridine orange is a metachromatic stain resulting in green colour for

ds nucleic acid (dsRNA and dsDNA) whereas ss nucleic acid (ssRNA and ssDNA) appears flame-red when excited with UV light at 254 nm. In contrast to RNA preparations containing a dsRNA-genome of the bacteriophage (BRL 5907), Flu vRNA preparations appeared not to contain detectable amounts of dsRNA (Fig 4.2.4E).

I concluded that ssRNA from Influenza virus particles is sufficient to stimulate IFN-α/β. This IFN production does not require dsRNA or virus replication as generally assumed for activation of cytoplasmic PRRs.

4.2.5. IFN induction by Flu vRNA is blocked by NS1

As shown in Fig 4.2.2 the NS1 protein blocks IFN-α/β induction from Flu and SeV. I tested whether the IFN-α/β response to single-stranded Flu vRNA is sensitive to NS1. Compared to an appropriate control, expression of NS1 in 293T cells markedly reduced activation of the IFN-β promoter triggered by Flu vRNA transfection (Fig 4.2.5). A mutant NS1 (NS1 R38A K41A), which bears two point mutations in the dsRNA binding domain, loses its ability to bind dsRNA (Donelan et al., 2003; Min and Krug, 2006) and a recombinant influenza virus bearing the same point mutations in its NS1 has been reported to induce higher levels of IFN-α/β than a corresponding virus with an intact NS1 (Donelan et al., 2003). I considered the possibility that the NS1 protein may exert its activity by interacting with single stranded RNA and that this may require a functional dsRNA-binding domain. Consistent with this hypothesis wt NS1 was much more potent in blocking IFN-β promoter activation after Flu vRNA transfection than a mutant NS1 (NS1 R38A K41A) (Fig 4.2.5). The expression of wt and mutant NS1 was similar (Fig 4.2.5), excluding the possibility that the observed effect was simply due to differences in expression levels. Choon-Ping Tan in our laboratory found that wt NS1 but not the mutant NS1 was able to bind to beads coated with ssRNA (Pichlmair et al., 2006), demonstrating that NS1 can interact with ssRNA.

Collectively these data suggest that NS1 can bind to ssRNA and block IFN-α/β induction triggered by the ssRNA genome of influenza virus and both effects depend on the integrity of the dsRNA binding site.

4.2.6. *RIG-I is the cytoplasmic sensor for Flu vRNA*

NS1 binds to RIG-I and specifically blocks IFN-α/β triggered by the RIG-I dependent viruses Flu and SeV, which may suggest that responses blocked by NS1 are RIG-I dependent. Consistent with this, activation of the IFN-β promoter by Flu vRNA was inhibited by expression of a dominant negative RIG-I-helicase construct when compared to an empty control plasmid (Fig 4.2.6A). Also, reducing intracellular levels of RIG-I by means of siRNA in human 293HEK or mouse 3T3 cells reduced activation of the IFN-β promoter by Flu vRNA to comparable levels as the control SeV (Fig 4.2.6B). IFN-β triggered by EMCV, in contrast, was unchanged in cells that received siRNA for RIG-I, consistent with the notion that EMCV induces IFN-α/β via MDA5 (Fig 4.2.6B). SiRNAs were designed to be species specific; therefore hRIG-I siRNA served as unspecific control for mRIG-I siRNA in murine cells and vice versa in cells of human origin. Unspecific effects mediated through siRNA transfection could be excluded, as cells receiving unspecific siRNA were similarly responsive as cells not receiving siRNA (Fig 4.2.6C).
I concluded that RIG-I is responsible for sensing Flu vRNA.

4.2.7. *Similar to Flu vRNA, in vitro transcribed RNA induces IFN-α/β*

Flu vRNA is uncapped and contains phosphorylated 5' ends (5'PPP), a remnant of the transcription process. Notably, phosphorylated 5' termini in siRNA and ssRNA generated by viral RNA polymerases *in vitro* have been reported to induce IFN-α/β responses when transfected into cells (Kim et al., 2004). I confirmed that *in vitro* transcribed RNA elicits an IFN-α response when transfected into murine BM-DC or NIH3T3 cells (Fig 4.2.7A, B) and activates the IFN-β promoter in NIH3T3 cells and human HEK293 cells (Fig 4.2.7C, D). As reported, treatment of *in vitro* transcribed RNA with Calf intestinal phosphatase (CIP), which removes the 5' triphosphate group, completely abolished activation of the IFN-β promoter (Fig 4.2.7C, D). Long dsRNA generated by annealing of *in vitro* synthesised sense and antisense GFP-RNA induced IFN-α in NIH3T3 cells, as reported (Kato et al., 2006) (Fig 4.2.7E).

Surprisingly, IFN-α induction by dsGFP-RNA was highly sensitive to CIP treatment (Fig 4.2.7E).

From these experiments I concluded that, unlike any other RNA tested (Fig 4.2.4B), Flu vRNA and *in vitro* transcribed RNA are both inducing IFN-α/β when transfected into cells. *In vitro* transcribed ssRNA and *in vitro* generated dsRNA require the presence of a 5'triphosphate group to induce IFN-α/β.

4.2.8. 5' phosphates present on Flu vRNA are critically involved in IFN-α/β induction

I proceeded to test whether Flu vRNA recognition also depends on the presence of phosphorylated 5' ends. Treatment of Flu vRNA with CIP completely abrogated its stimulatory properties, whether tested in an IFN-β reporter assay (Fig 4.2.8A) or by IFN-α and IL6 induction in BM-DC (Fig 4.2.8B). To exclude that CIP was contaminated or has an unspecific effect on the RNA, I incubated Flu vRNA with CIP in the absence or presence of increasing amounts of inorganic phosphate or EDTA, which both act as phosphatase inhibitors. As expected, inhibiting CIP restored the stimulatory activity of Flu vRNA to levels seen without phosphatase treatment (Fig 4.2.8C). CIP did not affect the ability of vRNA to stimulate TLR7-dependent IFN-α production from BM, which contains pDC (Fig 4.2.8D), showing that CIP treatment does not unspecifically degrade the RNA. Further, this suggests that 5' phosphorylation is not required for TLR7 activation.

Choon-Ping Tan in our laboratory could show that RIG-I has higher binding affinity to beads coupled to 5' triphosphorylated RNA as compared to beads bearing unphosphorylated version of the same RNA (Pichlmair et al., 2006), which further suggests that RIG-I binds and senses 5' phosphates.

In conclusion, the RIG-I agonist Flu vRNA induces cytokine responses in a 5'phosphate dependent manner.

4.2.9. RIG-I activating virus genomes induce IFN-α in a 5'PPP dependent manner

Many other ssRNA viruses belonging to the family of negative strand RNA viruses have uncapped genomes (Ball, 2001; Lamb and Krug, 2001), including vesicular stomatitis virus (VSV) (Rose and Whitt, 2001) that is recognised via RIG-I (Kato et al., 2005; Kato et al., 2006). Similar to Flu vRNA, transfection of vRNA from VSV activated the IFN-β promoter in HEK293 cells (Fig 4.2.9A) and elicited IFN-α from BM-DC (Fig 4.2.9B), which was completely abrogated by CIP treatment (Fig 4.2.9A, B). Picornavirus genomes like the one of EMCV do not have a 5' tri-phosphate but a covalently bound protein at the 5' terminus (Ball, 2001; Racaniello, 2001). Consistent with the lack of 5' phosphates, EMCV is recognised via MDA5 instead of RIG-I (Gitlin et al., 2006; Kato et al., 2006) and EMCV vRNA failed to induce a response when transfected into cells at amounts comparable to Flu or VSV vRNA (Fig 4.2.9A). At high doses, however, transfection of EMCV vRNA elicited IFN-α in NIH3T3 cells (Fig 4.2.9C), as might be expected from the fact that vRNA from positive strand RNA viruses is infectious (Ball, 2001; Racaniello, 2001).

These data suggest that cells use RIG-I to recognise phosphorylated 5' ends of uncapped ssRNA viral genomes and that RNAs not recognised by RIG-I bear 5'modifications or lack a 5' triphosphate.

4.3. Discussion

The ability to sense viral presence is critical to initiate innate- and adaptive immunity to viral infection (Akira et al., 2006; Iwasaki and Medzhitov, 2004; Pichlmair and Reis e Sousa, 2007). dsRNA produced during viral replication and transcription or present in the genome of dsRNA viruses is thought to constitute the major target of the RIG-I and MDA5 RNA helicases involved in cytosolic viral sensing (Kawai and Akira, 2006; Meylan et al., 2006). The ability to produce dsRNA after virus infection was already recognised in the 70's when a couple of publications suggested the presence of dsRNA after infection with various viruses (Merigan, 1970). Notably, there are no reports of dsRNA production from negative strand RNA viruses like Flu and SeV. Indeed, when compared to some positive strand RNA viruses like SFV and EMCV, the negative strand RNA viruses influenza A and SeV generate undetectable amounts of dsRNA in infected cells, if any (Fig 4.1.1 and (Pichlmair et al., 2006; Weber et al., 2006)). As experiments done by Oliver Schulz (Fig 4.1.1 and (Pichlmair et al., 2006)) and by Friedemann Weber (Weber et al., 2006) are essentially based on the same technique using monoclonal antibodies raised against dsRNA one cannot exclude that the antibody is selectively binding to products generated by positive strand RNA viruses. However, the absence of immunodetectable dsRNA in influenza virus infected cells correlates with the inability to stimulate TLR3 in $CD8^+$ DC (Pichlmair et al., 2006), strengthening the point that influenza virus infected cells only contain little amounts of dsRNA. Despite the apparent absence of dsRNA, however, influenza virus can elicit a very potent innate immune response involving production of IFN-α/β (Fig 4.2.1) by activating the cytoplasmic sensor protein RIG-I (Kato et al., 2006).

In this chapter, I describe dsRNA independent activation of a cytoplasmic PRR that leads to production of IFN-α/β and could constitute a major innate stimulus in cells infected with influenza virus: When transfected into cells, the uncapped single stranded RNA genome isolated from influenza virus particles (vRNA) acts as a powerful stimulator of IFN-α/β and IL6 (Fig 4.2.4). Dominant interfering particles

of VSV are known to be strong inducers of the IFN system, a phenomenon originally explained by the presence of dsRNA in these preparations (Marcus and Sekellick, 1977). However, Flu vRNA did not contain detectable dsRNA when tested by acridine orange staining (Fig 4.2.4E). I could exclude new generation of dsRNA as transfection of genomes derived from negative strand RNA viruses does not result in expression of detectable virus proteins and accumulation of progeny virus (Fig 4.2.4D, E), consistent with the conception that negative strand RNA is not infectious (Ball, 2001; Lamb and Krug, 2001). Further, transfection of ssRNA generated by viral polymerases *in vitro* resulted in a similar IFN-α/β induction (Fig 4.2.7).

Expression of a dominant negative protein and siRNA knockdown experiments suggest that RIG-I is involved in sensing Flu vRNA (Fig 4.2.6). I cannot entirely exclude the possibility that other proteins are involved, as cells receiving siRNA for RIG-I were still able to activate the IFN-β promoter albeit to lesser extent (Fig 4.2.6B, C). This is likely to be explained by insufficient reduction of the endogenous protein level. Testing this proved to be difficult as commercially available antibodies did not detect endogenous RIG-I (data not shown). It would be possible to clarify the requirement for RIG-I by using cells from mice genetically engineered to lack RIG-I (Kato et al., 2005).

I demonstrate that vRNA from influenza virus is not unique in its ability to activate RIG-I as vRNA from VSV, another RIG-I-dependent ssRNA virus, behaves in a similar manner (Fig 4.2.9B, C). Notably, the response to both vRNAs is abrogated by dephosphorylation with CIP, suggesting that RIG-I recognises viral 5' phosphorylated ssRNAs (Fig 4.2.8 and Fig 4.2.9B, C). I excluded the possibility that unspecific effects of the dephosphorylation process could influence IFN-α/β induction as CIP treated RNA was still able to stimulate TLR7 responses in BM (Fig 4.2.8D) and blocking CIP by phosphatase inhibitors preserved the stimulatory potential of vRNA (Fig 4.2.8C).

Transcription of 5' triphosphorylated RNA is not uncommon as all cellular polymerases transcribe RNA bearing 5' triphosphates. The absence of RIG-I from the nucleus (Fig 4.2.3B) and the requirement of signalling molecules partly tethered to mitochondria may explain why IFN responses are not initiated. Intriguingly, the 5'PPP is very often lost during processing and modification of cellular RNA.

Messenger RNA (mRNA), for instance, is transcribed by the cellular polymerase-II (pol-II) and enzymes that are part of the pol-II transcription complex mediate 'RNA-capping', a process that results in covalent binding of a 7-mehtyl guanosine nucleotide (Cap) at the 5' end of the RNA, therefore masking the 5' triphosphate group (Alberts et al., 2002). A nuclear protein, CRM1 (also called exportin 1), is involved in exporting large RNA fragments but this only happens in the presence of a Cap (Alberts et al., 2002). It is feasible to speculate that the nuclear RNA export machinery does not allow potential stimulatory RNA (bearing 5' triphosphates) to enter the cytoplasm and therefore contributes to avoid recognition of self-RNA. Transfer RNA (tRNA) is processed from a larger precursor tRNA that must be trimmed and covalently altered before allowed to exit from the nucleus (Alberts et al., 2002; Xiao et al., 2002). Therefore tRNA only bears a 5' monophoshate and this has been shown not to be sufficient to activate RIG-I (Hornung et al., 2006). Similar to tRNA, ribosomal RNA (rRNA), that represents the most abundant RNA species in the cell, undergoes a processing step before leaving the nucleus. Most rRNA subunits are transcribed as a single 13 kilo Bases precursor RNA that is cleaved and subject to extensive chemical modifications before leaving the nucleus (Alberts et al., 2002). Such chemical modifications include methylation of the 2'OH positions on nucleotide sugars and isomerization of uridine to pseudouridine nucleotides. Functions of these modifications are not understood but are thought to aid folding and assembly of the final rRNAs (Ferre-D'Amare, 2003). However, Hornung et al. and others (Hornung et al., 2006; Kariko et al., 2005) showed very elegantly that the presence of pseudouridine and other nucleotide modifications found in mammalian RNA abolishes IFN induction despite the presence of a 5' triphosphate. Notably, the 5S rRNA found in the cytoplasm carries a methyl group on the γ-phosphate of the 5' RNA terminus and I would expect that this kind of RNA might not allow RIG-I activation. Clearly, chemical modification of cellular RNA within the nucleus contributes to avoid initiation of antiviral processes elicited by cellular RNA.

Interestingly, in my hands, GFP-tagged RIG-I appears to reside in the cytoplasm (Fig 4.2.3B), which could be of importance to ignore newly transcribed 5'PPP-RNA generated in the nucleus. One could even speculate that orthomyxoviruses like influenza exploit this 'ignorance' by replicating in the nucleus (Lamb and Krug,

2001), although this may also have other reasons like the use of the cellular splicing machinery in order to process viral RNA (Lamb and Krug, 2001).

In vitro studies suggest that virus delivery into cells can induce IFN-α/β in the absence of virus replication and independent of TLR recognition (Collins et al., 2004; Hidmark et al., 2005; Isaacs and Lindenmann, 1957). Remarkably, IFN was originally discovered as a factor that is produced from chorio-allantois membranes infected with heat inactivated influenza virus (Isaacs and Lindenmann, 1957). As heat inactivated influenza virus cannot replicate I would suspect that the virus genome was the initiator for IFN production. Entry of virus particles therefore can deliver stimulatory nucleic acid that can be sensed by cytoplasmic PRRs. However, it is questionable whether the amount of virus entering a cell during a natural infection is sufficient to activate an IFN response and I would rather expect that virus replication is necessary to amplify the agonist.

Orthomyxoviruses such as influenza generate two types of RNA. Viral mRNA is capped at the 5' end because the viral polymerase actively scavenges host cell mRNA caps to use as primers for mRNA synthesis (Krug et al., 1979; Lamb and Krug, 2001). At later stages of the infectious cycle, the polymerase switches to making primer-independent full-length transcripts of template RNA which are then replicated into vRNA, again in a primer-independent manner (Lamb and Krug, 2001). This primer-independent replication process results in 5' triphosphorylated genomic RNA (Ball, 2001; van Dijk et al., 2004) and other RNA species of shorter length (personal communication George Brownlee). However, influenza virus RNA requires accessory viral proteins to exit the nucleus: The nucleoprotein (NP) of influenza virus binds to virus RNA and at the same time can interact specifically with CRM1, the nuclear export factor that is needed for mRNA transport through the nuclear pore (Elton et al., 2001). Thus, through direct interaction with cellular proteins involved in nuclear export, the triphosphorylated virus genome and possibly other viral RNA species gain access to the cytoplasm. However, whether the viral RNA genome or other viral RNA species are recognised by RIG-I is currently unknown. In fact, the virus polymerase complex is still attached to the 5' end of the virus' genomic segments and may potentially protect the genomic viral RNA from being recognised by RIG-I (Basler and Garcia-Sastre, 2007). In that regard, it was

shown that viral transcripts are produced early in the replication cycle of measles virus and these may be sensed via RIG-I (Plumet et al., 2007).

For influenza virus, the viral RNA (i.e. the viral genome and possibly other RNA species) exported from the nucleus, contains uncapped 5' ends bearing tri-phosphorylated ribonucleotides (Lamb and Krug, 2001). My data would indicate that this type of RNA can be recognised by cytosolic RIG-I unless this is prevented by the NS1 protein (Fig 4.2.2). Others and myself found that the NS1 protein binds and inhibits RIG-I dependent interferon responses (Fig 4.2.2)(Guo et al., 2007; Mibayashi et al., 2007). The dsRNA-binding site of NS1 appears to be important to exert this activity as a dsRNA binding mutant (NS1 R38A K41A) partly looses its interferon antagonistic function (Pichlmair et al., 2006). Interestingly, Choon-Ping Tan could show that association of NS1 with ssRNA also requires a functional dsRNA-binding domain (Pichlmair et al., 2006). It is feasible to speculate that NS1 may be recruited to nascent viral RNA molecules present within the nucleus and the NS1 protein could accompany the viral RNA into the cytosol. This model may be supported by the fact that NS1 is found predominantly in the nucleus and in the cytosol of infected cells (Fig 4.2.3 B) and that a dsRNA-binding mutant NS1 (A/WSN/33) has cytoplasmic rather than nuclear localisation when expressed in the context of a virus infection. Although the mechanism by which NS1 inhibits RIG-I activation is not known, the two proteins can be found in a complex (Fig 4.2.3A, C) and it is therefore possible that NS1 prevents RIG-I from accessing the 5' triphosphate. Although expression of RIG-I and NS1 is sufficient to form a complex (Fig 4.2.3A), the presence of 5'PPP RNA significantly enhances its stability (Pichlmair et al., 2006). The co-immunoprecipitation results could be explained by various models: (i) As NS1 and RIG-I can bind ssRNA the reason for them co-immunoprecipitating could simply be based on both proteins binding the same RNA molecule. This is an unlikely scenario, as it would not explain why NS1 inhibits RIG-I responses (Fig 4.2.2). Alternatively, (ii) the NS1 protein could bind first to triphosphorylated RNA and thereby prevent RIG-I activation. (iii) NS1 could be recruited to a complex of RIG-I and 5'PPP RNA in a dsRNA binding domain dependent manner and thereby disturb the function of RIG-I. (iv) RIG-I changes its conformation and exposes the CARD after binding to its ligand (Saito et al., 2007). It

is possible that the NS1 is recruited to the exposed CARD and thereby prevents activation of the downstream signalling molecule IPS-1. The latter hypothesis would also be supported by the fact that NS1 can block IFN induction elicited by expression of the RIG-I CARD domain (Mibayashi et al., 2007). Collectively, my data and published observations suggest that the binding of RIG-I and NS1 can occur in a steady state (Fig 4.2.2 and (Mibayashi et al., 2007)) but that the presence of a RIG-I agonist can facilitate this association (Pichlmair et al., 2006). I want to emphasise at this stage that all my experiments were done with the NS1 protein of the PR8 strain of influenza virus (A/PR8/34) and that strain specific differences may occur (Kochs et al., 2007). Like many other IFN inhibitors (Andrejeva et al., 2004; Garcia et al., 2007; Kim et al., 2003; Smith et al., 2001), the NS1 protein definitely has multiple functions.

The presence of 5' triphosphates on virus genomes is a remnant of the virus replication process. Viruses that replicate and transcribe their genome in a primer-independent manner bear 5'PPP (van Dijk et al., 2004). In contrast, picornaviruses such as EMCV, use a small protein primer that covalently binds the 5' end of the vRNA via a O^4(5'-uridylyl) tyrosine linkage and thereby protect the uncapped 5' uridylic acid (Ball, 2001; Racaniello, 2001). The unavailability of a phosphorylated 5' end may explain why the vRNA of picornaviruses such as EMCV only elicits a minimal response when transfected into cells (Fig 4.2.9A, C) and, therefore, why the virus cannot be recognised via RIG-I (Gitlin et al., 2006; Kato et al., 2006). Other viruses like some members of the *bunyaviridae* and *bornaviridae* use a different replication strategy that results in a genome bearing only monophosphates (Kolakofsky and Hacker, 1991; Schneider et al., 2007) and these virus genomes do not activate RIG-I (personal communication Friedemann Weber). The replication mechanisms used by these viruses may constitute a way to subvert the IFN-α/β response and could be of eminent importance for viruses that do not have known interferon antagonistic proteins. It may be that some persisting viruses like Borna disease virus evolved this mechanism to remain undetected from the innate immune surveillance (Schneider et al., 2007).

One might wonder why NS1-containing influenza virus, which blocks RIG-I activation, does not engage the MDA5 pathway like EMCV? As shown here and

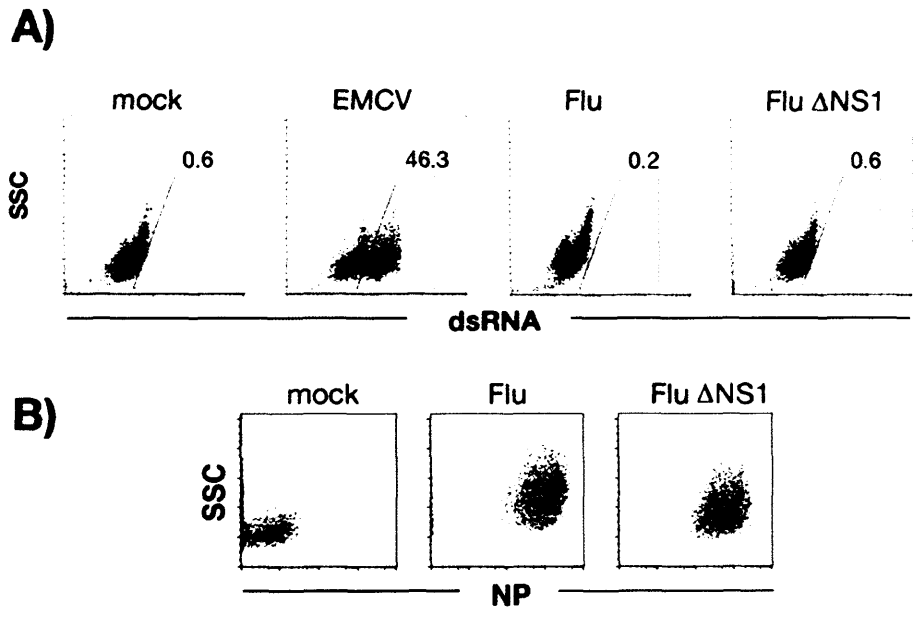
elsewhere, the answer could lie in the differential ability of the two viruses to generate dsRNA (Fig 4.1.1 and (Pichlmair et al., 2006; Weber et al., 2006)). MDA5 mediates responses to poly-I:C (Gitlin et al., 2006; Kato et al., 2006) and may therefore act as a dsRNA sensor, which would mean that it can only be activated by dsRNA-generating viruses such as EMCV. This notion is complicated by the lack of IFN induction from de-phosphorylated *in vitro* transcribed dsRNA (Fig 4.2.7E), which suggests that MDA5 may recognise dsRNA in the context of another, yet undefined virus specific structure that may also be found in poly-I:C. Notably, RIG-I does not mediate IFN-α/β responses to poly-I:C *in vivo* (Gitlin et al., 2006; Kato et al., 2006; Rothenfusser et al., 2005; Yoneyama et al., 2005; Yoneyama et al., 2004) and its reported ability to recognise *in vitro* synthesised dsRNA (Kato et al., 2006) is probably due to the presence of triphosphates at the 5' terminus rather than the double stranded nature of the stimulus (Fig 4.2.7E). It is therefore tempting to speculate that MDA5 recognises virally produced dsRNA whereas RIG-I acts primarily as a ssRNA sensor (Fig 4.3.1). However, further work is needed to elucidate the role dsRNA structures for RIG-I activation.

The entry of ssRNA viruses into cells can trigger innate responses in the absence of virus replication (Collins et al., 2004; Hidmark et al., 2005; Isaacs and Lindenmann, 1957) and synthetic ssRNA has long been know to induce IFN (Baron et al., 1969; Billiau et al., 1969). Yet, the role of ssRNA in activating cytosolic PRRs has been largely ignored until a recent report highlighted the IFN-inducing potential of synthetic RNAs bearing 5' phosphates (Kim et al., 2004). Here I propose that virus recognition in the cytoplasm can be accomplished by RIG-I sensing of ssRNA with 5' phosphorylated termini (Fig 4.3.1). Of course, it remains possible that the activation of RIG-I by ssRNA is potentiated by local double stranded regions, such as the panhandle secondary structures found at the ends of the influenza genome and local dsRNA formation of the non-translated region of HCV (Lamb and Krug, 2001; Saito et al., 2007). Likewise, even though NS1 can bind ssRNA, I do not exclude that local dsRNA formation contributes to its inhibitory activity (Wang et al., 1999). Nevertheless, my results argue for an ability of the innate immune system to sense uncapped ssRNA in the cytoplasm. Added to the recent discovery of mechanisms for sensing cytoplasmic DNA (Ishii et al., 2006; Stetson and Medzhitov, 2006a; Yasuda

et al., 2005) via DAI and possibly other proteins (Takaoka et al., 2007), this finding extends our understanding of the repertoire of antiviral defense strategies and suggests a remarkable parallel between cytosolic and endosomal viral recognition, with MDA5, RIG-I and the cytosolic DNA PRRs constituting functional homologues of TLR3, -7/8 and -9.

4.4. Figures

Figure 4.1.1



This figure was kindly provided by Oliver Schulz

Figure 4.1.1: Virus infection does not necessarily generate dsRNA

Vero cells were left uninfected or infected with influenza virus (Flu), influenza virus lacking the NS1 protein (FluΔNS1) or EMCV for six hours. Cells were then stained for dsRNA (A) or Flu nucleoprotein (NP) (B). This Figure was kindly provided by Oliver Schulz.

Figure 4.2.1

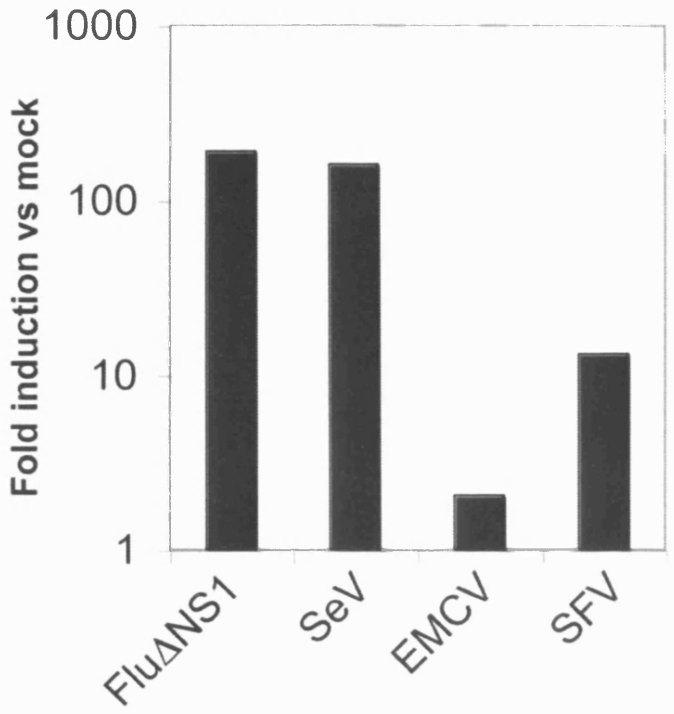


Figure 4.2.1: Activation of the IFN-β promoter by various viruses

HEK293 cells were co-transfected with an IFN-β firefly luciferase reporter plasmid and a control plasmid encoding renilla luciferase under control of a constitutive promoter (see material and methods for details). 24h later cells were left uninfected or infected with the indicated viruses at a MOI of 1 and luciferase activity analysed 16h later. All reporter assays in this thesis show the fold induction of the ratio between firefly- and renilla luciferase from stimulated cells as compared to the ratio of uninfected or untreated cells. One representative of three independent experiments is shown.

Figure 4.2.2

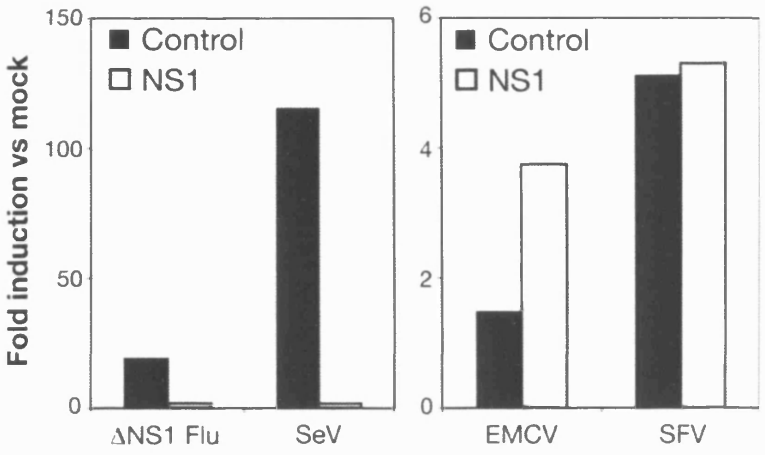


Figure 4.2.2: Flu NS1 inhibits activation of the IFN-β promoter in a virus specific manner

HEK293 cells co-transfected with IFN-β reporter plasmids together with NS1-encoding plasmid or control empty vector, as indicated. 24h later cells were infected with the indicated viruses at an MOI of 1 and luciferase activity analysed 16h later. Shown is the fold induction of the ratio between firefly- and renilla luciferase as compared to the ratio of uninfected cells. One representative of four independent experiments is shown.

Figure 4.2.3

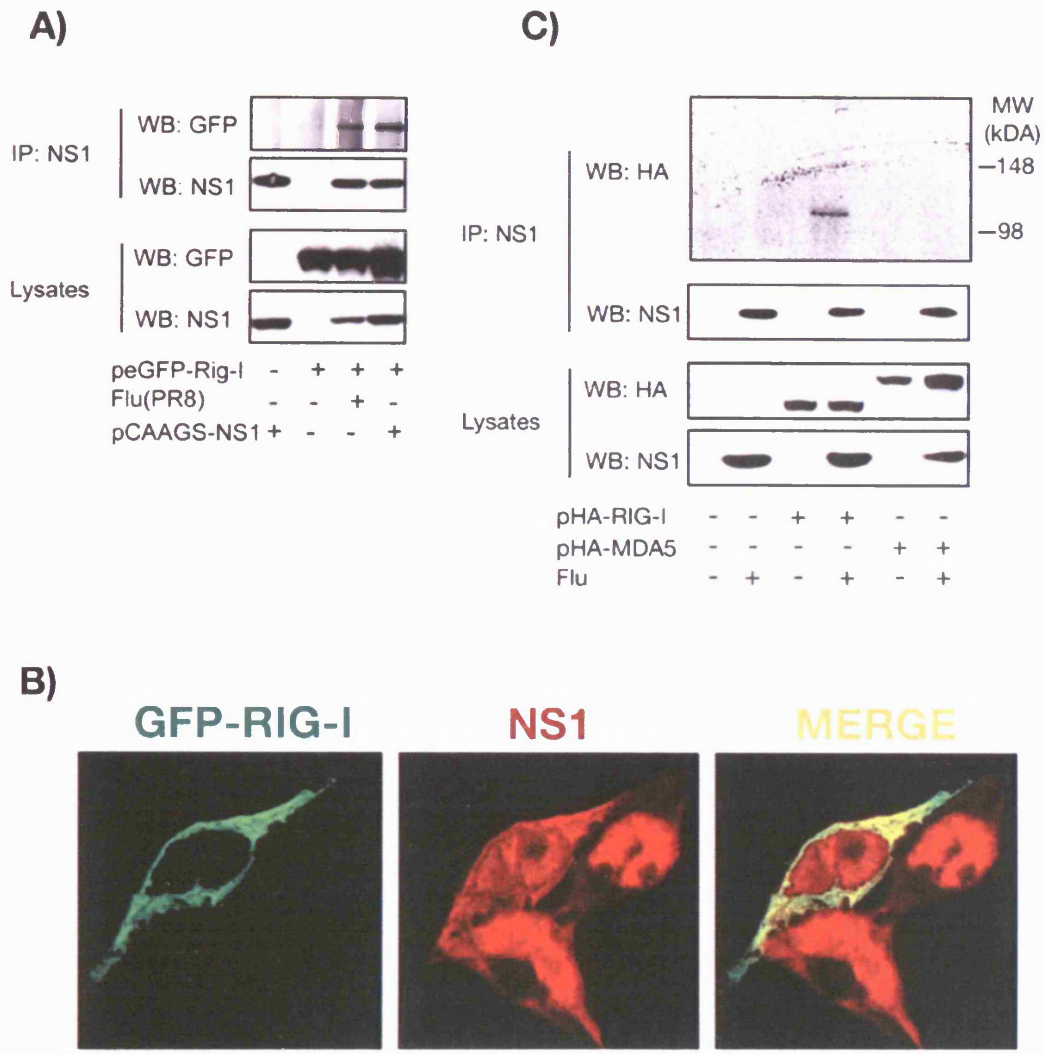


Figure 4.2.3: Flu NS1 associates with RIG-I

293T cells were transiently transfected with pGFP-RIG-I (A) or with pHA-RIG-I or pHA-MDA5 (C) and 12h later infected or not with influenza virus (MOI 1), as indicated. At 24h, cells were lysed and lysates subjected to immunoprecipitation with an anti-NS1 antibody. (A) and (C) show western blots (WB) for the presence of NS1 and GFP (A) or NS1 and HA (C) in total cell lysates (lower panels) or after immunoprecipitation (IP) with anti-NS1 antibody (upper panels). All detected proteins had the expected molecular size. (B) Confocal analysis of 293T cells transfected with pGFP-RIG-I for 16h and infected with influenza virus (MOI 2) for another 24h. Cells were fixed and stained for NS1. Panels show GFP-RIG-I (green, left panel), NS1 (red, middle panel) and the merged image (yellow, right panel).

Figure 4.2.4

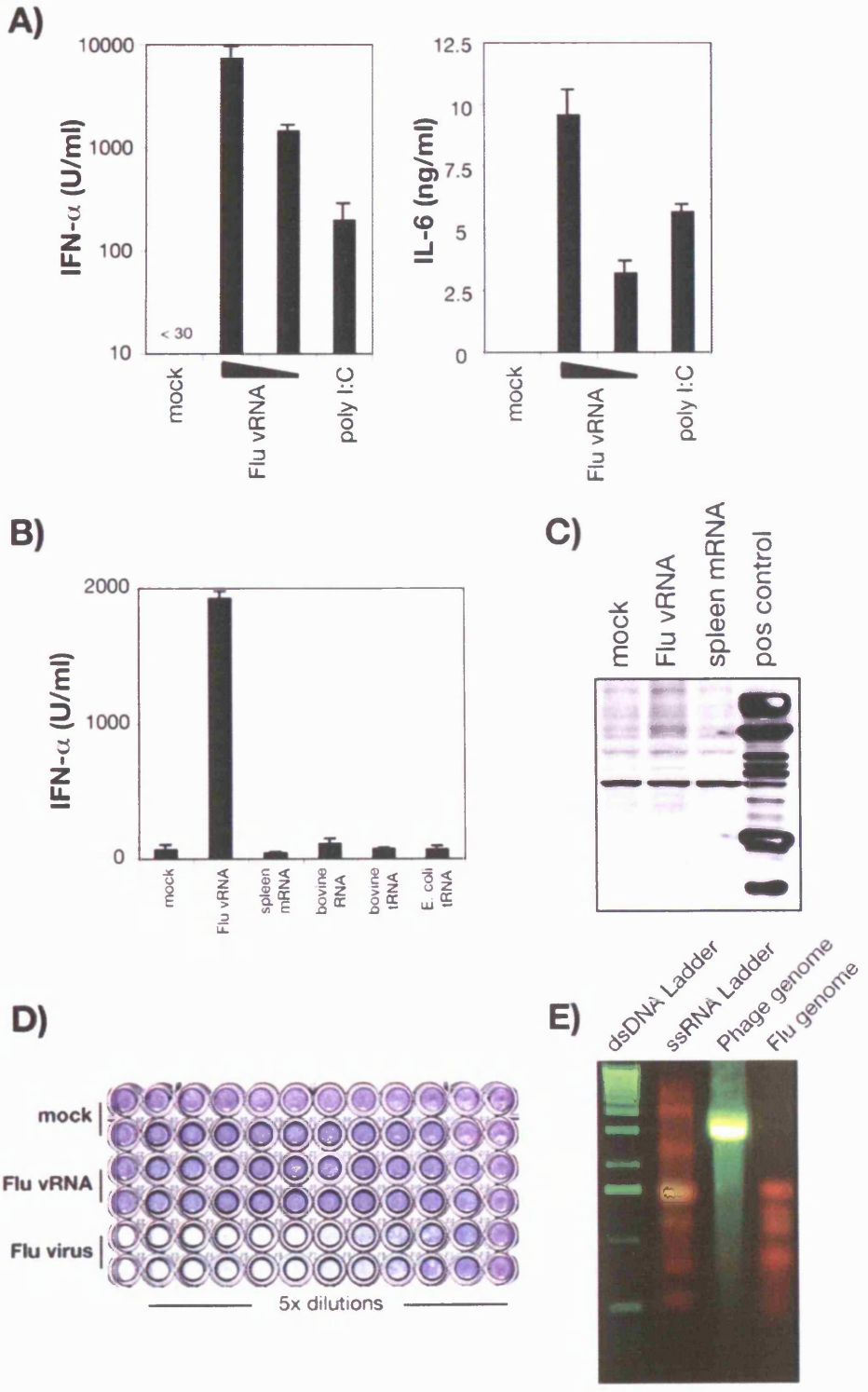

A)
IFN-α (U/ml)
10000
1000
100
10
< 30
mock
Flu vRNA
poly I:C
IL-6 (ng/ml)
12.5
10
7.5
5
2.5
0
mock
Flu vRNA
poly I:C
B)
IFN-α (U/ml)
2000
1000
0
mock
Flu vRNA
spleen mRNA
bovine RNA
bovine tRNA
E. coli tRNA
C)
mock
Flu vRNA
spleen mRNA
pos control
D)
mock
Flu vRNA
Flu virus
5x dilutions
E)
dsDNA Ladder
ssRNA Ladder
Phage genome
Flu genome

Figure 4.2.4: The ssRNA genome of influenza virus induces IFN-α/β and IL6

(A, B) BM-DC were mock treated or transfected with Flu vRNA (1μg and 0.2μg) and poly-I:C (0.5μg), respectively, (A) or with 0.2μg of the indicated RNAs (B). IFN-α (A, B) and IL6 (A) were measured by ELISA after over night incubation. n.d., not detectable. (C, D) 293HEK cells were transfected with 0.2μg of the indicated RNA or infected with influenza virus (MOI 1). 16h later supernatant was collected for (D) and cells lysed for western blot to detect viral proteins using a polyclonal antibody raised against influenza virus (C). (D) Supernatant of transfected cells (mock, Flu vRNA) or control influenza virus (Flu virus) was serially diluted and used to treat Vero cells. Shown are cells stained with giemsa 48h after treatment. (E) Acridine orange staining of the indicated nucleic acid separated on a 1% agarose gel. The blue channel has been removed by Adobe Photoshop. All shown experiments have been repeated for three or more times.

Figure 4.2.5

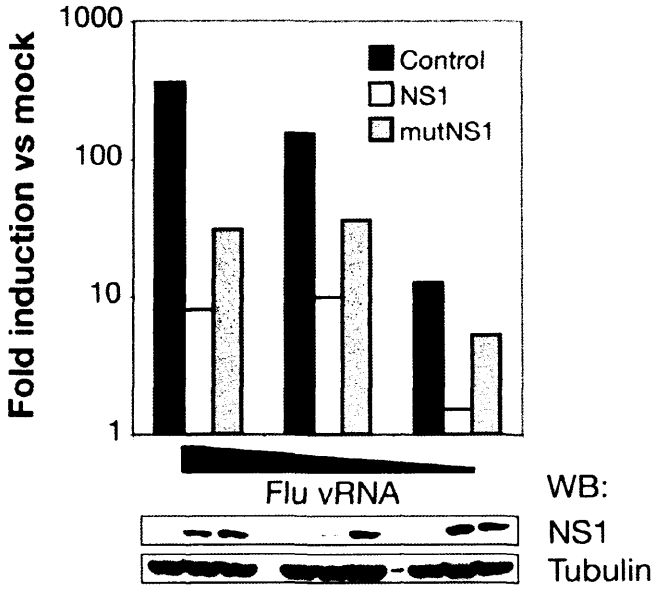


Figure 4.2.5: NS1 inhibits IFN-α/β induction elicited by Flu vRNA

Induction of IFN-β luciferase activity in HEK293 cells co-transfected with IFN-β reporter plasmids and NS1 expression plasmids (NS1 or mutNS1) or empty vector (control). 24h later, cells were transfected with flu vRNA (0.2, 0.04 or 0.008μg). Cell lysates were prepared at 38h and used to measure luciferase activity and perform western blot for NS1 and a loading control (Tubulin). The experiment was done three times, one representative experiment is shown.

Figure 4.2.6

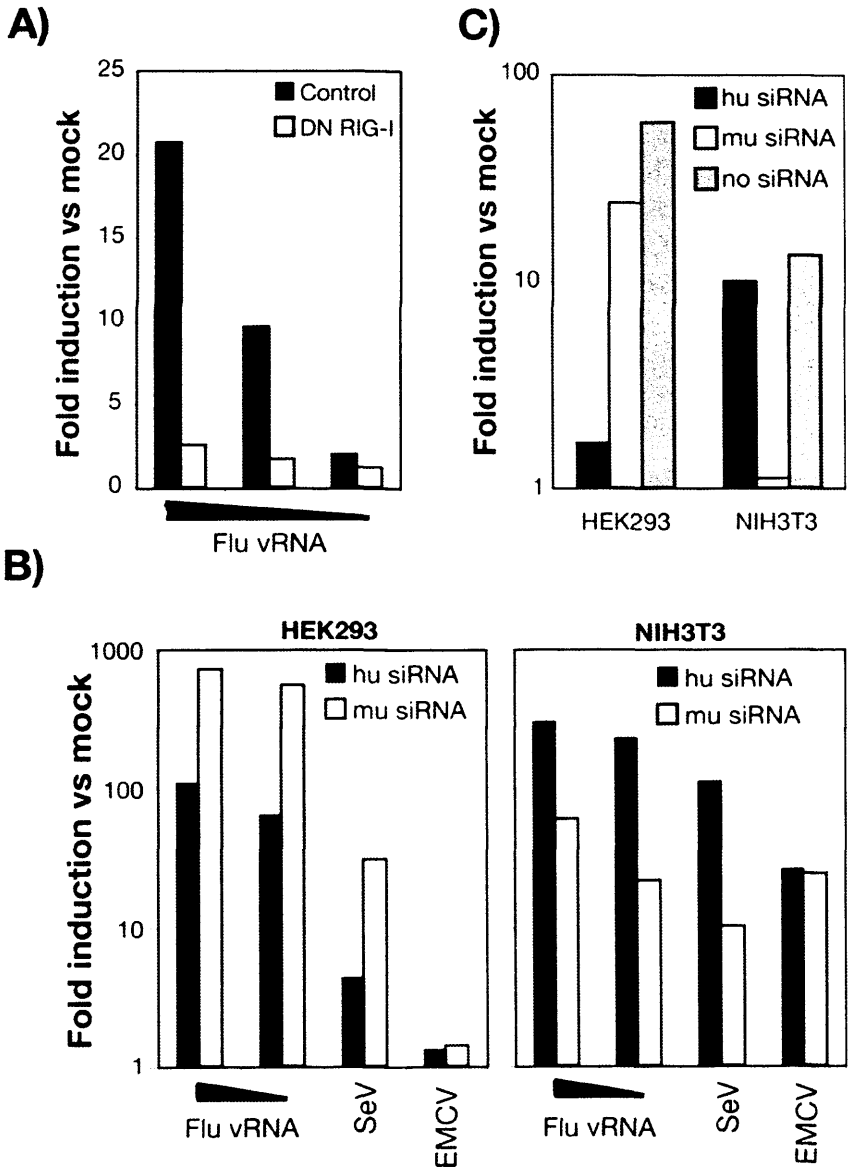

A)
Fold induction vs mock
25
20
15
10
5
0
Control
DN RIG-I
Flu vRNA
C)
Fold induction vs mock
100
10
1
hu siRNA
mu siRNA
no siRNA
HEK293
NIH3T3
B)
HEK293
NIH3T3
Fold induction vs mock
1000
100
10
1
hu siRNA
mu siRNA
Flu vRNA
SeV
EMCV

Figure 4.2.6: Inhibition of RIG-I reduces the IFN response to Flu vRNA

(A) Inhibition of IFN-β reporter activation by Flu vRNA through transfection of a dominant negative version of RIG-I. HEK293 cells were co-transfected with IFN-β reporter plasmids and a plasmid encoding dominant negative RIG-I (DN RIG-I) or an empty vector control (control). 48h later cells were transfected with 0.5, 0.1 and 0.02μg Flu vRNA and luciferase activity was tested 16h later. Shown is the fold induction of the ratio between firefly and renilla luciferase compared to untreated control cells. One of three repeat experiments with similar results is shown. (B, C) Human HEK293 cells and mouse NIH3T3 were co-transfected with IFN-β reporter plasmids and siRNAs specific for mouse (mu siRNA) or human RIG-I (hu siRNA) (B, C), or the IFN-β reporter plasmids only (no siRNA) (C). 72h later, cells were transfected with Flu vRNA (B: 0.2 or 0.04μg and C: 0.04μg) or were infected with SeV or EMCV (both MOI 1) where indicated. Luciferase activity was measured at 86h. The graphs show induction of IFN-β reporter activity compared to unstimulated control cells. siRNA experiments were repeated 4 times, two representative experiments are shown.

Figure 4.2.7

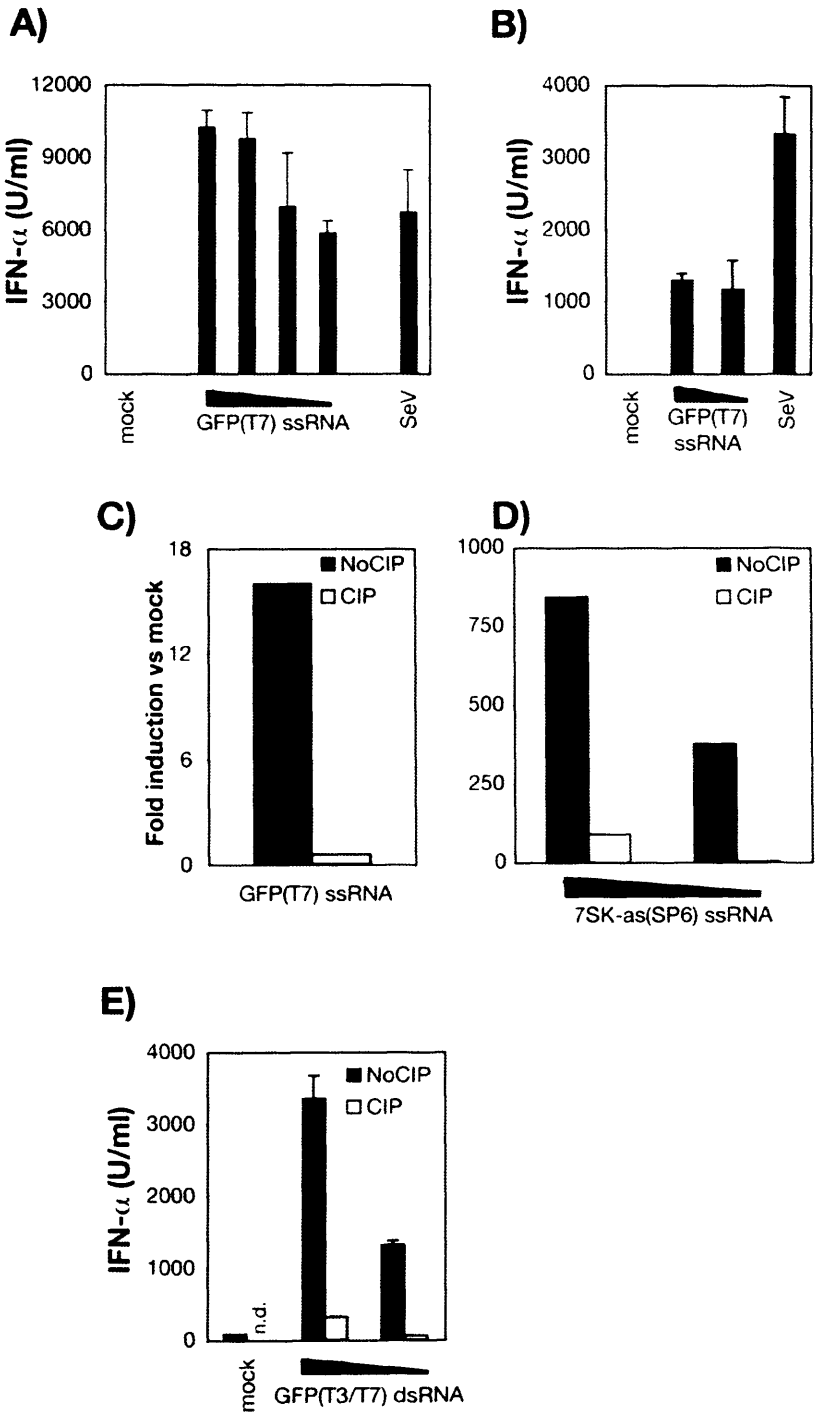

A)
12000
9000
6000
3000
0
IFN-α (U/ml)
mock
GFP(T7) ssRNA
SeV
B)
4000
3000
2000
1000
0
IFN-α (U/ml)
mock
GFP(T7) ssRNA
SeV
C)
18
12
6
0
Fold induction vs mock
NoCIP
CIP
GFP(T7) ssRNA
D)
1000
750
500
250
0
NoCIP
CIP
7SK-as(SP6) ssRNA
E)
4000
3000
2000
1000
0
IFN-α (U/ml)
n.d.
NoCIP
CIP
mock
GFP(T3/T7) dsRNA

Figure 4.2.7: IFN responses to *in vitro* transcribed RNA in human and mouse cells

(A) BM-DC or (B) NIH3T3 cells were transfected with varying doses of GFP ssRNA (A: 5, 1, 0.2, 0.04μg and B: 1, 0.2μg) or infected with SeV as positive control (A). IFN-α in over night culture supernatants was measured by ELISA. (C, D) NIH3T3 (C) or HEK293 (D) cells were transfected with IFN-β reporter plasmids. 24h later cells were transfected with RNA transcribed by T7 polymerase (GFP(T7)) (0.2μg) (C) or SP6 polymerase (7SK-as(SP6)) (0.5 and 0.16μg) (D) that had been either mock treated (NoCIP) or treated with calf intestinal phosphatase (CIP). Luciferase activity was measured at 38h. Graphs show fold activation of IFN-β promoter as compared to untreated control cells. (E) NIH3T3 cells were transfected with 1 and 0.2μg *in vitro* transcribed dsRNA (GFP(T3/T7)) and IFN-α accumulation after over night stimulation was measured by ELISA. One representative experiment of three (C, E) to five (A, B, D) is shown.

Figure 4.2.8

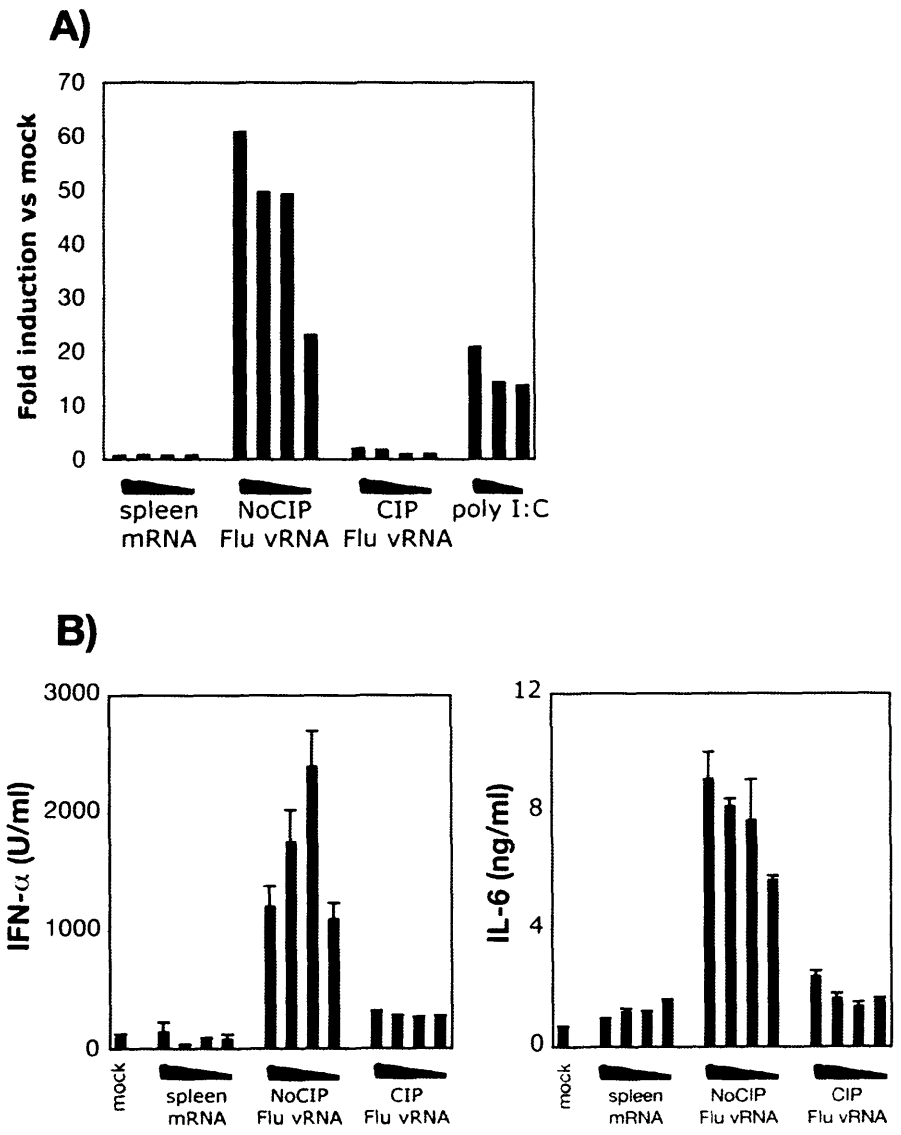

A)
Fold induction vs mock
70
60
50
40
30
20
10
0
spleen mRNA
NoCIP Flu vRNA
CIP Flu vRNA
poly I:C
B)
IFN-α (U/ml)
3000
2000
1000
0
IL-6 (ng/ml)
12
8
4
0
mock
spleen mRNA
NoCIP Flu vRNA
CIP Flu vRNA

Figure 4.2.8

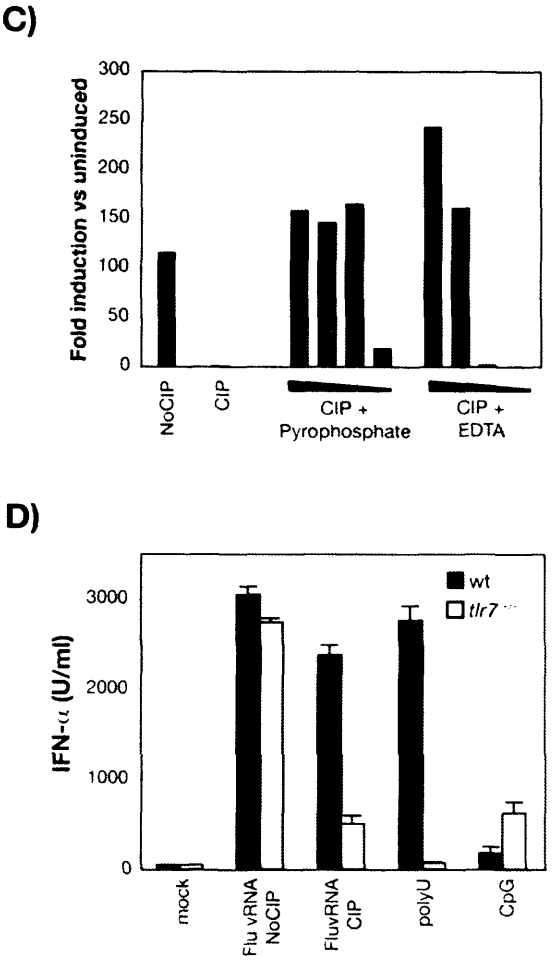


Figure 4.2.8: Flu vRNA induces IFN in a 5'phosphate dependent manner

HEK293 transfected with IFN-β reporter plasmids (A) or BM-DC (B) were transfected with different amounts (0.6, 0.2, 0.06. 0.02μg) of mouse spleen mRNA or the same amount of Flu vRNA (A, B) or poly-I:C (2, 1, 0.5μg) (A). RNAs were pre-treated (CIP) or not (NoCIP) with calf intestinal phosphatase. Luciferase activity (A) or IFN-α and IL6 (B) were measured after over night culture. (C) HEK293 cells were transfected with IFN-β reporter plasmids and stimulated 24h later with Flu vRNA that had been either mock treated or treated with CIP in the absence or presence of pyrophosphate (50, 10, 2, 0.4mM) or EDTA (250, 50, 10, 2mM). Luciferase activity was measured at 38h. (D) Total bone marrow cells ($1x10^6$ per cavity 96-well plate) from C57BL/6 (wt) or *tlr7*$^{-/-}$ mice were treated with complexes of lipofectamine2000 and 0.2μg of Flu vRNA and 1μg of poly-U, respectively, or with CpG (0.5μg/ml). IFN-α accumulation in over night culture supernatants was measured by ELISA. One of four (A, B) or two (C, D) experiments with similar results is shown.

Figure 4.2.9

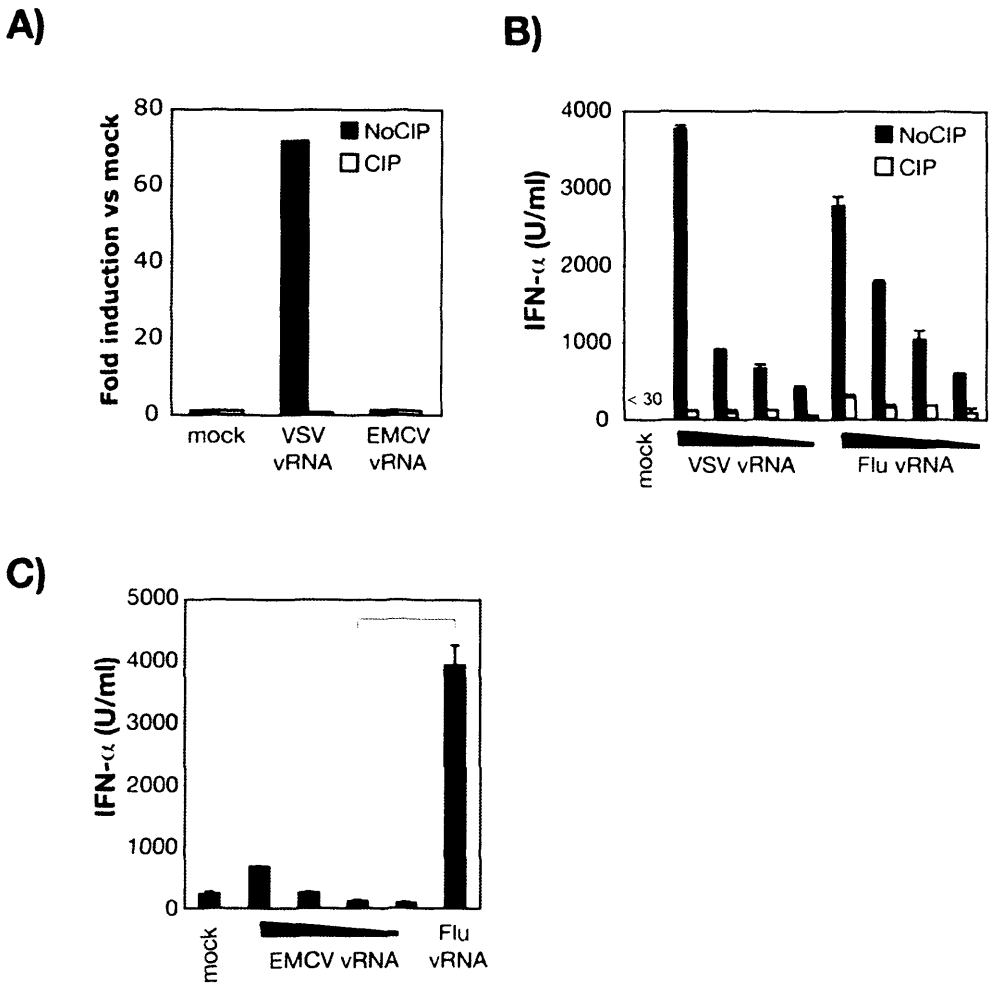


Figure 4.2.9: IFN-α/β induction by genomic RNA from other viruses

(A) HEK293 transfected with IFN-β reporter plasmids for 24h were treated with 0.2μg EMCV vRNA or vRNA from $8x10^7$ pfu VSV that was not (NoCIP) or was treated with CIP. At 38h cells were lysed and luciferase expression analysed. The graph shows induction of IFN-β luciferase as compared to mock treated cells. (B) IFN-α production in BM-DC transfected with vRNA from VSV and Flu that was not (NoCIP) or was treated with CIP. Cells received vRNA corresponding to $8x10^7$ pfu VSV and 5-fold serial dilutions thereof and 0.6, 0.2, 0.06 or 0.02μg of Flu vRNA, respectively. IFN-α accumulation in the cell supernatant was measured by ELISA after over night incubation. In panel (C) BMDC were treated with various doses of EMCV vRNA (5, 1, 0.2, 0.04μg) or Flu vRNA (0.2μg) and IFN-α in the supernatant was measured by ELISA after over night incubation. The bracket indicates transfections with similar amounts of vRNA. Experiments were repeated twice (A, B) or four times (C).

Figure 4.3.1

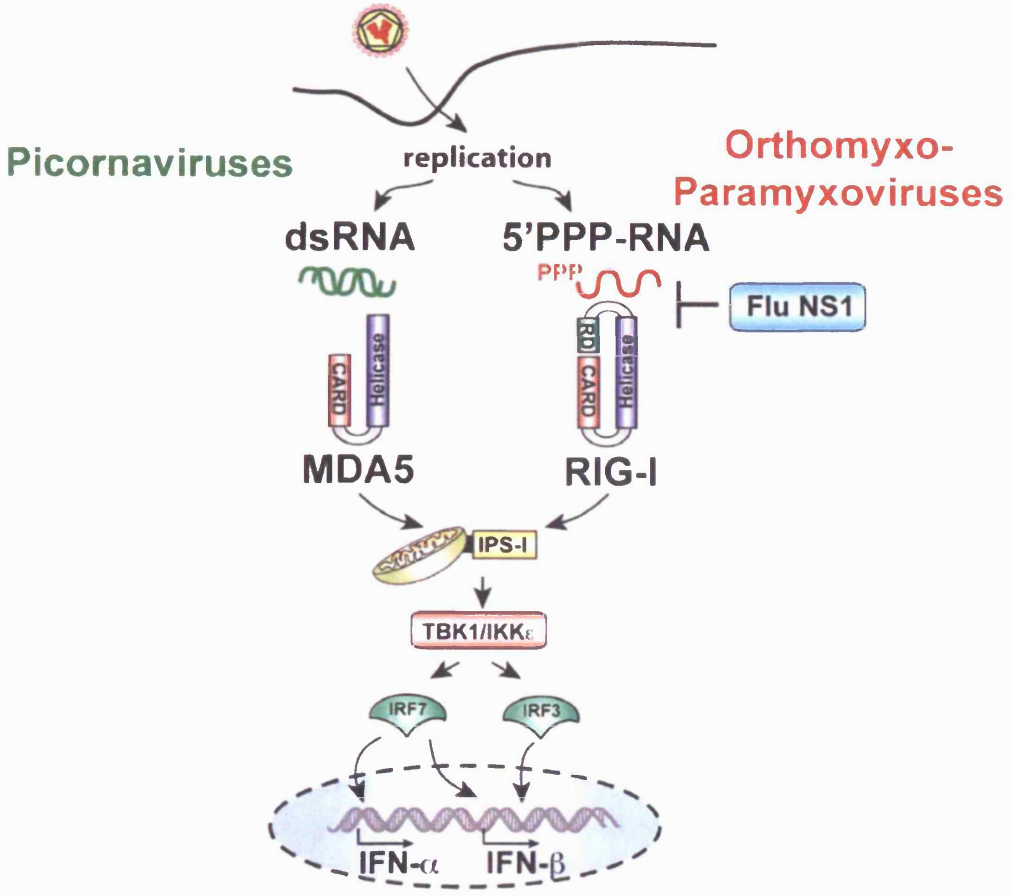


Figure 4.3.1: Proposed model for IFN induction through activation of cytoplasmic PRRs

Virus infection delivers agonists for MDA5 and RIG-I. The replication process may be necessary to generate sufficient amount of agonist although virus entry may be sufficient. RIG-I is activated by triphosphorylated RNA and this activation can be inhibited by the influenza NS1 protein. MDA5 may or may not recognise dsRNA. RIG-I and MDA5 signal via IPS-1 and the canonical interferon induction pathway to modulate transcription of IFN genes.

CHAPTER 5: Final Discussion and Concluding Remarks

Antiviral programs are mainly initiated through recognition of virus-associated nucleic acid that can be distinguished from cellular nucleic acid by two criteria: a specific signature present on nucleic acid and/or atypical localisation within the cell (Basler and Garcia-Sastre, 2007; Bowie and Fitzgerald, 2007; Pichlmair and Reis e Sousa, 2007; Yoneyama and Fujita, 2007). TLRs recognise nucleic acid that is delivered into the endosomal compartment during the viral life cycle. Similarly, the cytoplasmic presence of DNA, which exclusively localises in the nucleus under normal conditions, can be sensed by DNA recognition receptors. Specific structural features on viral RNA that include long double-strandedness and the presence of 5'phosphates mark viral RNA as foreign and initiates an innate antiviral response.
Here I discuss the current knowledge and 'unknowns' in viral recognition and also present some further thoughts.

5.1. TLR recognition of recombinant lentiviruses

TLRs3, -7, -8, and -9 recognise nucleic acid in the endosome (Fig 5.1.1). Normally, nucleic acid is not present in the endosome but degraded by nucleases, either within the cell or in the extracellular milieu (Barton et al., 2006). However, nucleic acid present in virus particles is highly resistant to nucleases (e.g. Fig 3.2.10A) and endosomal uptake of the virus particle is believed to deliver nucleic acid to a compartment that allows sensing via TLRs (Bowie, 2007; Kawai and Akira, 2006). Consequently, the infection route should be critical for TLR- mediated recognition of viruses. Indeed, in murine pDC, viruses that infect the cell via the endosome like influenza, VSV and HSV-1 are strong activators of TLR7 and -9 (Diebold et al., 2004; Lund et al., 2003; Lund et al., 2004). Also, TLR7 responses are reduced if VSV is genetically engineered to bear the RSV-F protein that mediates fusion at the plasma membrane (Lund et al., 2004). If TLR9 is genetically engineered to localise

at the plasma membrane antiviral responses to HSV-1 are abolished (Barton et al., 2006), clearly demonstrating that TLR9 senses viruses in the endosome.

The ability of pDC to produce IFN-α was associated with their unique ability to retain CpG in the early endosome and cDC express IFN-α when treated with CpG that is experimentally formulated to be delivered into early endosomes (Honda et al., 2005a). Hence, IFN-α production through TLR9 activation requires localisation of the agonist in early endosomes. pDC and cDC are apparently intrinsically different in handling endosomal cargo and this difference can lead to cell type-specific innate immune responses after treatment with TLR9 ligands. It still remains to be shown whether this also applies to TLR7 and whether the unique ability of pDC to produce high amounts of IFN-α after virus infection is based on similar mechanisms, i.e. whether the viral genomic nucleic acid is localising in early endosomes of pDC but not in early endosomes of cDC.

As mentioned above, viral genomes are tightly packaged and thereby protected from degradation. Sensing viral genomic nucleic acid by TLRs would require releasing the virus' genomic material by degrading the virus particle, something that is only happening in late endosomes. Currently it is not clear how TLR7, -8 and -9 gain access to virus genomic material after virus uptake.

Viruses entering the cell via endosomes remain in this compartment for varying time (Smith and Helenius, 2004): Influenza virus, for instance, requires an acidification step at low pH to enter the cytoplasm (Sieczkarski and Whittaker, 2002) and this only takes place in late endosomes. Therefore, Influenza virus particles could theoretically interact with TLRs that are thought to be activated in early endosomes. Adenoviruses (AdV), in contrast, transverse the endocytic compartment very transiently; they leave the early endosome before endosomal maturation (Smith and Helenius, 2004). It is surprising, that AdV can be sensed by the TLR system, as an entering virus particle would leave the endosome at a very early stage of endosomal maturation and one may question whether the current model of TLR activation can accommodate this aspect of virus biology. Even more puzzling is the situation for viruses belonging to the paramyxo- (e.g. SeV) and retrovirus family. These viruses mainly fuse at the plasma membrane when infecting the cell. One could hypothesise that this route of infection may have evolved as a mechanism to circumvent TLR

signalling. Surprisingly, pDC stimulated with SeV can mount an efficient innate immune response and this clearly depends on endosomal acidification and TLR7 (Lee et al., 2007).

I aimed to evaluate the importance of the virus infection route by using standardised virus particles that only differ in their glycoproteins which mediate fusion of virus particles at the plasma membrane or virus entry through endosomes, respectively. In my hands, LV particles did not lead to detectable activation of the TLR pathway regardless of the surface protein present on the surface of the virus particle (Fig 3.2.2, 3.2.5 and 3.2.10). This was surprising; especially in the light of publications suggesting TLR activation by wild type HIV-I (Beignon et al., 2005; Fonteneau et al., 2004; Francis and Meltzer, 1993; Schmidt et al., 2005; Yonezawa et al., 2003). A more recent publication by Lee et al. may help to find an explanation for this apparent discrepancy. Lee et al showed that for some viruses like VSV and SeV, induction of IFN-α from pDC depends on the presence of TLR7 and the ability of the virus to replicate. Unlike UV-treated Influenza virus, UV-treated VSV and SeV are not stimulating IFN-α from pDC. The authors therefore hypothesised that replication is necessary for efficient recognition of VSV in pDC. Surprisingly, this still depended on TLR7 that senses endosomal nucleic acid. One way to deliver cytoplasmic contents into the endosome is autophagy (self-ingestion), a cellular process that is known to be important to recycle catabolic substances. By using PI3K inhibitors that block autophagy (and many other cellular processes) and pDC from mice deficient in ATG5, that is required for autophagy, the authors showed that autophagy could be involved in TLR7 recognition of VSV. Although this model is very appealing, it is not entirely clear how the cell might distinguish viral nucleic acid from cellular nucleic acid, as cellular contents including cytoplasmic RNA would also be delivered into the auophagosome. However, this work highlights the existence of remarkable cross-talk between the cytoplasmic and the endosomal compartment.

Might a similar phenomenon explain differences between my results and the ones obtained by Beignon and colleagues? Replication-competent lentivirus may be delivered into endosomes during the virus life cycle but not necessarily during the entry process. Indeed, HIV-I particles can be found in endosomes of infected cells

and the HIV envelope contains proteins found exclusively in the endosomal compartment. Further, the 'Late-domain' (PPPY motif) of the HIV gag protein interacts with the ESCRT-I system, which is implicated in inward budding into endosome-like vesicles and formation of multivesicuar bodies. Multivesicular bodies and HIV particles are released through exocytosis or can re-enter the endocytic machinery (Morita and Sundquist, 2004; Williams and Urbe, 2007). Intriguingly, Rhabdoviruses like VSV may also interact with the cellular ESCRT-I system through interaction with a similar L-domain on its matrix protein (Craven et al., 1999; Morita and Sundquist, 2004; Williams and Urbe, 2007). It would be interesting to understand whether inward budding is involved in delivery of virus particles from the cytoplasm into endosomes and therefore facilitate recognition of Retro- and Rhabdoviruses. I would speculate that this endosomal delivery method would be virus specific (possibly requiring a Late-domain) and could therefore serve to distinguish cellular from virus-derived nucleic acid.

It is feasible to speculate that the TLR system has evolved to recognise viruses at different stages of their life-cycle: viruses entering the cell are recognised in the endocytic compartment, viruses replicating in the cytoplasm may be sensed through autophagy and, additionally, viruses could possibly be detected when leaving the cell.

5.2. Cytoplasmic recognition of influenza virus

Virus entry and replication deliver molecules into the cytoplasm that can be sensed by cytoplasmic PRRs, which leads to initiation of an antiviral state. Per definition, a PRR recognises a PAMP that must be necessarily expressed by the pathogen and must not be present or not accessible in uninfected cells. dsRNA present in some virally infected cells serves this purpose as it is expected that dsRNA is not present in uninfected cells. However, the notion that dsRNA is capable to induce antiviral responses seems surprising when considering the fact that about 98% of transcribed RNA in mammals is non-coding and wide-spread expression of sense- and corresponding antisense RNA species has clearly been shown (Lavorgna et al., 2004; Mattick and Makunin, 2005; Yelin et al., 2003). However, upon transcription,

nuclear dsRNA would quickly be hyperedited by nuclear Adenosine deamination processes and edited RNA is selectively retained in the nucleus (Zhang and Carmichael, 2001) or degraded by a promiscuous nuclear endonuclease (Scadden and Smith, 2001). The presence of dsRNA in the nucleus and the lack of IFN-α/β induction at the same time implies that the cell either cannot respond to this kind of dsRNA and/or that the nucleus forms a specialised compartment not allowing initiation of IFN responses. The former point is supported by the fact that dsRNA not bearing 5'PPP does not stimulate activation of the IFN-β promoter (Fig 4.2.7E). Further, annealing polyriboadenylic : polyribouridylic acid (poly-A and poly-U) homopolymers to form poly-A:U appears not to result in RNA with stimulatory potential (Okahira et al., 2005) (and data not shown). This suggests that double stranded-ness is not sufficient to activate cytoplasmic PRRs and that special properties associated with poly-I:C are necessary to do so. dsRNA generated during virus replication may have properties of poly-I:C and not constitute perfectly matched dsRNA. That viral polymerases can generate products that are recognised by the antiviral defence system of the cell was demonstrated by Kim and colleagues who described that siRNA generated *in vitro* by phage polymerases can induce IFN when transfected into cells (Kim et al., 2004). Interestingly, *in vitro* transcription of RNA resembles generation of virus-derived RNA during an infection: Similar to phage polymerases, polymerases of some viruses infecting mammalian cells produce RNA bearing a triphospate group on the 5' terminus (Ball, 2001; van Dijk et al., 2004). Others and myself could show that this kind of RNA stimulates RIG-I in the cytoplasm (Hornung et al., 2006).

It would be interesting to investigate whether replicative products from MDA5-activating viruses like EMCV also bear a specific signature and have special potential to activate the innate immune system.

5.3. Redundancy in virus recognition

Most viruses may not be specific for a single PRR but activate a panoply of PRRs at the time: The "RIG-I dependent viruses" VSV and NDV tested in RIG-I deficient

cells elicit basal levels of IFN-α and JEV and SeV are decreased to a variable extent by MDA5 deficiency suggesting that some viruses may be recognized by more than one PRR (Kato et al., 2006). Influenza virus is thought to be a specific agonist for TLR7 that senses ssRNA and RIG-I that is activated through single-stranded triphosphorylated Flu vRNA (Diebold et al., 2006)(and results presented here). At the same time Flu infection leads to activation of the dsRNA activated proteins PKR (Bergmann et al., 2000; Lu et al., 1995) and TLR3, resulting in inflammatory reactions in the lung and immunopathology (Le Goffic et al., 2006; Le Goffic et al., 2007). Therefore, viruses preferentially activate a certain class of PRRs but activation of additional pathways may happen. It may well be, however, that dsRNA binding proteins are activated indirectly through other proteins as it has been demonstrated for PKR that can be positively regulated by the dsRNA binding protein PACT and melanoma differentiation associated gene -7 (MDA7) or negatively by p58IPK, TAR RNA binding protein (TRBP), nucleophosmin and Heat shock protein 90 and -70 (Garcia et al., 2007).

5.4. A mirror image of TLRs and RLRs?

Endosomal TLRs and cytoplasmic RLRs discriminate between viral and cellular nucleic acid. Startling similarities between both recognition systems begin to unravel (Fig 5.1.1): Downstream signal transduction in both, the TLR and the RLR system, for instance, relies on the same or very similar signalling molecules (Bowie and Fitzgerald, 2007; Kawai and Akira, 2007; Uematsu and Akira, 2007; Yoneyama and Fujita, 2007). TLR3 is activated by the dsRNA analog poly-I:C and dsRNA derived from ReoV and is thought to be a receptor for dsRNA (Alexopoulou et al., 2001). Likewise, based on similar experiments (i.e. IFN-α/β induction through poly-I:C transfection) cytoplasmic proteins were believed to sense dsRNA that is produced during virus replication (Field et al., 1967b; Merigan, 1970). However, some viruses do not produce substantial amounts of dsRNA and 5'PPP ssRNA can activate RIG-I. Therefore RIG-I may constitute a cytoplasmic analogue for TLR7 that senses ssRNA in the endosome (Diebold et al., 2004; Heil et al., 2004). The difference mainly lies in the mechanism both proteins utilise to discriminate between self from viral

ssRNA: Whereas RIG-I senses a structure present on viral RNA in the cytoplasm, TLR7 discriminates cellular from viral RNA on the basis of localisation within the endosome. Similarly, DAI is a cytoplasmic receptor that detects the presence of DNA in the cytoplasm (Takaoka et al., 2007) and therefore functionally resembles TLR9 that is sensing DNA in the endosome (Lund et al., 2003). Poly-I:C activates innate immune responses through MDA5 in the cytoplasm (Gitlin et al., 2006; Kato et al., 2006) or TLR3 in the endosome (Alexopoulou et al., 2001). Although little is known about the real agonist for both proteins, it is suspected that dsRNA present in poly-I:C preparations is recognised. This is supported by binding experiments showing that TLR3 and MDA5 can bind to dsRNA *in vitro* (Bell et al., 2006; Rothenfusser et al., 2005; Yoneyama et al., 2005). TLR3 recognises exogenously added poly-I:C and isolated ReoV dsRNA, but is only weakly activated by perfectly matched synthetic dsRNA (Okahira et al., 2005). A recent report by Marshall-Clarke and colleagues clearly shows that TLR3 can be activated by polyinosinic (poly-I) homopolymers (Marshall-Clarke et al., 2007), highlighting the possibility of ssRNA recognition through TLR3. It would be interesting to understand whether the similarities between TLR3 and MDA5 in terms of ligand requirements can be extended beyond the recognition of poly-I:C.

5.5. Future directions and concluding remarks

A lot of work has been done in the past 50 years to elucidate principles of virus recognition leading to induction of IFN-α/β. The discovery TLRs and RLRs as viral sensors opened a new avenue in the field of viral innate immune recognition.

Phosphate-bearing RNA binding and activating RIG-I is currently the only well-defined PAMP activating the cytoplasmic pathway. However, it still needs to be shown whether virus genomes or by-products of virus replication bearing 5'phosphates are sensed by RIG-I. The characterisation of the ligand(s) for MDA5 and the search for DAI-like receptors that sense cytoplasmic DNA will be in the centre of future research. It will be interesting to understand the interplay between cytoplasmic virus PRRs and whether viruses produce several PAMPs activating a set of cytoplasmic PRRs at a time.

TLRs and RLRs sense viral nucleic acid within distinct cellular compartments. These compartments can feed into each other, i.e. virus nucleic acid can be delivered from the endosome into the cytoplasm and vice versa. It is important to understand the cross talk between the cytoplasmic and the endosomal compartment in order to understand how PRRs get in contact with stimulatory nucleic acid (Schmid and Munz, 2007). In fact, this cross talk is reminiscent to the MHC system where cytoplasmic content is commonly presented on MHC-I and endosomal contents MHC-II. However, endosomal contents can also be presented on MHC-I through a process named cross-presentation. Integrating cell biological aspects in innate virus recognition mechanisms may play a central role to eventually understand antiviral responses to virus infections.

Clearly, much more work is needed to understand mechanistic details of host-virus interactions. Every piece that is added to the puzzle of innate immune recognition of viruses raises new questions and shows the remarkable sophistication of the host-pathogen interplay.

References

Publications based on work presented in this thesis are attached in the Appendix

Abe, A., Chen, S.T., Miyanohara, A., and Friedmann, T. (1998). In vitro cell-free conversion of noninfectious Moloney retrovirus particles to an infectious form by the addition of the vesicular stomatitis virus surrogate envelope G protein. J Virol *72*, 6356-6361.

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Appendix

For convenience, publications based on work presented in this thesis are attached

Pichlmair, A., Diebold, S.S., Gschmeissner, S., Takeuchi, Y., Ikeda, Y., Collins, M.K., and Reis e Sousa, C. (2007). Tubulovesicular structures within vesicular stomatitis virus G protein-pseudotyped lentiviral vector preparations carry DNA and stimulate antiviral responses via Toll-like receptor 9. J Virol *81*, 539-547.

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Tubulovesicular Structures within Vesicular Stomatitis Virus G Protein-Pseudotyped Lentiviral Vector Preparations Carry DNA and Stimulate Antiviral Responses via Toll-Like Receptor 9[▽]

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RIG-I–Mediated Antiviral Responses to Single-Stranded RNA Bearing 5′-Phosphates

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between cytosolic and endosomal viral recognition, with MDA5, RIG-I, and the cytosolic DNA receptor constituting functional homologs of TLR3, TLR7, TLR8, and TLR9. Similar to virologists, the innate immune system may therefore have learned to classify viruses by their genomes.

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Supporting Online Material

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Materials and Methods
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Figs S1 to S10
References

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Review

Innate Recognition of Viruses

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