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INDUCTION OF TYPE I INTERFERONS AND VIRAL IMMUNITY

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ABSTRACT

Virus-induced type I interferons (IFNα/β) are key mediators of innate immunity and important modulators of adaptive immunity. Early recognition of virus and induction of IFNα/β are important for limiting the spread of the virus. In paper I and II, we use RNA viruses, Semliki Forest virus (SFV) and Rotavirus, to investigate which viral functions and what cellular pathways are required for the induction of IFNα/β-production in murine bone marrow-derived myeloid dendritic cells (mDC). We show that both SFV and Rotavirus induce IFNα/β production via a toll-like receptor-independent pathway and IFNα/β induction in mDC by both viruses is largely dependent on IRF-3. Our data suggest that events during or downstream of viral entry, Pbut prior to viral replication are required for the activation of IFNα/β-production in mDC.

In paper III, we show that SFV provides an adjuvant effect on antibody responses against co-administered protein antigens. The adjuvant effect of SFV is abolished in mice lacking the IFNα/β receptor (IFNR-AR1$^{-/-}$ mice). In contrast, amplitude, longevity and composition of the antibody responses directed against virus-encoded antigens are intact in the absence of IFNα/β-signalling. Antibody responses against both the virus-encoded antigens and against co-administered antigens are also intact in MyD88$^{-/-}$ and TLR3$^{-/-}$ mice, in agreement with the observation that these mice are capable of IFNα/β induction in response to SFV. Further, we show that rSFV-induced antibody responses are dependent on T cell help and we suggest that the absence of IFNα/β-signalling in the IFNR-AR1$^{-/-}$ mice leads to insufficient priming of T helper cells by DC. These results show that virus-induced IFNα/β can act as a potent adjuvant for antibody responses against co-administered protein antigens, but that IFNα/β are not required for the induction of immune responses against virus-encoded antigens.

In paper IV, we show that CD8+ T cell responses directed against SFV-encoded antigens are enhanced in the absence of IFNα/β-signalling. MHC class I tetramer staining demonstrated that the number of antigen-specific CD8+ T cells is lower both in blood and spleen of SFV-immunized wildtype mice compared to in SFV-immunized IFN-AR1$^{-/-}$ mice. The number of IFNγ-producing CD8+ T cells in spleen was also lower in wildtype mice than in mice lacking the IFN-AR1. Wildtype and IFN-AR1$^{-/-}$ mice immunized with *ex vivo*-infected wildtype mouse embryonic fibroblasts cells gave similar results. These data suggest that IFNα/β signalling restricts the CD8+ T cell responses to virally encoded antigens, in contrast to its previously shown enhancing effect on cross-presentation of protein-based antigens.

LIST OF PUBLICATIONS

I. **Hidmark ÅS**, McInerney GM, Nordström EK, Douagi I, Werner KM, Liljeström P, Karlsson Hedestam GB. Early alpha/beta interferon production by myeloid dendritic cells in response to UV-inactivated virus requires viral entry and interferon regulatory factor 3 but not MyD88.
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II. Douagi I, McInerney GM, **Hidmark ÅS**, Miriallis V, Johansen K, Svensson L, Karlsson Hedestam GB. Role of interferon regulatory factor 3 in type I interferon responses in rotavirus-infected dendritic cells and fibroblasts.
J Virol. 2007 Mar;81(6):2758-68.

III. **Hidmark ÅS**, Nordström EK, Dosenovic P, Forsell MN, Liljeström P, Karlsson Hedestam GB. Humoral responses against coimmunized protein antigen but not against alphavirus-encoded antigens require alpha/beta interferon signaling.
J Virol. 2006 Jul;80(14):7100-10.

IV. **Hidmark ÅS**, Douagi I, Nordström EK, Dosenovic P, Karlsson Hedestam GB. CD8+ T cell responses against alphavirus-encoded antigens are enhanced in the absence of IFNα/β signaling.
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LIST OF ABBREVIATIONS

aa	amino acid
AP	Activating Protein
APC	Antigen Presenting Cell
APRIL	A Proliferation-Inducing Ligand
BLYS	B lymphocyte stimulator
CCR	CC-chemokine Receptor
CpG	Unmethylated cytosine and guanine nucleotides separated by a phosphate
DC	Dendritic Cells
ds	double-stranded
eIF2α	eukaryotic Initiation Factor 2α
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELISPOT	Enzyme-Linked ImmunoSpot
ENV	Human Immunodeficiency virus envelope
ER	Endoplasmatic Reticulum
FACS	Fluorescent-Activated Cell Sorting
Flt3-L	Fms-like tyrosine kinase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HIV	Human Immunodeficiency Virus
i.v.	intra venously
IFN	Interferon
IFN-AR	Interferon α/β Receptor
IFN-AR1	Interferon α/β Receptor Subunit 1
Ig	Immunoglobulin
IKK	IKappaB Kinase
IL	Interleukin
IPS-1	Interferon-beta Promoter Stimulator 1
IRF	Interferon Regulatory Factor
ISGF3	Interferon-Stimulated Gene Factor 3
ISRE	Interferon-Stimulated Response Element
MDA5	Melanoma Differentiation Associated gene 5
mDC	myeloid Dendritic Cell
MEFs	Mouse embryonic fibroblasts
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
MTT	3-(4, 5-dimethyl-2-thiazolyl)-2, 5-dihphenyl-2H-tetrazolium bromide, a Formazan dye
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear Factor-kappa B
NK	Natural Killer

NSP	Non-Structural Protein
OD	Optic Density
PAMPs	Pathogen-associated molecular patterns
pCD	plasmacytoid Dendritic Cell
PKR	Protein Kinase R
poly(I:C)	polyriboinosinic:polyribocytidylic acid
PRRs	Pattern Recognition Receptors
RIG-I	Retinoic acid-Inducible Gene I
s.c.	sub cutaneously
SFC	Spot-Forming Cell
SFV	Semliki Forest virus
SIN	Sindbis virus
STAT	Signal Transducers and Activators of Transcription
TAP	transporter associated with antigen processing
TBK-1	TANK-binding kinase 1
T_{CM}	Central memory T cell
TCR	T Cell Receptor
T_{EM}	Effector memory T cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF6	Tumor necrosis factor Receptor−Associated Factor 6
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRIF	Toll/IL-1 Receptor domain-containing adaptor inducing IFN-beta
UV	Ultra Violet (light)
VEEV	Venezuelan Equine Encephalitis Virus
VP	Viral Protein

1 AIM

Virus infection is a potent stimulant of the adaptive immune responses. The magnitude and nature of these immune responses are consequences of early innate signals induced by the host upon recognition of virus and viral infection. IFNα/β is an important group of early innate cytokines induced during most viral infections, limiting viral spread and affecting the cells of the immune system in various ways. The aim of this thesis is to understand the pathways by which RNA viruses induce IFNα/β and to investigate what effects these cytokines have on the induction and shaping of adaptive immune responses.

2 INTRODUCTION

2.1 VIRUS INFECTION

Viruses are obligate intracellular parasites. Outside the cell they exist as particles called virions. Virions range in size from about 30 nanometres in diameter for the smallest viruses to the 230 nanometres of vaccinia virus. The virion consists of a protein capsid protecting the viral genome and in some cases a cell-derived lipid bi-layer envelope, with protruding viral glycoproteins. The genome can consist of either DNA or RNA and encodes relatively few proteins (3-100 depending on the virus). The viral proteins are needed for viral replication and building up the structure of the virion. Upon infection, the virion attaches to the surface of the host cell, usually by binding to a specific cell surface molecule that determines the specificity of the infection. Once inside the cell, the virions are uncoated, releasing the viral genome. DNA viruses can be further divided into those that have their genes on a double-stranded DNA molecule, e.g. smallpox virus, and those that have their genes on a molecule of single-stranded DNA, e.g. Adeno-Associated Virus.

RNA viruses exist in four distinct groups:

- Viruses with genomes consisting of single-stranded sense RNA that can act directly as a messenger RNA (mRNA). These are also called positive-stranded RNA virus. Examples of positive-stranded viruses are poliovirus and hepatitis C virus.

- Viruses with genomes that consisting of single-stranded anti-sense RNA; that is, RNA which is the complement of mRNA. These are also called negative-stranded RNA virus. Examples of negative-stranded viruses are measles, Ebola and Newcastle disease virus.

- Viruses with genomes consisting of several segments of double-stranded (ds) RNA, for example Reoviruses.

- Retroviruses, with genomes consisting of positive RNA strands that are converted by a virus-encoded reverse transcriptase into a double-stranded DNA genome (termed a provirus), which can integrate into the host cell chromosomal DNA. Human immunodeficiency virus (HIV) is an example of a retrovirus.

2.1.1 Semliki Forest virus

In our studies we have used Semliki Forest virus (SFV) of the Alphavirus genus as a model virus for induction of innate and adaptive immunity. Alphaviruses are mosquito borne single-stranded sense RNA viruses of the Togavirus family with birds and rodents serving as natural reservoirs. For humans, the laboratory strains of SFV and the closely related Sindbis virus (SIN) are considered safe (136), but SFV causes lethal encephalitis in mice and has been used as a model for viral neuropathogenesis (10, 32). SFV and SIN are amongst the best-characterized alphaviruses and both have been extensively used as model viruses in studies of molecular virology for three decades.

2.1.1.1 SFV structure and replication

Alphavirus particles consist of an icosahedral inner capsid protein, enveloped by a lipid bi-layer, from which viral spike proteins E1 and E2 protrude. The structure of an alphavirus particle, Venezuelan equine encephalitis virus (VEEV), is shown in Figure 1b. The alphaviral particles contain a single-stranded sense RNA genome of approximately 11-13 kb. The first open reading frame of SFV, constituting about two thirds of the genome, encodes the RNA replicase, a polyprotein consisting of non-structural proteins 1-4 (Nsp1-4) (Figure 1a).

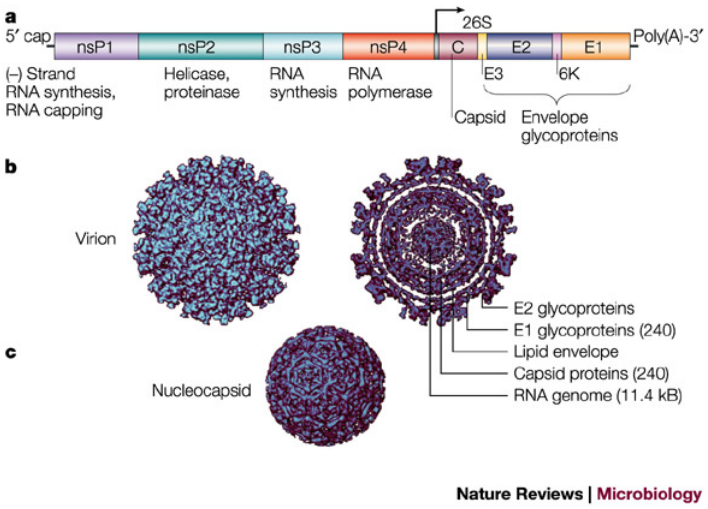


Figure 1. Genomic organisation and structure of Alphaviruses (a) Genomic organisation. (b-c) The structure of alphavirus particles (146).

The replicase is gradually auto-proteolytically processed and this processing changes the function of the replicase. Early after translation, the replicase complex synthesizes the negative strand from the genomic mRNA but as the replicase polyprotein gets further cleaved, it gains the capacity to synthesise sense RNA from the anti-sense template. There are two different mRNAs made from the negative strand; a full-length genome (42S RNA) and an RNA from a sub-genomic promoter exposed about two thirds down on the negative strand, producing a 26S RNA. The 26S RNA encodes the structural proteins of SFV and is produced in high copy number (73, 136). The SFV structural proteins consist of capsid and spike proteins E3, E2, 6k and E1, which are translated as a polyprotein. During translation, the capsid is auto-proteolytically cleaved off from the nascent polypeptide, which is thereafter translocated into the endoplasmic reticulum (ER). The spike proteins are further processed by the cellular proteases signal peptidase and furin in ER and in the Golgi respectively and translocated to the plasma membrane, from where the viral particles bud.

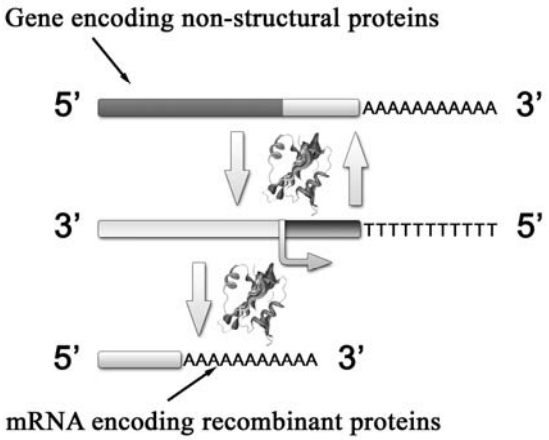


Figure 2. Alphavirus replicons. The replicase gene encoded by first two thirds of the replicon mRNA is translated. The replicase protein complex makes a negative strand template of the mRNA, which is used to make new copies of the positive mRNA. The subgenomic promoter is exposed on the negative strand. From this promoter, subgenomic mRNA is produced, encoding a recombinant protein of choice.

2.1.1.2 Alphavirus vectors

The broad host range of alphaviruses and their efficient cytoplasmic gene expression in a variety of cell types has prompted the development of expression vectors based on the genomes of SFV, SIN and VEEV (86, 115, 150). The sub-genomic 26S promoter of alphaviruses can be used to drive the expression of any foreign gene, which then replaces the coding region of the structural proteins (Figure 2). Plasmid DNA, with the viral genome expressed from a mammalian promoter, or in vitro produced viral mRNA can be transfected into cells where it establishes infection. These vectors can also be packaged into recombinant SFV (rSFV) particles, indistinguishable from wildtype viral particles, by co-transfection with helper RNA molecules (helpers) encoding the structural proteins (86) (Figure 3). To prevent recombination leading to formation of replication-competent virus, the RNA encoding capsid and spike can be divided up on two separate helper molecules (133). The helpers have a large deletion within the replicase gene, but they can be replicated in trans by the viral replicase encoded by the vector RNA. Upon replication, the helpers express viral structural proteins from sub-genomic promoters present on the viral RNA. Only the full-length vector RNA contains the sequence in the nsp2 region of the replicase gene required for packaging in the viral particles (149) and consequently no RNA genome encoding the structural proteins is packaged. Thus, when rSFV particles produced by this system infect new cells there is

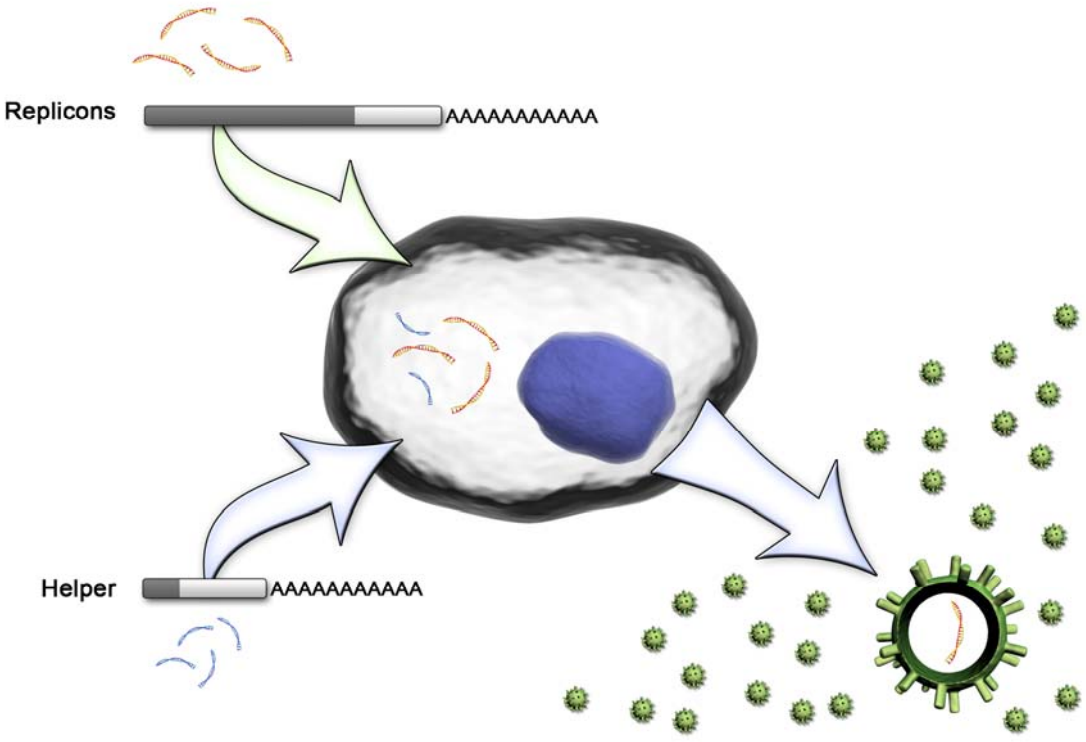


Figure 3. Production of suicidal rSFV viral particles. The replicon mRNA is transfected into the cell together with helper mRNA. The replicase translated from the replicon makes negative strand RNA copies of both the replicon and the helper mRNA. The replicase also makes subgenomic mRNA from both constructs. The subgenomic mRNA from the helper encodes SFV structural viral proteins; spikes and capsid. Only the replicon mRNA is packaged into the rSFV particles. When a rSFV particle infects a new cell, there is no helper mRNA to make new viral particles and thus the infection is limited to that cell.

no production of new viral particles; i.e. the infection is suicidal. Infection of mammalian cells with the SFV as well as with rSFV leads to a stress response, during which the host-cell protein synthesis is efficiently shut-down by induction of phosphorylation of eIF2α (eukaryotic initiation factor 2α) (79, 99) and eventually to cell death through apoptosis (43). In addition to their use in basic research, recombinant alphavirus vectors are being developed for the use as vaccine vehicles and as vectors for gene therapy.

2.1.2 Rotavirus

Rotaviruses belong to the Reoviridae family. There are seven major groups of rotaviruses, of which three (groups A, B, and C) infect humans, causing vomiting and diarrhoea. Rotavirus infections are the most common cause of severe diarrhoea in young children, killing over half a million children every year in developing countries. The development of a safe rotavirus vaccine has previously suffered serious drawbacks. After efforts by a large number of parties, the world's first rotavirus vaccine, Rotashield™, consisting of a live attenuated rhesus macaque strain, was licensed for use in 1998. Initially, no serious adverse effects of the vaccine were detected and it was found it to be 80 to 100% effective at preventing severe rotavirus diarrhoea. Soon a few rare cases of a bowel obstruction called intussusception (when the bowl folds over upon itself, like a telescope) were found among some infants during the first 1-2 weeks after vaccination. Initially it was estimated that RotaShield® vaccine increased the risk for intussusception by 1 or 2 cases per 10,000 infants vaccinated and the vaccine as withdrawn from the market 1999. However, when a larger set of data was evaluated, the increased risk was found to be much lower, about 1:30-40,000, but the vaccine would never re-emerge on the market. Two new oral live-attenuated vaccines against rotavirus infection (Rotarix®, manufactured by GlaxoSmithKline; and RotaTeq®, manufactured by Merck & Co., Inc.) were licensed in Europe and in the US in 2006. The Rotarix vaccine is derived from a single human strain of rotavirus while RotaTeq has components from 5 different bovine and human strains. Both companies claim their vaccine is safe and will protect against severe rotavirus gastroenteritis caused by a broad set of different rotaviruses.

An infectious rotavirus particle consists of three layers of protein (triple-layered) around a double-stranded dsRNA genome and has a diameter of 75-100 nm. Rotaviruses infect the cells of the intestinal epithelium and the triple-layered protein coat makes the viral particles resistant to the low pH of the stomach and to digestive enzymes in the gastrointestinal tract. The rotavirus genome consists of 11 RNA segments, which encode 6 structural (VPs) and 6 non-structural (NSPs) proteins. RNA-dependent RNA polymerase VP1 and capping enzyme VP3 are carried within the inner protein layer of the rotavirus particle, which is formed by an icosahedral shell of VP2. VP2 binds both the RNA and VP6 of the intermediate layer. When taken up into the endosome, the proteins of the third layer (VP7 and the VP4 spike) disrupt the endosomal membrane. Ca^{2+} influx into the endosome drives the disassembly of the third outer layer, revealing a double-layer particle with large open channels reaching into the viral genome at the centre of the particle. When the double-layer particles reach the cytoplasm, the replicase generates viral mRNA transcripts from the double-stranded viral genome. The viral mRNAs are then exported out from the viral particles to the

cytoplasm to be translated. Hiding the genomic viral dsRNA most likely serves to prevent activation of innate pathways triggered by dsRNA in the cytoplasm.

2.2 INNATE IMMUNE RESPONSES TO VIRAL INFECTIONS

The immune responses to viral infection consist of early innate responses, which induce and shape the later adaptive defence systems. The innate immune system comprises the cells and mechanisms that defend the host from infection by other organisms, in a non-specific manner. Detection of virus and other pathogen and triggering of innate immune responses occur when pathogen-associated molecular patterns (PAMPs) are recognised by pattern recognition receptors (PRRs). PAMPs are typically conserved structural and functional features of pathogens, essential for their persistence or proliferation. Viral PAMPs are primarily genomes and replication intermediates, but there are examples of how other components, such as viral capsid or glycoproteins can trigger PRRs (17, 40, 143). The innate immune response consists of the complement system, specialised cells and signalling molecules called cytokines. The complement system consists of set of serum proteins that upon activation (by PAMPs or the binding of antibodies) initiate a proteolytic cascade, resulting in proteins binding to the surface of pathogens, lysing their lipid membrane and labelling them for phagocytosis. Phagocytotic cells, such as macrophages and dendritic cells (DC), have the capacity to engulf, or phagocytose, infectious material and debris from dead cells and constitute an important component of the innate immune system. Natural killer (NK) cells can recognise and kill cells infected by pathogens, such as DNA viruses of the Herpes family, independently of pre-existing immunity. The cells of the innate immune response are activated by the recognition of PAMPs, which also induce the secretion of cytokines, small molecules that bind to receptors on surface of cells, to initiate signalling cascades modulating the functions of the target cells. Viral infections rapidly stimulate DC to produce early innate cytokines such as interleukin 12 (IL-12) and type I interferons (IFNα/β). IL-12 is an immune-regulating cytokine and the biologically active form is the heterodimeric IL-12 p70, composed of the IL-12 p35 and IL-12 p40 chains. IFNα/β have, in addition to immune regulatory effects, direct antiviral effects, preventing viral infection and replication. DC thus act as sentinels, alerting and activating other parts of the immune system before the infection has had the chance to spread. Chemotactic cytokines, or chemokines, induced during infection are also important for attracting more cells to the area, causing inflammation.

2.2.1 Recognition of viral infection

2.2.1.1 Toll like receptors

Toll-like receptors (TLRs) have emerged as an important group of PRRs in vertebrates over the last decade (2, 3, 16, 66). TLRs are evolutionarily conserved molecules that share homology with the Toll-receptor in Drosophila melanogaster, which stimulates the production of antimicrobial proteins and plays a role inducing anti-fungal immune responses (84, 101). TLR ligands include bacterial lipopolysaccharide (detected by TLR4) (114), bacterial lipoproteins and lipoteichoic acids (detected by TLR2) (5), flagellin (detected by TLR5) (48), unmethylated CpG DNA of bacteria and viruses (detected by TLR9) (53), dsRNA (detected by TLR3) (4) and single-stranded viral

RNA (detected by TLR7/8) (28, 50, 93). TLRs 3, 7, 8 and 9 are mainly localized in the endosomal membrane and seem to be specialised in detection of PAMPs associated with viral infection, recognising nucleic acids of viral genomes (15). Although these nucleic acids are not unique to viruses, the basis of recognition rather seems to be that they are in the "wrong" cellular compartment (109). The TLRs detecting nucleic acids are expressed by distinct DC populations: plasmacytoid DC (pDC) express high levels of TLR7 and TLR9 but not TLR3, while TLR3 is expressed in myeloid DC (mDC) (9). dsRNA is a common bi-product of viral replication and transcription, which normally does not occur within cells. Purified viral RNA from reovirus and synthetical (polyriboinosinic:polyribocytidylic acid, poly(I:C)) dsRNA can induce signalling via by TLR3 (4), but currently there is no evidence that TLR3 would be involved in the initial recognition of incoming viruses (125). It is rather possible that TLR3 has a function in detecting dsRNA leaking out from apoptotic virus-infected cells. It has been suggested that TLR7 can be activated by some RNA viruses through an exogenous pathway, where the RNA of viral particles is detected in the endosome after digestion of the viral envelope and capsid proteins by host cell enzymes (28, 50, 93). In addition, it has recently been shown that TLR7 can be activated by endogenous RNA, taken up from the cytoplasm into the endosome by the process of autophagy (82), a process that seems to be vital for the recognition of some RNA viruses by TLR7. Thus, infected pDC expressing TLR7 are equipped with a pathway that enhances direct recognition of viral infection in these cells, independently of the availability of viral particles or viral RNA released from infected cells upon lysis. With the exception of TLR3, and to some extent TLR4, the intracellular domains of TLRs require the adapter protein MyD88 (Myeloid differentiation factor 88) to transmit their signal.

Since the various TLRs are expressed on different cell-types, their activation can lead to the transcription of different cytokines. IL-12 is induced by signalling through TLRs mainly on monocytes and DCs (2, 3). In particular, simultaneous stimulation with several TLRs has been shown to have a synergistic effect on the induction of IL-12 (38, 106). IL-12 induces proliferation and production of IFNγ by T cells and NK cells, thereby shaping the subsequent adaptive immune response (145). Some of the TLRs induce IFNα/β, particularly in pDC, which have been described as natural interferon producers and can produce high levels of IFNα/β (88). Although there is little evidence that TLRs are required for an effective antiviral defence against RNA viruses, it has been shown that TLR3 and TLR9 are both needed to control infection with mouse cytomegalovirus (138). Also, it has been shown that TLR9-mediated activation of pDC is required for the innate defence against challenge with HSV-2, a similar virus (94).

In addition to PAMPs, there is also a growing list of host-derived immune-stimulators, such as heat-shock proteins and uric acid. These host-derived immune-stimulatory signals originate from the tissue damage caused by viral infection and they can act as potent danger signals (37, 128).

2.2.1.2 Intracellular recognition of virus

In addition to TLRs, a class of cytoplasmic sensors of viral infection has been identified. RNA helicases RIG-I (Retinoic acid-inducible gene I) (154) and MDA5 (Melanoma differentiation associated gene 5) (8), belonging to the DExD/H-box

helicase family, have been described to directly bind viral RNA and induce a set of antiviral genes (137). Recently, it was shown that poly(I:C) is a ligand for MDA5 but not RIG-I (42, 69), whereas long dsRNA was found to activate RIG-I but not MDA-5 (69). Further, it has been demonstrated that recognition of RNA by RIG-I requires a 5' triphosphate group (58, 112). RIG-I specifically recognises the viral RNA genomes of Paramyxoviridae, Flaviviridae, Rhabdoviridae and Orthomyxoviridae (69, 137, 154), while MDA5 has been shown to recognise picornavirus RNA (69). Fibroblasts and mDC require RIG-I or MDA5 for the induction of IFNα/β in response to RNA virus (68).

PKR (Protein kinase R) is a sensor of viral infection that binds dsRNA in the cytoplasm and phosphorylates eIF2α (142). This leads to inhibition of cellular translation, including inhibition of synthesis of viral proteins. It has been suggested that induction of IFNα/β by dsRNA is regulated by PKR (29, 153). However, $PKR^{-/-}$ mouse embryonic fibroblasts (MEFs) are not defective in their IFNα/β production in response to infection with Newcastle disease virus, Sendai virus or vesicular stomatitis virus (6). Thus a majority of published data suggests that RIG-I rather than PKR mediates the recognition of virus in the cytoplasm that induces the induction of IFNα/β (19, 51, 68, 85, 137, 154). Recently, an additional pathway sensing dsDNA in the cytoplasm, independently of TLRs and RIG-I, has been described (63, 135). The importance of this pathway for induction of IFNα/β has been described for both cytoplasmic bacterial and viral DNA but the actual PRR remains to be identified.

2.2.2 Type I Interferons – Interferon α/β

IFNα/β are central mediators of antiviral responses and are produced by various types of cells after viral infection. IFNα comprises at least 12 genes, while there is just one gene for IFNβ in the human genome. Additional type I IFNs, such as the IFNλ-family, (76) and IFN, ω, ε, κ, δ, and τ have also been described (111). Though all type I IFNs appear to have some degree of antiviral properties, IFNε rather seems to play a role in reproductive function in placental mammals (111). The structure of the type I IFNs is a bundle of α-helices, kept together by at least one disulfide bond. IFNβ and IFNα4 are the earliest type I IFNs produced by fibroblasts upon viral infection (95).

2.2.2.1 Induction of IFNα/β

IFNα/β can be induced either by intracellular recognition of virus by one of the cytoplasmic receptors described above or through recognition of viral components via one of the membrane-bound TLRs. When viral genomes in the cytoplasm are recognised by RIG-I or MDA5, signals are transmitted through their N-terminal CARD (Caspase recruitment domain) of these proteins. Through their CARD domains, RIG-I and MDA5 interact with the mitochondrial CARD-containing protein IPS-1 (Interferon-beta Promoter Stimulator 1), also called VISA, MAVS or Cardif (71). IPS-1 transmits the signal to TBK1 (TANK-binding kinase 1) and IKKε (IKappaB Kinase ε), the two kinases responsible for the phosphorylation of IRF-3 and IRF-7 (33, 129). The virus-stimulated IFNβ expression is synergistically mediated by the activation of the transcription factors nuclear factor kappa B (NF-κB), IRF-3 (interferon-regulatory factor 3) and activating protein 1 (AP-1) (74, 75).

TLR3 induces IRF-3 activation in a different manner from that of cytoplasmic recognition of virus infection (30). Upon dsRNA binding, TIR adaptor molecule Toll/IL-1 receptor domain-containing adaptor inducing IFNβ (TRIF) is recruited to the intracellular domain of TLR3. TBK1, recruited directly to TRIF, mediates IRF-3 activation (151, 152). Thus the signalling pathways of the intracellular receptors and that of TLR3 converge on the IRF-3 kinase TBK1. These events are schematically illustrated in Figure 4.

Ligand-binding to TLR7, TLR8 or TLR9 recruits MyD88 and adaptor molecule tumor necrosis factor receptor-associated factor 6 (TRAF6) to its intracellular domain, where these proteins interact with and activate IRF-7 (70). pDCs produce large amounts of IFNα/β, mainly IFNα, in response to TLR7 or TLR9 engagement. This ability is most likely due to the constitutive expression of IRF-7 in pDC's, permitting these cells to rapidly respond with high levels of IFNα without prior IFNα/β signalling (65, 72, 140). However, mDC have also been shown to be potent producers of IFNα/β in response to infectious viral particles, at least in the murine system (7, 29, 54, 56, 89, 90, 119, 121).

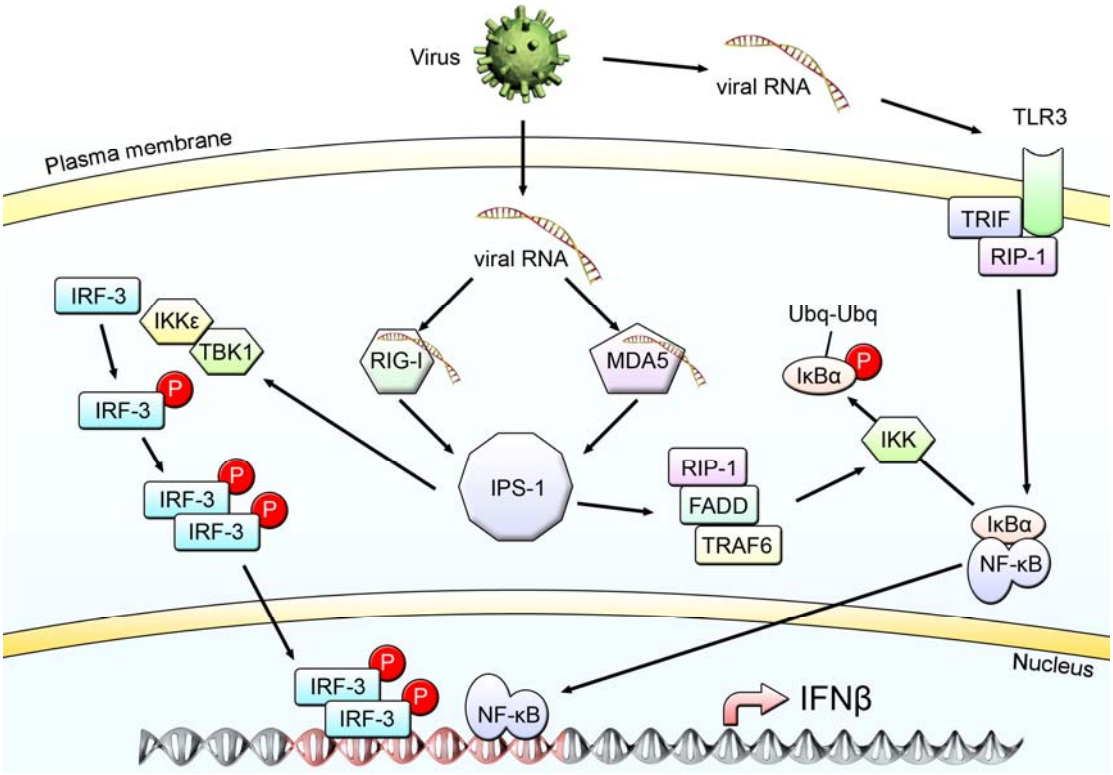
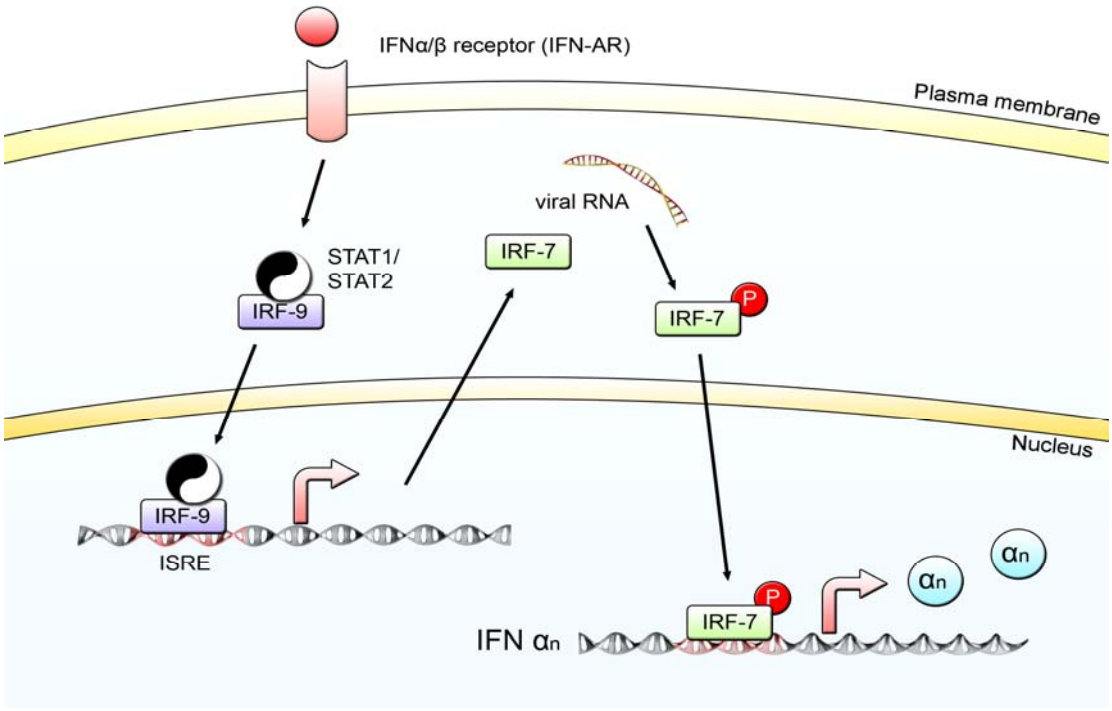


Figure 4. Induction of IFNα/β by virus. IFNα/β transcription is induced by recognition of viral PAMPs. IFNα/β can be induced either by signalling through TLR3 (recognising extracellular dsRNA taken up into the endosome) or RIG-I/MDA5 (recognises intracellular viral/dsRNA). TLR3 signalling activates a TRIF-dependent pathway, which leads to the nuclear translocation of NFκB and activation of the IRF-3 kinase TBK1. RIG-I/MDA5 activates IRF-3 kinases TBK1 and IKKε via IPS-1, which also is also required for nuclear translocation of NFκB. When IRF-3 is phosphorylated it forms dimers, which translocate to the nucleus where, together with NFκB (and other transcription factors), it induces the transcription of IFNβ.

2.2.2.2 IFNα/β Signalling

IFNα/β exert their biological effects through the common receptor IFN-AR, which is a hetero-dimeric receptor composed of IFN-AR1 and IFN-AR2 subunits. Signalling through the ubiquitously expressed IFN-AR results in the induction of an antiviral state in the cell. Ligand binding to the IFN-AR leads to phosphorylation of Janus kinases, which in turn induce the hetero-dimerisation of STAT1/STAT2 (25, 132). IFNα/β and the IFNγ signalling pathways can crosstalk and act in synergy with each other since STAT1 also mediates signalling through the IFNγ receptor, adding to the complexity of the system (139, 141). STAT1/STAT2 associates with the IFN regulatory factor 9 (IRF-9) to form the IFN-stimulated gene factor 3 (ISGF3)-complex, which is translocated into the nucleus (Figure 5). ISGF3 binds to upstream regulatory consensus sequences of IFNα/β-inducible genes (IFN-stimulated response elements, ISRE) and initiates transcription. A large number of genes involved in the host defence against viruses are regulated by ISRE in their promoters. ISRE regulates the expression of the transcription factor IRF-7, which in turn directs the transcription of IFNα. The location and activity of IRF-7 is regulated by phosphorylation, by the same virus-activated kinases as IRF-3, rendering it active only in infected cells. Thus cells that have been primed via signalling through the IFN-AR can rapidly produce all IFNα subtypes in addition to IFNβ, thus amplifying the IFNα/β signalling. The great variety of different genes regulated by IFNα/β signalling makes this a complex system.

Figure 5. IFNα/β signalling. After secretion, IFNα/β bind to the IFNα/β receptor (IFN-AR), present on most cells. Signalling through IFN-AR leads to phosphorylation and dimerisation of STAT1 and STAT2. Dimerised STAT1/STAT2 binds IRF-9 to form the ISGF3 complex. ISGF3 translocates to the nucleus, where it binds to ISRE in the promoter several genes involved in antiviral defence and activates transcription. Amongst other genes, ISGF3 regulates the transcription of IRF-7, which is induced upon IFNα/β signalling (see Figure 4). IRF-7 is phosphorylated through the same mechanisms as IRF-3 in response to virus infection. Phosphorylated IRF-7 drives the transcription of several other IFNα genes ($IFN\alpha_n$).



2.2.2.3 The IFNα/β bioassay

The IFNα/β bioassay is a classical method for measuring levels of type I IFNs. The ability of recombinant IFNα/β to protect cells from virus infection is used to quantify the amount of biologically active IFN in a sample. For measuring mouse IFNα/β, L929 cells (also called L-cells) are incubated with serial dilutions of a known concentration of recombinant IFNα/β in parallel with dilutions of samples containing unknown levels of IFNα/β. When challenged with a virus inducing cell death, cells that have been incubated with samples containing sufficiently high levels of IFNα/β will be protected against virus infection and survive whereas cells incubated with more dilute concentrations will be susceptible to infection and undergo apoptosis. When the sample is pre-incubated with specific anti-IFNα/β antibodies, the protective effect is abolished. The living cells can be visualised using an MTT substrate and the OD can be plotted. The level of IFNα/β in the sample can be calculated from the curves obtained with the known concentration.

2.3 INDUCTION OF ADAPTIVE IMMUNITY AGAINST VIRUS

In addition to limiting viral spread, cytokines induced by innate pathways also shape the adaptive immune responses against the virus. This dual role of the innate immune responses can sometimes complicate the investigation of their effect on the adaptive immune responses. Understanding how innate signals induced early during infection contribute to the induction of adaptive immune responses is critical for rational development of new vaccines and other immune-therapies.

2.3.1 Antigen presentation

All nucleated cells present a selection of peptides, generated from the proteins produced in the cell (endogenous proteins), on cell surface Major Histocompatibility Complex (MHC) class I. The peptides are generated by the cytoplasmic proteosome complex and transported in the ER via transporter associated with antigen processing (TAP) for loading on MHC class I molecules, which are then transported to the cell surface (21). CD8+ T cells constantly monitor the peptides bound to MHC class I for specific peptides they recognise. Although all cells present peptides bound to MHC class I, mainly DC activate (prime) naïve CD8+ T cells, by virtue of their expression of co-stimulatory molecules.

In addition to presentation of endogenously derived peptides, DC can present peptides generated from exogenously derived proteins on MHC class I and prime CD8+ T cells in a process termed cross-priming (14, 130). Immature DC and macrophages are constantly sampling the environment, taking up foreign material into endosomes, a process that is enhanced immediately upon TLR signalling (147). Cross-priming of exogenously derived antigens has been shown to be an important pathway for inducing CD8+ T cell responses against pathogens that do not themselves infect DC, as recently reviewed by Rock and Shen (117). Peptides derived from exogenous proteins in the endosome can access the MHC class I presentation pathway through different mechanisms. Exogenous protein can be brought from the endosome into the cytoplasm where it reaches the MHC class I molecules through the same way as do endogenous

proteins (77, 118). In addition, the endosomes themselves may be associated with, or contain, ER components necessary for exporting, processing, importing and loading peptides for MHC class I presentation (1, 59). Peptides derived from exogenous protein can also be presented on MHC class I through a vacuolar pathway, which does not require TAP or the proteosome. In the TAP-independent pathway, peptides are generated in the endosome by the protease cathepsin S and then either directly loaded on MHC class I molecules in the endosome or in the ER as a result of ER-endosomal fusion (117). The consequence of these pathways is the same, permitting DC to activate CD8+ T cells to antigens derived from the exogenous environment.

In addition to the expression of MHC class I, professional antigen presenting cells (APC), such as DC, macrophages and B cells all express MHC class II molecules on their surface and have the ability to present peptides to CD4+ T cells. MHC class II molecules present in endosomes can fuse with lysosomes, where proteins taken up from the surrounding (exogenous proteins) are digested by proteases activated by the low pH. Digestion results in peptides which are loaded on MHC class II molecules which are transported to the cell surface, as the lysosome fuses with the plasma membrane. APC also present endogenous proteins on MHC class II by taking in components of the cytoplasm to the lysosome in a process termed macroautophagy (27). Recently, this pathway has been shown to be important for antigen presentation to CD4+ T cells (124).

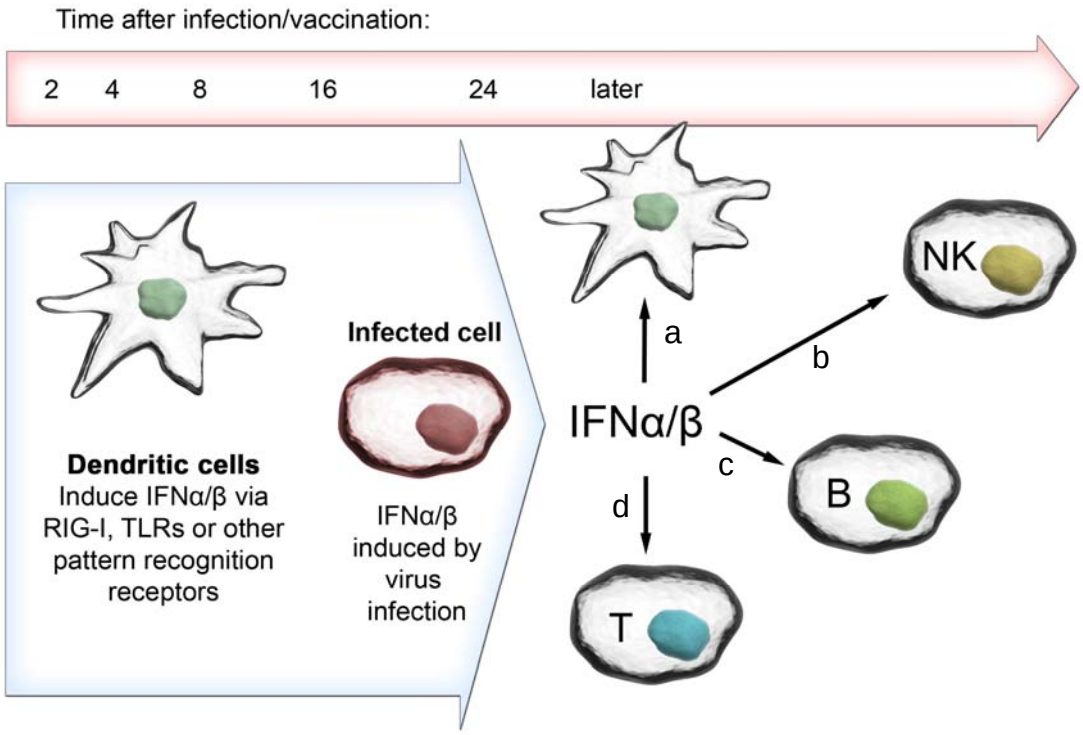


Figure 6. Effects of IFNα/β signalling. Early during infections, as early as 2 h after the encounter with virus, DC produce IFNα/β. When IFNα/β-primed cells become infected, the IFNα/β response is amplified as a result of IRF-7 upregulation. IFNα/β also have specific effects on cells of the immune system. IFNα/β signalling leads to a) maturation of DC, inducing the expression of co-stimulator molecules, b) activation and induction of cytotoxicity of NK cells, c) Upregulation of B cell survival and differentiation factors, d) enhances priming and survival of T cells.

Upon maturation, DC up-regulate their expression of CC-chemokine receptor 7 (CCR7), which controls homing to secondary lymphoid organs, causing the cells to migrate from the peripheral tissue to draining lymph nodes, where they interact with B and T cells (35). In response to PAMPs, DC induce the production of cytokines that stimulate and shape the adaptive immune responses against available antigens. In this thesis, I focus on IFNα/β and IL-12, cytokines produced early during viral infection as these have been shown to be important for the development of adaptive immune responses against virus. These cytokines can act both at the level of the DC itself, modifying its function, or on T, B and NK cells. However, numerous other cytokines, such as IL-6, IL-15 and TNFα also contribute in this process, either directly of by affecting the outcome of IFNα/β and IL-12 signalling. IFNα/β can have either stimulating or suppressing effects on the induction of immune responses, depending on the cytokine environment. It has been shown that IFNα/β can function as an adjuvant for adaptive immune responses during viral infection (80). It is also known that IFNα/β are important for DC maturation, leading to up-regulation of MHC molecules, chemokine receptors and co-stimulatory molecules CD40, CD80 and CD86 on the cell surface (37, 64, 91, 103) (Figure 6). IFNα/β can stimulate DC to take up and present antigen to and prime naïve $CD8^+$ and $CD4^+$ T cells (80). Moreover, IFNα/β-induced IL-15 has been shown to prolong the half-life of activated $CD8^+$ and $CD4^+$ T cells and enhance their proliferation (144, 156), but IFNα/β can also act directly to maintain and stimulate the activated T-cells (96).

2.3.2 Induction of T cell responses

Both pathogen-derived and host cell-derived immune-stimulatory molecules act on DC, causing them to up-regulate co-stimulatory molecules on their surface and to mature into APC (49). Priming of naïve T cells occurs when a mature DC encounters a T cell with a T cell receptor (TCR) that recognises a peptide presented on the major histocompatibility complexes (MHCs) of the DC. If a T cell recognises a peptide on an immature DC, the T cell becomes anergic; functionally inactivated and unable respond, even if the peptide is presented later with full co-stimulation (134).

Naïve T cells are circulating in peripheral blood and lymph until they encounter mature DC presenting a peptide that can bind to the specific TCR of the naïve T cell. Priming of T cells occurs is a process that can be divided into three phases (102). The first encounters between the naïve T cells and the antigen-presenting DC are rapid and brief and the T cells sample several different DC in their surrounding. Thereafter the interactions with the antigen-presenting DC become more stable, lasting generally for more than 30 minutes, forming clusters. If the DC present a specific antigen recognized by the naïve T cell, the interaction causes the T cell to up-regulate activation markers and maintain the interaction with the DC. The third phase, initiated 24 hour after the initial contact, is characterized by rapid division and cytokine secretion by the T cells.

2.3.2.1 Development of cytotoxic T cells

Activation of naïve CD8 T cells to undergo clonal expansion and develop effector and memory functions require a number of signals. First, a peptide from the antigen needs to be presented to the naïve T cell on MHC class I molecules present on a mature DC.

Although all cells express MHC class I, only APC, and primarily mature DC, express co-stimulatory molecules required for priming naïve T cells (127). In addition to co-stimulatory molecules and antigen presentation it has been shown that efficient CD8+ T cell priming requires either IFNα/β or IL-12, especially when there low levels of antigen is available (22-24).

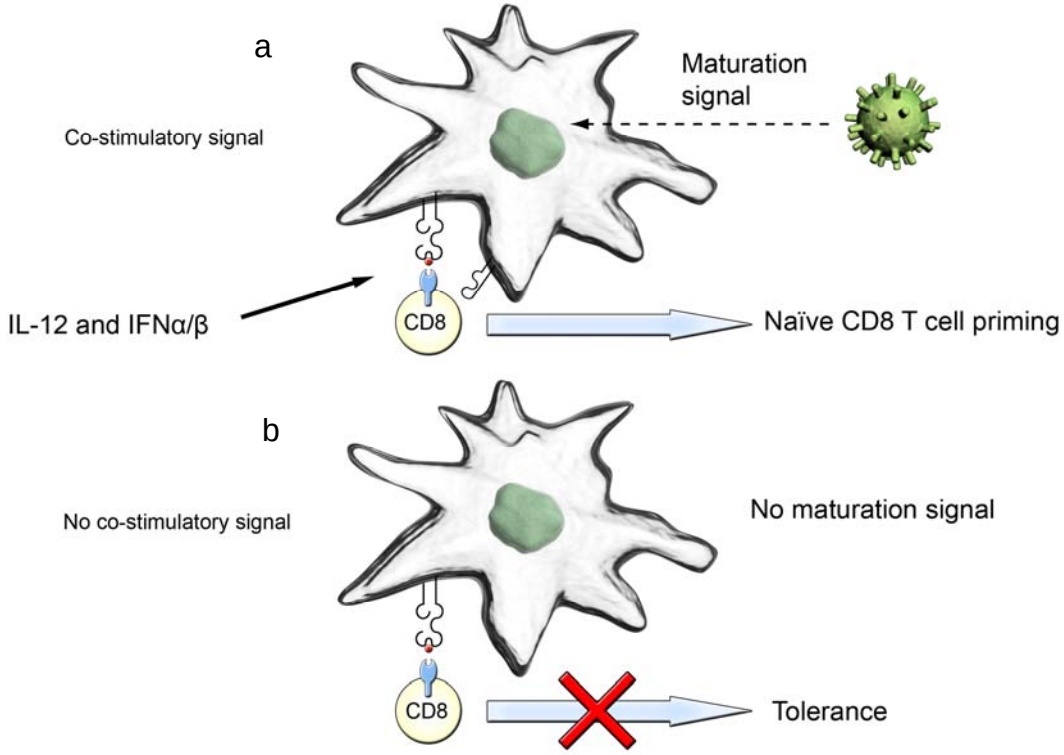


Figure 7. Maturation of DC. (a) DC need a signal to become a mature APC capable of activating immune responses. The maturation signal can be a pathogen, such as a virus, or cytokines. Mature DC up-regulate co-stimulatory molecules on their surface. IL-12 and/or IFNα/β are also required to efficiently prime naïve CD8+ T cells. (b) If the DC presents antigen without providing co-stimulation, the T cells become anergic.

Primed CD8+ T cells rapidly up-regulate activation markers such as CD69, CD44 and IL-2 receptor CD25. The expression of CD69 and CD25 is transient, while the expression of CD44 on antigen-experienced cells remains high (102). Activated, or antigen-experienced CD8+ T cells can be divided into two categories; effector and memory T cells. CD8+ effector T cells are in a state of "activation-readiness" and have the ability to kill cells that present their specific peptide on MHC class I. The effector T cells are characterised by their ability to rapidly secrete IFNγ upon recognition of the peptide/MHC class I complex and by the loss of the expression of CD62 ligand (CD62L) on their surface. CD62L, also known as L-selectin, is a member of a family of adhesion/homing receptors expressed on naïve T cells and is, together with CCR7 essential for lymphocytes to enter lymph nodes (18, 35). After priming and the initial phase of clonal expansion, antigen-specific CD8+ T cell population go through a contraction phase in which the majority (about 90%) of activated effector T cells undergo apoptosis. During the contraction phase, effector cells gradually develop into memory T cells in a process that takes several weeks after clearance of the antigen.

Depending on the stage of their development, memory T cells can be divided into effector memory (T_{EM}) and central memory (T_{CM}) T cells, defined by their surface expression of lymphocyte homing receptors CD62L, CD44 and CCR7 (122, 148). This division is mainly valid in human T cell biology, but translates to some extent to the murine system. T_{EM} express receptors for migration to inflamed tissues, where they can rapidly gain effector function, while T_{CM} cells are "true" long lasting memory cells with the ability to proliferate upon re-encounter of antigen (122). T_{CM} cells, expressing lymphocyte homing receptors, are mainly found in the lymph nodes, blood and spleen, while T_{EM} cells are found in non-lymphoid tissues (such as the gut, lung and liver), blood and spleen (97). T_{CM} and T_{EM} cells have different functions, since their distribution and kinetic of their ability to regain effector functions are different, but the consensus is that it is the CD62L expressing T_{CM} that confer protection against re-infection (155). It is argued that a strong priming signal predominantly drives the CD8+ T cell response towards and T_{EM}, and that the subsequent T_{CM} is then delayed (83, 148).

2.3.2.2 Induction of helper T cells

CD4+ T cells, also called T helper cells, recognise MHC class II on the surface of APC. Upon priming, surface receptor CD40 ligand expressed by T helper cells can bind CD40 on the surface of B cells and induce proliferation and differentiation of the B cell into antibody-producing plasma cells. Depending on the signals the T cell receives during priming, naïve T helper cells can differentiate into either T_H1 cells, characterized by their production of IFNγ, or T_H2 cells producing for example IL-4 and IL-10. This classification is mainly used in the murine system. IFNγ produced by the T_H1 T helper cells stimulates the proliferation and differentiation of the antigen-specific cytotoxic CD8+ T cells. T_H2 type responses induce primarily antibody-mediated immunity, where IL-4 produced by the T_H2 T helper drives B cell proliferation and differentiation into IgG1-producing plasma cells. The T_H1 type T helper cells can also induce antibody production by switching B cells into producers of antibodies, which generally are of IgG2a subclass. This distinct division of immune responses into T_H1 and T_H2 biased functions is likely too simplistic and has lately been challenged by new findings (116).

2.3.3 Induction of B cell responses

Antibody production after infection or vaccination provides the first line of defence against infection by the pathogens. In addition to the presence of antibodies, resting antigen-specific memory B cells can respond to infection by quickly dividing and differentiating into antibody-secreting plasma cells. Antibodies, or immunoglobulins (Ig), consist of variable, heavy and light chains. Highly diverse variable chains recognise the antigen, while the heavy constant chain determines the effector function of the antibody. Initially, upon stimulation by antigen, B cells secret antibodies with heavy constant chains M and D that can bind and neutralise pathogens. As the B cells differentiate, the heavy chains are substituted with G, A or E in a process called class switching. Ig with heavy chains G, A or E can trigger a large array of actions of the immune response, such as binding Ig receptors present on various cells of the immune system and activating complement. When naïve B cells are stimulated by antigen and T-cell help, they proliferate at the margins of the T-cell zones in lymph nodes and spleen. Once activated, B cells differentiate either into short-lived plasma cells, or migrate into the lymph node B cell follicles. In the follicles T helper cells drives the

formation of germinal centres, where B cells carrying receptors with high affinity for the antigens are selected and stimulated by T helper cells. B cells are stimulated, by CD40-CD40 ligand interaction and cytokines provided by antigen-specific T helper cells, to differentiate into memory B cells or long-lived plasma cells.

In addition to priming T helper cells, required for most B cell responses, DC can also stimulate B cells in a more direct fashion. DC activated by pathogens secrete IL-12, which can drive naïve B cell proliferation and differentiation into plasma cells (31). Activated pDC can also stimulate B cells to differentiate into plasma cells, independently of T helper cells (113). This stimulation is, at least in part, mediated by IFNα/β secreted by the pDC. In the presence of IL-6, IFNα/β induce already activated B cells to differentiate into Ig-secreting plasma cells (67, 81). IFNα/β can also affect the survival of B cells. IFNα/β-signalling triggers up-regulation of BLyS (B lymphocyte stimulator) and APRIL (a proliferation-inducing ligand), two major B cell survival factors expressed by monocytes and DC (45, 104). Stimulation of B cells through BLyS and APRIL has been shown to contribute to CD40-independent Ig class-switch, decreasing the need for T cell help (87). Thus, innate signals and especially IFNα/β may be important for the outcome of B cell stimulation during viral infection.

3 RESULTS AND DISCUSSION

I present here a brief overview and an extended analysis of the work contained within this thesis. I have included some additional results, omitted from the papers, to broaden the discussion. I also attempt the put my earlier paper in the new light of recently published findings in the field.

3.1 PAPER I

Our first study focuses on what viral functions are required for the induction of IFNα/β and what cellular pathways mediate the IFNα/β induction. SFV is a potent inducer of IFNα/β (61, 62), which induce anti-viral responses that inhibit SFV replication (36) and are important for controlling SFV infection *in vivo* (105). Though most cell types have the potential to induce IFNα/β upon viral infection, pDC have been shown to be extraordinary potent producers of IFNα/β following recognition of virus (65, 72, 140). There are also reports showing that mDC can produce IFNα/β in response to virus infection (29, 56, 89, 90, 119, 121). In paper I of this thesis, we demonstrated that murine bone marrow-derived GM-CSF matured mDC are a source of IFNα/β in response to rSFV.

We initially studied the induction of IFNα/β in both FLT3L (fms-related tyrosine kinase 3 ligand) and GM-CSF (granulocyte/macrophage colony stimulating factor) matured DC cultures (41). The FLT3L cultures contained typically about 30% pDC, defined by their expression of CD11c and CD45RB/ B220, while the remaining cells were mainly mDC expressing CD11c and CD11b. The GM-CSF matured cultures did not contain any detectable pDC. In fact, previous studies have shown that GM-CSF directly prevents the generation of pDC in bone marrow-derived cultures (41). We found that the FLT3L-matured cultures had a similar capacity to produce IFNα/β in response to high doses of rSFV, while there were distinct differences between the culture systems in the induction of IFNα/β in response to pI:C and CpG. The pDC-containing FLT3L-matured cultures responded with considerably higher production of IFNα/β in response to CpG, in agreement with reports of their expression of TLR9 (78). Both cultures responded to the presence of pI:C, which is partly mediated through TLR3 and partially through cytosolic receptors (4, 19, 51, 68, 85, 137, 154). The GM-CSF matured mDC cultures responded to pI:C with high levels of IFNα/β, consistent with the expression of TLR3 on these cells (9).

Since IFNα/β production by mDC in response to virus is less well characterized than that of pDC, we focused our further investigation on mDC. In paper I we have investigated what functions of rSFV were required to stimulate the mDC to produce IFNα/β. By specifically blocking viral replication through UV-inactivation of the virus, we showed that replication-incompetent virus particles induced IFNα/β in mDC cultures, but not in primary MEFs. mDC have primarily been described to respond to viral replication while the ability to respond to replication-inactivated virus has ascribed to pDC (28, 92). The only report of replication-inactivated virus stimulating IFNα/β production from mDC had concerned DNA virus (119).

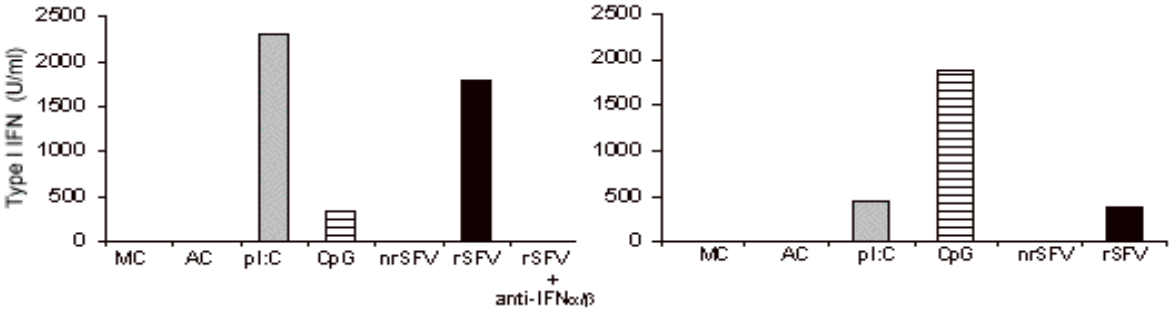


Figure 8. Levels of IFNα/β in GM-CSF vs FLT3L stimulated DC cultures in response to various stimuli.

The DC culture differentiated in the presence of GM-CSF contained 80-90 % mDC and no pDC. The DC culture differentiated in the presence of FLT3L contained 20-30 % pDC. The remaining cells were mostly mDC of immature phenotype. The cultures were incubated with different stimuli (see materials and methods section of Paper I) for 24h. IFNα/β in the supernatants was measured using the IFNα/β bioassay. When IFNα/β-containing samples from the mDC cultures stimulated with rSFV were pre-incubated with a specific anti-IFNα/β antibody, the IFNα/β-activity was abolished, showing the specificity of the bioassay.

We found that the ability of rSFV to induce IFNα/β in mDC cultures and *in vivo* required a fusion-competent virus. We used an rSFV vector packaged using a mutated helper RNA, where a furine recognition sequence of the spike polyprotein had been changed into an α-chymotrypsine site (13). This spike mutation produced incompletely processed and virtually non-infectious viral particles (nrSFV). nrSFV, which is incapable of the spike rearrangements required for viral fusion, did not induce IFNα/β in mDC cultures or *in vivo*. After the nrSFV particles had been rendered infectious, by *in vitro* proteolytic processing by α-chymotrypsine, the mutant viral particles regained the ability to stimulate production of IFNα/β. Also, viral particles in which the structural proteins had been cross-linked by high energy UV-irradiation were incapable of inducing IFNα/β production in mDC cultures or *in vivo* (data not shown). Collectively we show that a replication-inactivated, but fusion competent virus could induce IFNα/β-production in mDC but not in MEFs, while a fusion incompetent virus did not induce IFNα/β in either cell-type, or *in vivo*.

We had several hypothesises as to how the mDC but not MEFs could respond to the virus with IFNα/β induction at a stage prior to viral replication. The first question to be addressed was if the mDC carried a TLR that was responsible for detecting the virus prior to replication. pDC have been described to require MyD88 for the induction of IFNα/β via TLR7/8 in response to virus (28, 70). To address the involvement of these TLRs we used mice lacking MyD88 and we investigated if MyD88 was required for the IFNα/β induction in response to rSFV, either *in vivo* or in GM-CSF matured DC cultures. The ability of mDC to respond to UV-inactivated virus was not compromised in MyD88$^{-/-}$ mDC cultures and MyD88$^{-/-}$ mice were also fully competent of IFNα/β-production in response to i.v. inoculation with replication competent rSFV. Thus, our results show that mDC can detect rSFV via an alternative, MyD88-independent pathway, different from the MyD88-dependent endosomal pathway described to be essential for induction of IFNα/β in response to virus in pDC. The ability of the

MyD88 deficient mice to respond with IFNα/β production to rSFV-inoculation shows that this alternative pathway has a physiological relevance.

MyD88 has been reported to induce IFNα/β production through a signal cascade involving IRF-7, but not IRF-3 (70). Since the alternative pathway for induction of IFNα/β, the RIG-I dependent pathway, requires IRF-3 we investigated if IRF-3 was required for induction of IFNα/β by rSFV. IRF-3 is also the transcription factor mediating the IFNα/β production in response to signalling via TLR3 and 4 (30). We found that mDC lacking IRF-3 were defective in the induction of early IFNα/β in response to both rSFV and UV-rSFV. Thus we concluded that IRF-3 was required for induction of IFNα/β in mDC cultures in response to both replication-competent and replication-inactivated rSFV. Thus, rSFV induces IFNα/β through an IRF-3 dependent pathway such as TLR3, 4 or RIG-I. TLR3 has been reported to be a receptor specific for dsRNA and not for ssRNA (98), which is the content of the rSFV viral particles. Our results suggest that events during or downstream of viral fusion stimulate IFNα/β production by mDC in response to UV-inactivated virus and suggest that cytosolic recognition of incoming virus prior to replication mediates this response. The recognition of the entry-competent UV-inactivated viral particle occurs at a stage prior to replication and production of dsRNA replication intermediates, and thus we conclude that dsRNA is not required for IFNα/β induction by mDC in response to rSFV. It is therefore unlikely that TLR3 is required for the recognition and subsequent IFNα/β induction by rSFV. The lack of IFNα/β in response to the fusion-incompetent nrSFV particles demonstrated that the ability of the virus to induce IFNα/β was dependent on a viral function (fusion) and thus was not due to any potential contaminants of the viral preparations. The reagents used for proteolytic activation did not induce IFNα/β production in itself or activated the mDC.

The remaining and most interesting hypothesis was that viral RNA motifs are recognised inside the cytoplasm by RNA helicases, such as RIG-I. Our results suggest that a pathway upstream of IRF-3, which leads to IFNα/β production in response to incoming non-replicating virus genomes, is operative in mDC but not in MEFs. Since RIG-I is required for the induction of IFNα/β in response to Sendai virus, vesicular stomatitis virus, Newcastle disease virus (68, 85) and hepatitis C virus (137), we consider it likely that rSFV is recognised by the same mechanism. If the RIG-I pathway is engaged in the recognition of rSFV RNA prior to replication, it still remains to be explained why MEFs and DC respond differently to UV-inactivated virus, since RIG-I is expressed by both mDC and MEFs (137). The difference in IFNα/β-production in response to UV-inactivated virus between mDC and MEFs could possibly be due to a difference in the level of expression of RIG-I. An alternative and explanation for the difference between mDC and MEFs in their ability to induce IFNα/β upon incubation with replication incompetent virus could be that mDC are more responsive to signalling through the RIG-I pathway. RIG-I is IFNα/β-induced and mDC have been reported to maintain a level of constitutive IFNα/β signalling (46) and it has been shown that DC have higher constitutive levels of other IFNα/β-induced proteins (120, 121). The difference between MEFs and DC can also be due to higher levels of other molecules participating in the IFNα/β-induction downstream of RIG-I. One hypothesis, that mDC are in a constant IFNα/β-primed state and thus more sensitive to the recognition of virus, is consistent with the view that DC are early sentinels for virus infection and that

it is difficult to generate a productive infection of mDC by many viruses, including rSFV.

Another possibility is that mDC but not MEFs have additional cytoplasmic RNA helicases recognising viral RNA motifs in mDC that may be more sensitive in recognising motifs of incoming viral genomes. Several of the possibilities remain to be tested, and solving these questions is important for understanding the early detection of virus and induction of an innate antiviral response.

In conclusion, we have shown that mDC, but not MEFs, can be potent producers of IFNα/β in response to non-replicative virus. The pathway through which mDC induce IFNα/β in response to recognition of RNA virus is different from the TLR7/8 and MyD88/IRF-7 mediated signalling described for pDC. Instead, induction of IFNα/β in mDC in response to virus is mainly mediated by the transcription factor IRF-3. Our results show that there are alternative pathways for inducing an anti-viral state in response to early virus infection. Our findings imply that mDC may act as sentinels for detecting incoming viruses prior to establishment of viral replication and production of viral gene products, which could counteract the IFNα/β induction.

3.2 PAPER II

Rotaviruses are entereroviruses of the Reoviridae family, dsRNA viruses causing severe diarrhoea in small children. Significant effort has been put into developing vaccines against rotavirus infection. In 2005, two oral vaccines based on live attenuated viruses, have been licensed for use. However, little is known about how these vaccines, or natural infection, induce protective immunity. DCs are central for recognition of pathogens and for induction of adaptive immune responses. Human DC have previously been shown to mature and induce IL-6 in response to rotavirus infection (107), but whether rotavirus induced IFNα/β production in DC had not, to our knowledge, been studied. Early reports describe rotavirus as a poor inducer of IFNα/β, although rotavirus replication is sensitive to the actions IFNα/β in cell culture (100). The ability of rotavirus to induce IFNα/β has recently come into focus, with reports of rotavirus NSP1 interacting with and inducing degradation of IRF-3, 5 and 7 (11, 12, 44). Degradation of IRFs efficiently prevents the induction of IFNα/β in virus-infected cells and is an evasion strategy of rotavirus, which is lost during passage of the virus in cell culture, due to spontaneous deletions in the gene encoding NSP1 (11, 110).

Since we have previously shown that DC can recognize and induce IFNα/β at a stage prior to viral gene transcription, we were interested in investigating if DC had retained the ability to respond with IFNα/β production to early rotavirus infection. We found that murine bone-marrow derived DC could be infected by rotavirus and that there was viral protein synthesis in these cells, as shown by their expression of VP2 and VP6 after incubation with infectious virus. However, the infection did not seem to be productive in terms of particle formation and/or release, since no progeny virus could be detected in the supernatant of the DC cultures. The DC could indeed produce low levels of IFNα/β in response to infectious triple-layered particles, but not in response to the non-infectious double-layered particles. DCs exposed to infectious virus also up regulated co-stimulatory molecules on their surface in an IFNα/β dependent manner, showing

that although only low levels of IFNα/β were produced by rotavirus infected DCs, these levels were sufficient to activate the cells. We also confirmed that the strain of rotavirus used in our experiments (a macaque strain) was capable of degrading murine IRF-3, as shown by the lack of IRF-3 staining in rotavirus infected MEFs but not in mock-infected control MEFs.

There were at least two possible explanations for why DC but not MEFs produced IFNα/β in response to rotavirus infection. Either the degradation IRF-3 was incomplete in the infected DC or the IFNα/β production was not mediated by IRF-3. At the time our study was performed, the report describing degradation of IRF-5 and IRF-7 by NSP1 (12) was not yet published, thus we considered it possible that one of these transcription factors could mediate IFNα/β production in DCs in the absence of IRF-3. To investigate if IRF-3 was required for IFNα/β production in DC, we compared the levels of IFNα/β from rotavirus-infected wt and IRF-$3^{-/-}$ DC. We found that the IFNα/β levels were markedly reduced in IRF-$3^{-/-}$ DC compared to wt control DC, both when measured by the IFNα/β bioassay and by a commercial IFNβ ELISA. We therefore concluded that IRF-3 at least in part mediates IFNα/β induction in response to rotavirus in DC. We next investigated to which extent IRF-3 was degraded in the rotavirus-exposed DCs. By Western blot analysis we found that there was no detectable reduction in the IRF-3 levels at 24 and 48h post infection, even though VP2 was readily detected in cell lysates from infected DC. When rotavirus-infected DC were assayed by immunofluorescence, we detected VP6 in a high proportion of the cells, but only a very low proportion of the cells were positive for NSP4, another non-structural rotavirus protein. Since structural proteins are produced in higher copy numbers than non-structural proteins it is possible that we were at the level of detection for NSP4 and therefore only detected NSP4 in some of the VP6 positive cells. Together with the results described above, showing a lack of progeny virus from the infected DC, these data suggested that the viral infection was aborted at an early stage in DC, thus limiting the production of viral proteins including NSP1. So far, we have not had access to an antibody against NSP1, but it would be interesting to stain for NSP1 to confirm that it is expressed in DC. It therefore remained unclear if the IRF-3 detected by Western Blot originated from cells with ongoing rotavirus infection or from uninfected cells in the culture. Further immunofluorescence studies to attempt to co-localize NSP1 and IRF-3 in infected DC would be valuable to further address these questions.

To investigate of viral protein production affected the induction of IFNα/β from DCs, we treated cells with UV-inactivated virus. We found that DC cultures stimulated with UV-inactivated virus produced significantly higher levels of IFNα/β. This was consistent with a previous report showing that UV-inactivated, but not heat-inactivated, rotavirus induces higher amounts of IFNα/β in Macaque kidney cells than the replication-competent virus (100). This result strongly suggested that a virally encoded IFNα/β antagonist was expressed in DC infected with replication-competent rotavirus, but not in cells exposed to UV-inactivated rotavirus. The obvious candidate for such an antagonist is NSP1, even though we have not been able to formally demonstrate that NSP1 is expressed in these cells.

Since binding of TLR3 with purified reovirus dsRNA can activate IFNα/β transcription (4) and since TLR3 requires IRF-3 for IFNα/β induction (30), it was possible that

TLR3 might contribute to the IFNα/β-induction in DC. Also, TLR7/8 might mediate IFNα/β production in response to virus infection, either by recognition of viral mRNA in the cytoplasm of infected cells (82), or by uptake of viral particles into the endosomes (28, 50, 93). To investigate if IFNα/β production in rotavirus-infected DC was mediated through TLRs, we set up cultures from $TLR3^{-/-}$ and $MyD88^{-/-}$ mice. We found that both replication-competent and UV-treated rotavirus-stimulated similar levels of IFNα/β production from $TLR3^{-/-}$ and $MyD88^{-/-}$ DC cultures as from their respective wt control cultures. This suggested that IFNα/β production by mDC in response to rotavirus infection was not mediated by TLR signalling. Similar experiments in the $MyD88^{-/-}$ and $TRIF^{-/-}$ double knock-out mice are required to definitely determine if TLRs are involved in IFNα/β induction in response to rotavirus and such experiments are now possible thanks to the recent generation of such mice (57). The results from the $TLR3^{-/-}$ and $MyD88^{-/-}$ DC cultures confirm that the production of IFNα/β is significantly enhanced when the cells are exposed to UV-inactivated virus, consistent with a suppressive role of NSP1 in these cells.

The molecular details of IFNα/β induction in response to rotavirus remain to be investigated. Especially, it remains unknown which viral structure is recognised by the cells. Since rotavirus has a genome consisting of dsRNA, TLR3 was a candidate receptor for triggering IFNα/β production in response to rotavirus, however our results suggest that this pathway is not used. In fact, the great majority of studies that have investigated the role of TLR3 in IFNα/β induction in response to RNA virus and the importance TLR3 in antiviral defences in general have failed to show a role for TLR3, as also discussed by Schröder and Bowie (125). Furthermore, if viral RNA genomes were accessible for recognition by TLRs in endosomes, for example after degradation of viral particles by endosomal/lysosomal proteases, we would have expected the non-infectious double-layered particles to also induce IFNα/β as they also have dsRNA packaged, but they did not. It is possible that double-layered particles bind to DCs with a lower affinity than triple-layered particles as they lack some of the structural proteins and therefore may taken up by DC less efficiently. Nevertheless, collectively our results suggest that IFNα/β induction requires rotavirus to enter cells and release its genome in the cytosol and induce the response in a TLR-independent manner. Interestingly, these are the same conclusions as were drawn from similar experiments using rSFV. The exact nature of the ligand and the pathway that induces IFNα/β during rotavirus infection of DCs thus remains undetermined. As yet, there are no reports in the literature on the role of RIG-I or MDA5 in IFNα/β induction by rotavirus, but this will no doubt soon be addressed using cells that lack these molecules.

3.3 PAPER III

In the third study, the aim was to elucidate the importance of IFNα/β for inducing antibody responses during virus infection. Most viral infections stimulate potent adaptive immune responses to clear the virus and protect from re-infection. Previous reports have suggested that virus infection can enhance immune responses elicited against unrelated co-administered protein antigens (6, 26, 52, 123, 131). The mechanisms by which viruses provide adjuvant signals to co-administered proteins were not addressed in these studies. Also, it is not clear if the signals that drive immune responses against virus-encoded antigens are the same as those that promote immune

responses to co-administered antigens. To address this in a well-controlled system, we performed co-immunization experiments using rSFV particles expressing model antigens and unrelated purified protein antigens. We measured immune responses against virus-encoded antigens and against the protein antigens in a series of experiments as described in paper 3 of this thesis. We were primarily interested in elucidating the importance of pathways associated with IFNα/β induction. IFNα/β have been described to have potent adjuvant effects on adaptive immune responses (80, 81) and we have demonstrated that rSFV induces high levels of IFNα/β *in vivo* (55).

We demonstrate that rSFV provides a strong adjuvant effect on antibody responses against the co-administered proteins. When mice or rabbits were immunized either with recombinant protein antigens alone or with the antigens mixed with rSFV, the specific IgG response to the protein antigens was significantly higher if rSFV was present. The presence of rSFV also skewed the type of antibody response towards a T_H1 response (primarily IgG2a), as opposed to the T_H2 response (primarily IgG1) seen when protein alone was used for immunisation. An adjuvant effect on the antibody responses was also measurable when the protein antigen and rSFV were immunised at separate sites, suggesting that the virus-induced signals could act at a distance. We hypothesised the adjuvant effect was dependent on soluble cytokines and we proceeded to investigate if virus-induced IFNα/β contributed to this effect. We found that the adjuvant activity on co-administered antigen was completely abolished in mice lacking IFN-AR (IFN-AR1$^{-/-}$), suggesting that IFNα/β-signalling is critical for the effect. In contrast, IFNα/β-signalling was not required for the antibody responses against the virus-encoded antigens. These results point to a clear difference in the requirements for raising immune responses to virus-encoded antigens and purified protein antigens in this experimental system.

To investigate if the adjuvant effect of rSFV required viral replication, we immunised mice with recombinant protein alone or with protein mixed with UV-inactivated rSFV (UV'rSFV-NP). UV-inactivation was performed as in paper I, rendering the virus non-replicative while preserving the integrity of the structural proteins. UV'rSFV-NP could also provide an adjuvant effect to the antibody response against the recombinant protein, although slightly lower (data not shown). This is consistent with the results in paper I, where we show that UV'rSFV induces slightly lower levels of IFNα/β *in vivo*, as compared to the replication competent rSFV (54). A virus subjected to a harsher UV-treatment (UV''rSFV-NP) did not have an adjuvant effect on the antibody responses against β-gal, consistent with that this virus does not induce IFNα/β because this virus is entry-incompetent. No antibodies against the virus-encoded NP antigen could be detected in mice immunised with either UV''rSFV-NP or UV''rSFV-NP, showing that the UV-treatments had indeed severely impaired the ability of this virus to replicate.

Further, we show that $CD4^+$ T cell help is required for eliciting specific antibodies against both the virus-encoded and the co-immunised protein antigen during rSFV immunization. Induction of a T helper response is dependent on antigen presentation by appropriately activated DC. Since signalling through TLRs can directly stimulate the ability of DC to present antigen to T cells (147) and, for some TLRs, leads to the induction of IFNα/β, which activate DC, we investigated if the immune-stimulatory

effect of viruses was dependent on TLRs. Antibody responses to both virus-encoded antigens and co-administered protein antigens were independent of signalling via MyD88 and TLR3, which have both been implicated to play a role in the induction of adaptive immune responses against viruses (20, 126). In agreement with the dependence on IFNα/β for the adjuvant effect on antibody titres against the co-administered, both MyD88$^{-/-}$ and TLR3$^{-/-}$ mice were fully capable of responding IFNα/β-production in response to rSFV inoculation *in vivo* (54). Soon after the publication of paper III, studies in mice lacking both TRIF and MyD88 showed that TLR-signalling is not required for the elicitation of antibody responses against various antigens using different adjuvants (39, 108). This is in line with our observation using the rSFV system.

The antibody responses against both the vector-encoded and the co-immunized antigens were abolished in the mice lacking CD4, a surface molecule essential for a functional interaction between the TCR present on CD4$^+$ T cells and MHC class II molecules. This suggests that other innate signals induced during rSFV infection cannot compensate for the lack of T cell help to generate an antibody response. In addition, the IFNγ CD8$^+$ T cell response against the virus-encoded antigen was defective in the CD4$^{-/-}$ mice, indicating that the CD4$^+$ T cells are important also for the priming of the cellular arm of the antigen-specific immune response (Figure 9).

However, when we performed a chromium release assay for cytotoxicity, after 6 days of re-stimulation of splenocytes with an MHC class I peptide, the response of the CD4$^{-/-}$ mice was similar to that of wt mice (Figure 9b). It is possible that the extended re-stimulation masks defects in the priming of T cells in the CD4$^{-/-}$ mice observed in the ELISPOT assay (20 h stimulation with the peptide) (Figure 9a). Since T helper cells are primed and stimulated by DC, we hypothesised that the adjuvant effects provided by virus-induced IFNα/β were acting on DC, enhancing their ability to prime naïve T

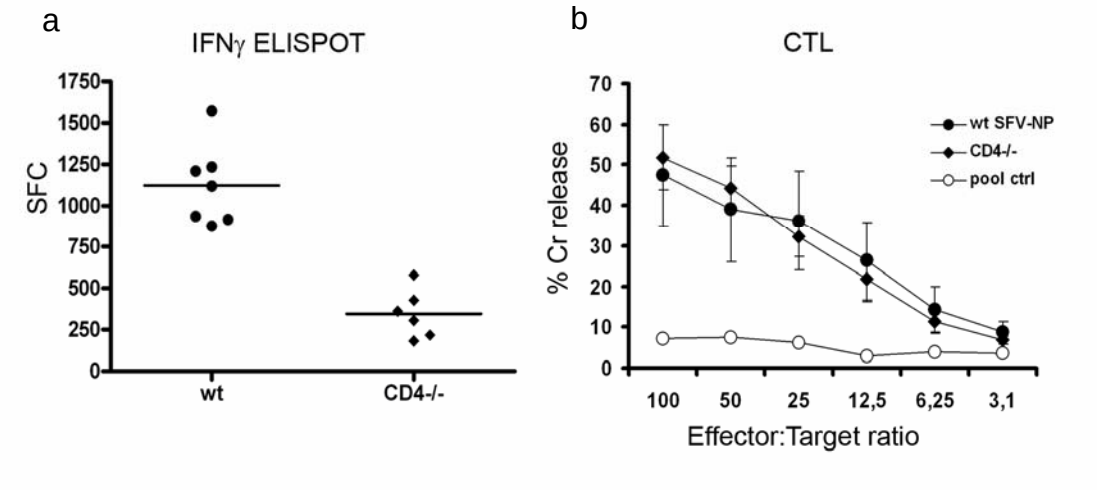


Figure 9. CD4+ T cell help is required for full CD8+ T cell responses. Splenocytes from wt and CD4$^{-/-}$ mice immunised with 10^6 IU of rSFV-NP were re-stimulated with MHC class I NP-peptide ASNENMETM a) in an IFNγ ELISPOT and expressed as spot-forming cell/10^6 cells (SFC). b) in a chromium release cytotoxicity assay (CTL).

cells. To investigate if IFNα/β contributed to the stimulation of DC following incubation with rSFV, we analysed the expression of co-stimulatory markers on mDC from wt and IFN-AR1$^{-/-}$ mice. We found that virus-exposed mDC up-regulated their surface expression of CD40 and CD86, while no up-regulation of co-stimulatory markers could be seen on mDC from IFN-AR1$^{-/-}$ mice. Neither did mDC stimulated with rSFV-infected MEFs up-regulate co-stimulatory markers in the absence if IFNα/β. Thus this in vitro system does not provide an explanation for how T helper cells are stimulated by DC in the absence of IFNα/β.

As mentioned above, antibody responses directed against virus-encoded antigens were intact in IFN-AR1$^{-/-}$ mice. This suggests that other signals associated with infected cells are sufficient to drive immune responses against virus-encoded antigens.

3.4 PAPER IV

During the work with paper III, where we compared antibody responses in wt and IFNR-AR1$^{-/-}$ mice, we also measured CD8+ T cell responses against rSFV-encoded antigen. We consistently observed a higher frequency antigen-specific IFNγ-producing CD8+ T cells in the spleen of immunized IFNR-AR1$^{-/-}$ mice compared to in wt control mice. Considering our findings in paper I showing that DC are not readily susceptible to infection by rSFV, thus making direct priming of CD8+ T cells unlikely, we considered it likely that CD8+ T cell responses against rSFV-encoded antigens were the result of cross-priming, a hypothesis also supported by the work of Huckriede et al. (60). Since IFNα/β has been reported to enhance cross-priming (80), the enhanced CD8+ T cell responses in the IFNR-AR1$^{-/-}$ mice was a very intriguing observation. Another observation apparently at odds with the enhanced CD8+ T cell responses detected in the IFNR-AR1$^{-/-}$ mice was our results described in paper III, which show that rSFV, and rSFV-infected cells, failed to stimulate mDC to up regulate co-stimulatory markers in the absence of IFNα/β (paper III, Fig 7).

To investigate if the increased number IFNγ-producing cells observed in rSFV-NP immunised IFNR-AR1$^{-/-}$ mice was due to a larger amount of antigen-specific CD8+ T cells, we assayed blood and spleen for NP-specific CD8+ T cells using tetramer staining. In agreement with earlier results, IFNR-AR1$^{-/-}$ mice showed an increased proportion of NP-specific CD8+ T cells both in blood and in spleen. This result also showed that the enhanced responses observed in spleen could not be explained by redistribution of antigen-specific T cells to the spleen of IFNR-AR1$^{-/-}$ mice. A possible explanation for these results is that the IFNR-AR1$^{-/-}$ mice are more susceptible to rSFV infection due to their compromised innate anti-viral response, which could lead to infection of a larger number of cells or to a higher production of antigen per infected cell, which in turn could influence the antigen-specific CD8+ T cell responses. We proceeded with two experiments to investigate if this could be the case. First, we addressed whether an increased number of infectious particles (and thus an increased number of infected cells) would lead to an enhanced CD8+ T cell response. We immunised both wt and IFNR-AR1$^{-/-}$ mice with 10^6 or 10^7 infectious units (IU) of rSFV-NP and compared the proportions of antigen-specific IFNγ-producing CD8+ T cells between the two regiments. We found that already at a 10^6 IU, a plateau level of CD8+ T cell responses had been reached and the plateau level remained almost

threefold higher in IFNR-AR1$^{-/-}$ mice immunised with 10^7 IU. Second, to remove the variable of how much antigen was produced in immunised wt versus IFNR-AR1$^{-/-}$ mice, we infected wt MEFs in vitro with rSFV-NP and we washed off the cell-free virus from the cells. The MEFs were then divided in two equal portions and used to immunise wt and IFNR-AR1$^{-/-}$ mice and CD8+ T cells were examined as before. We found that the difference between these mice in regards to IFNγ-production and number of antigen-specific CD8+ T cells were similar to that observed after rSFV particle immunisation. From this data we concluded that the increased CD8+ T cell responses against viral antigen in the absence of IFNα/β signalling are not due to increased antigen levels in the IFNR-AR1$^{-/-}$ mice.

Since elevated CD8+ T cell responses in IFNR-AR1$^{-/-}$ mice were observed after a single immunisation with rSFV-NP, we attributed this effect to enhanced CD8+ T cell priming in the absence of IFNα/β-signalling. To explain these data, we hypothesised that IFNα/β-activated NK cells may regulate T cell priming in wt mice by the mechanism described by Hayakawa et al. (47). Hayakawa et al. showed that DC were eliminated by NK cells in a TNF-related apoptosis-inducing ligand (TRAIL)-dependent manner, a process that decreased the induction of cytotoxic T cells. Since the cytotoxicity of NK cells can be regulated by IFNα/β, we speculated that the IFNα/β induced during rSFV immunisation contributed to this process. To address this we performed a similar experiment to that performed by Hayakawa et al, where an anti-TRAIL antibody was used to block this pathway prior to and during immunisation. When TRAIL signalling was blocked in wt mice, we observed no detectable effect on T cell responses, neither on the number of specific CD8+ T cells nor on the number of IFNγ-producing T cells upon re-stimulation (data not shown). We thereafter proceeded to deplete NK cells using an anti-NK1.1 antibody, which binds NK1.1 on the surface of NK cells from B6 mice. We noted a tendency toward increased CD8+ T cell responses in NK cell depleted mice in two repeated experiments, although the increase was not significant (Figure 10). As an alternative approach to NK cell depletion, we used a depleting anti-CD122 antibody, TMβ-1. This antibody targets a subunit of the IL-2/15 receptor beta chain, which is present on NK cells, but is also up-regulated on activated CD8+ T cells (156). The benefit with this antibody is that in contrast to the anti-NK1.1 antibody, it can be used to deplete NK cells in the IFNR-AR1$^{-/-}$ mice that are on Sv129 background. TMβ1-depleted wt SV129 mice showed a significantly lower specific CD8+ T cell response compared to non-depleted SV129 mice post rSFV immunisation. This difference might be caused by unintentional depletion of activated TMβ1-expressing T

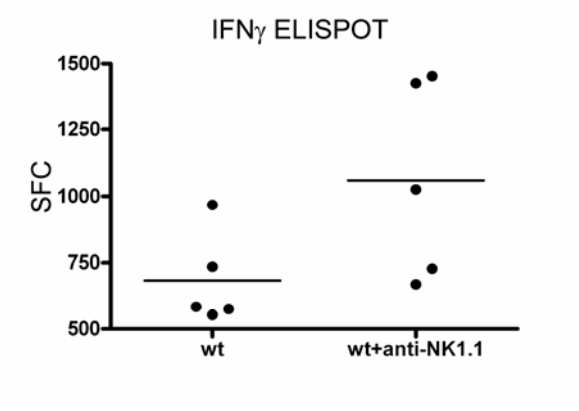


Figure 10. Effect of depletion of NK cells on CD8+ T cell responses against rSFV-encoded antigen. Wt mice received 200 μg of depleting anti-NK1.1 antibody 48 h before immunisation with 10^6 IU of rSFV-NP. After 12 days, splenocytes were re-stimulated with MHC class I NP-peptide ASNENMETM in an IFNγ ELISPOT and expressed as spot-forming colonies/10^6 cells.

cells by remaining anti-TMβ1 antibody circulating in the depleted rSFV-immunized mice. Interestingly, the difference between deleted and non-depleted mice was not observed in the IFNR-AR1$^{-/-}$ mice. It remains to investigate what impact IFNα/β-activated NK cells have on the T cell priming during rSFV immunisation and we are currently perusing this question.

We also investigated if the CD4+ T cell response against rSFV-encoded NP was enhanced in IFNR-AR1$^{-/-}$ mice. Recombinant NP has previously been used to stimulate T cells for detection of specific IFNγ response in macaques immunized with rSFV-NP (A. Mörner, personal communication). Using recombinant protein for re-stimulation of T cells has previously been shown to primarily stimulate CD4+ T cells (34). We detected no IFNγ response from rSFV-NP immunised mice upon restimulation of splenocytes with recombinant NP protein, although the same mice responded with a high frequency of IFNγ-producing cells in response to the MHC class I peptide previously used. Since the CD8+ T cell response against rSFV-NP in mice lacking CD4 was markedly reduced compared to the CD8+ T cell in wt mice, we concluded that although there should reasonably exist an NP-specific CD4+ T cell response we could not detect it using this experimental approach.

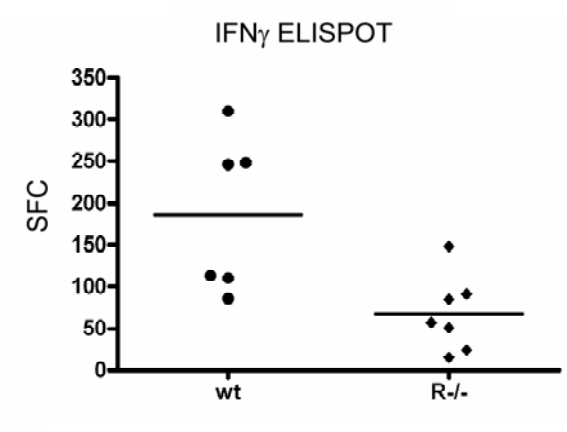


Figure 11. IFNα/β signalling is required for full CD4+ T cell responses against rSFV-encoded antigen. Splenocytes from rSFV-ENV-immunised wt or IFN-AR1$^{-/-}$ mice (R-/-) were re-stimulated with recombinant HIV ENV protein in an IFNγ ELISPOT and expressed as spot-forming colonies/10^6 cells (SFC).

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In another attempt to study CD4+ T cell responses in IFNR-AR1$^{-/-}$ mice, we used an rSFV vector encoding the HIV-1 envelope glycoprotein gp120 (rSFV-ENV), against which we have previously measured a specific CD4+ response (34). Upon immunisation of wt and IFNR-AR1$^{-/-}$ mice with rSFV-ENV we observed a significantly lower CD4+ T cell response in the absence of IFNα/β signalling (Figure 11). Further studies are required to examine the effect of IFNα/β signalling on CD4+ T cell responses during rSFV-immunisation.

Instead, we focused our investigations on characterising the ratio between effector and memory CD8+ T cells in rSFV-NP immunised wt and IFNR-AR1$^{-/-}$ mice, since strong signals during priming (e.g. abundant antigen and/or co-stimulation) have been described to favour the development of effector responses (83). We used the CD62L marker to distinguish between effector and memory cells as CD62L is expressed on naïve T cells and is then lost as the CD8+ T cell is primed and gains effector function, to later be regained as the T cell develops into central memory T cells. We assayed surface expression of CD62L on NP-specific CD8+ T cells from wt and IFNR-AR1$^{-/-}$ mice immunised with an equal number of rSFV-NP infected wt MEFs, and thus an equal amount of antigen. We found that at day 12 post-immunisation, a significantly smaller proportion of antigen-experienced IFNR-AR1$^{-/-}$

CD8+ T cells expressed CD62L, as compared to their wt counterparts (data not shown). Thus, not only did the IFNR-AR1$^{-/-}$ mice raise more virus-specific CD8+ T cells at this time after immunisation, but they also had a more pronounced effector phenotype.

Since 12 days after immunisation is insufficient time for memory cell development, we immunised mice with rSFV-NP and assayed blood at day 24 for NP-specific (tetramer positive) CD8+ T cells expressing CD62L. Similarly to the results observed at day 12, we found that a significantly larger proportion of the NP-specific IFNR-AR1$^{-/-}$ CD8+ T cells lacked expression of CD62L on their surface. We further asked what consequence this large proportion of effector type CD8+ T cell response in the IFNR-AR1$^{-/-}$ mice

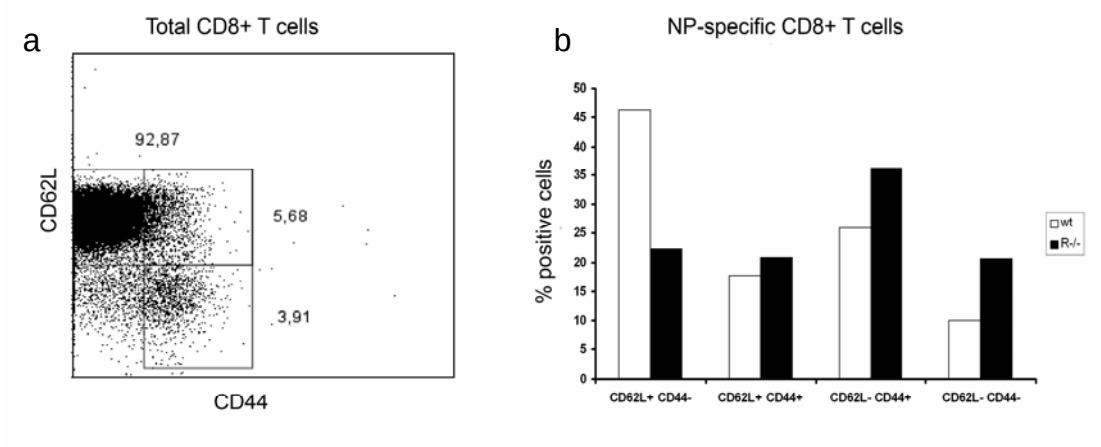

Figure 12. The development of rSFV-specific (CD44+ CD62L+) T_{CM} is not dependent on IFNα/β signalling. a) FACS scatterplot showing CD44 and CD62L expression on the total CD8+ T cell population from an rSFV-NP-immunised IFN-AR1$^{-/-}$ mouse, 24 days post immunisation b) Proportions of NP-specific peripheral blood CD8+ T cells from rSFV-NP-immunised wt (wt, white staples) or IFN-AR1$^{-/-}$ mice (R-/-, black staples) expressing of CD44 and CD62L on their surface, 24 days post immunisation. The staples represent an average of 6 mice per group.

would have for the development of a central memory response in these animals. In order to define the T_{CM} population, we examined the CD8+ T cells for both CD62L and CD44 expression (148). Interestingly, we found that although a smaller proportion of the antigen specific CD8+ T cell population in the IFNR-AR1$^{-/-}$ mice expressed CD62L, a larger part of these cells also expressed CD44. Thus, we found no defect in the formation of a T_{CM} population, as defined by antigen-specific CD8+ T cells expressing both CD62L and CD44, as there was no difference in the proportion of double positive cells between wt and IFNR-AR1$^{-/-}$ mice (Figure 12). Upon analysis of recall responses, we found that more than a year after the rSFV-NP priming, recall responses were similar in wt and IFNR-AR1$^{-/-}$ mice, indicating that the T_{CM} population observed at day 24 post immunisation could expand and gain effector functions. We conclude that the induction of virus-specific CD8+ T cell effector responses are enhanced in the absence of IFNα/β signalling, while formation of memory responses is not compromised. This is an important conclusion since it has been argued that overly strong stimulation during T cell priming might lead to exhaustion of the T cell response and compromise the ability of proliferation upon recall (83).

4 CONCLUSIONS

There are two major conclusions from my thesis. First, viruses can stimulate mDC to produce IFNα/β via TLR-independent pathways. For both SFV and rotavirus, this is dependent on viral entry but not strictly dependent on viral replication. When Paper I was published, the intracellular receptors for sensing viral genomes in the cytoplasm of cells, RIG-I and MDA5, were not identified. It has later been confirmed viral replication is not required to activate signalling by these proteins as shown in a number of viral systems. What still remains unknown is why DCs have a higher capacity than other cells, such as fibroblasts, to respond to non-replicating (UV-inactivated) virus. One possible explanation is that DC differ from MEFs in regards to the signalling pathways that trigger IFNα/β induction, for example they may have a higher constitutive expression of molecules such as RIG-1 or other proteins involved in initiating a IFNα/β response. Second, we have learnt that the effects of IFNα/β on shaping adaptive immune-responses depends on if the antigen is encoded by a virus or if it is provided as recombinant protein during using virus as an adjuvant. Virus-induced IFNα/β provide an adjuvant effect on co-administered protein antigens, similarly as the effect reported by some vaccine adjuvants that are also dependent on IFNα/β induction. In contrast, for virus-encoded antigens expressed in the context of an infected cell, both antibody responses and CD8+ T cell responses are elicited in the absence of IFNα/β signalling. We even found that the induction of antigen-specific CD8+ T cells is enhanced in the absence of IFNα/β signalling. This differs markedly from the adjuvant effects of IFNα/β reported for CD8+ T cell responses induced by cross-presentation of protein antigen reported by others. Our results suggest that further studies of the role of IFNα/β for modulating adaptive immune responses during viral infection are warranted.

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