From DEPARTMENT OF MICROBIOLOGY, TUMOR AND CELL BIOLOGY
Karolinska Institutet, Stockholm, Sweden

VIRAL VECTORS, IN INNATE AND ADAPTIVE IMMUNITY

Tanja I. Näslund

Karolinska Institutet

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ABSTRACT

Recombinant viral vectors and DNA vectors induce strong immune responses in animal models. However, in clinical trials, the generation of immune responses is less robust, suggesting that further optimization and a deeper understanding of nucleic acid-based vaccines are required. Improvements, such as combining the vaccine vectors in heterologous prime boost regimens and/or using vectors that do not induce strong immune responses against the vector itself may enhance immune responses against the antigen-of-interest. In Paper I, we performed head-to-head comparisons of adenovirus, ALVAC and Semliki Forest virus (SFV) vectors, in homologous and heterologous prime boost regimens. The recombinant viral vectors were evaluated for their potency to generate T cell responses and to protect against a tumor challenge. We show that the memory T cell response induced by the different immunization regimens were distinctly different and that protection against a tumor challenge was more dependent on the quality of the response than the magnitude.

The potency of the nucleic acid-based vaccines depends on the activation of innate signaling pathways. In Paper II-IV, we investigated innate signaling pathways activated by different viral vectors and the role of these pathways for induction of T cell responses. The CD8α^+ DCs play a major role in cross-priming of anti-viral T cells. This dendritic cell subtype phagocytoses apoptotic bodies, expresses high levels of toll-like receptor (TLR) 3 and has a unique ability to cross-present exogenously derived cell-associated material. In Paper II, we investigated the role of TLR3 expression in the CD8α^+ DCs and its relevance for cross-priming of T cells. We show that dsRNA activates CD8α^+ DCs to cross-prime T cells via TLR3.

In addition to TLRs, the cytoplasmic RNA receptors RIG-I and MDA5, expressed by most cell types, are detectors of viral infection. It was initially suggested that both RIG-I and MDA5 recognize double-stranded RNA (dsRNA) intermediates generated in the cytoplasm during viral infection in the host cell. However, negative-sense RNA viruses do not generate detectable levels of dsRNA in infected cells, thus these viruses may be recognized via alternative non-self signatures. In Paper III, we show that RIG-I is a receptor for single-stranded RNA molecules bearing 5'-phophates, illustrating one of the differences between RIG-I and MDA5 virus recognition.

The alphavirus replicon-based DNA (DREP) vectors induce superior immune responses in comparison to conventional DNA (convDNA) vectors in animal models. We hypothesized that DREP vectors induce potent innate signaling pathways that account for the immunogenic properties of these vectors. In Paper IV, we investigated T cell responses in mice deficient in innate signaling pathways, including TLR3, TLR9, MyD88, IRF3 and the interferon α/β receptor (IFN-AR1), after SFV viral and DNA based vector immunization. We show that IFN-AR1 and IRF3, but not detectably the other molecules, influence the T cell response induced by these vectors.

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I. **T.I. Näslund**, C. Uyttenhove, E. K. L. Nordström, D. Colau, G. Warnier, M. Jondal, B. J. Van den Eynde, and P. Liljeström. 2007. Comparative prime-boost vaccinations using Semliki Forest virus, adenovirus, and ALVAC vectors demonstrate differences in the generation of a protective central memory CTL response against the P815 tumor. *J Immunol* 178:6761-6769.

II. Schulz, O., S. S. Diebold, M. Chen, **T. I. Näslund**, M. A. Nolte, L. Alexopoulou, Y. T. Azuma, R. A. Flavell, P. Liljeström, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433:887-892.

III. Pichlmair, A., O. Schulz, C. P. Tan, **T. I. Näslund**, P. Liljeström, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314:997-1001.

IV. **T.I. Näslund**, L. Kostic, M. Chen, E.K.L. Nordström and P. Liljeström. 2009. Role of innate signalling pathways in alphavirus replicon immunization. *Manuscript.*

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LIST OF ABBREVIATIONS

AIM2	Absent in melanoma 2
APC	Antigen presenting cell
BHK	Baby hamster kidney cell
BM-DC	Bone-marrow derived dendritic cell
convDNA	Conventional DNA
CpG	Unmethylated cytosine and guanine nucleotides separated by a phosphate
CTL	Cytotoxic T cell
DAI	DNA dependent activator of IRFs
DC	Dendritic cell
DREP	Alphavirus replicon-based DNA
dsRNA	Double stranded RNA
eIF2α	Eukaryotic translation initiation factor 2α subunit
EMCV	Encephalomyocarditis virus
Flu	Influenza A virus
Gas6	Growth-arrest-specific 6
HEV	High endothelial venules
IFN	Interferon
IFN-AR1	Interferon α/β Receptor
IL	Interleukin
IPS-1	Interferon-β promoter stimulator 1
IRF	Interferon regulatory factor
LGP-2	Laboratory of genetics and physiology-2
LN	Lymph node
LPS	Lipopolysaccharide
MDA5	Melanoma differentiation associated gene 5
MHC	Major histocompatibility complex
MΦ	Macrophage
MVA	Modified Ankara virus
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor-kappa B
NOD	Nucleotide-binding oligomerization domain
nsP	Non-structural protein
2'-5' OAS	2'-5' oligoadenylate synthetase
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
pDC	Plasmacytoid dendritic cell
PKR	Protein kinase R
PolyI:C	Polyriboinosinic:polyribocytidylic acid
ProS	Protein S
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like family
RNaseL	Ribonuclease L

SeV	Sendai virus
SFV	Semliki Forest virus
SIN	Sindbis virus
SOCS	Suppressor of cytokine signaling
ssRNA	Single-stranded RNA
TAM	Tyro3/Axl/Mer
TCR	T cell receptor
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
Th	T helper cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta
VEE	Venezuelan encephalitis virus
VLA-4	Very late activation antigen-4
VREP	SFV particle
VSV	Vesicular stomatitis virus

INTRODUCTION

Vaccines are the most cost-effective way to combat disease. In vaccination, immunogens are administered to a subject to stimulate the immune system to produce effector components. This type of vaccine includes peptides, proteins and live attenuated pathogens. Vaccination programs worldwide have successfully eradicated smallpox, and reduced the incidence of several severe infectious diseases, such as polio and measles. However, effective vaccines against numerous infectious diseases, including HIV and malaria, and other diseases, such as many cancers, are still lacking.

Use of nucleic acid-based vaccines, represented by DNA and recombinant viral vectors, are being considered as the next generation of vaccines. These vaccine vectors carry RNA or DNA encoding specific antigens. In a vaccinated subject, these antigens are expressed in host cells and induce an immune response in a similar way as a natural viral infection. By mimicking a viral infection, these vaccine vectors activate both the innate and adaptive arms of the immune system. The innate immune system is activated within minutes to hours after vaccination and serves to initiate and shape an adaptive immune response towards the antigen expressed by the vaccine vector. The adaptive immune response generates specific T and B cells, which recognize the antigen encoded by the vaccine vector. This is a process that takes several weeks. A subset of the antigen-specific cells creates a memory pool that may survive for a lifetime. These memory cells will quickly respond upon a secondary encounter with the same antigen, for example after exposure to the pathogen the vaccine was directed against. In this thesis, I have studied the induction of both innate and adaptive immunity by viral or DNA vectors.

INNATE IMMUNITY

Viruses and other pathogens express common and conserved structures referred to as pathogen-associated molecular patterns (PAMPs). These PAMPs are recognized by the pattern recognition receptors (PRRs) (1-6). The PRRs are found both in serum and in cells of different origin (Table I). Among the PRRs, the cytoplasmic PRRs and the toll-like receptors (TLRs) are the main detectors of viruses. The cytoplasmic PRRs are found in most cell types. In contrast, the TLRs are mainly expressed on cells that are part of the innate and adaptive immune systems, such as macrophages (MΦs), dendritic cells (DCs), monocytes, natural killer (NK) cells, neutrophils, T cells and B cells (2). Activation of TLRs and cytoplasmic PRRs involved in viral detection lead to production of pro-inflammatory cytokines (TNFα, IL-6 and IL-12) and type I interferons (IFNs). The role of these cytokines is to stimulate and enhance the immune response. For instance, TNFα increases the influx of cells into the infected area via up-regulation of selectin expression on endothelial cells. Selectin expression facilitates entry of cells expressing selectin ligands, such as neutrophils, monocytes, DCs and lymphocytes. Moreover, TNFα, in combination with PRR activation, induce migration of DCs to draining lymph nodes (LNs). IL-12 activates NK cells and promotes the differentiation of helper T cells, whereas IL-6 activates lymphocytes and induces fever along with TNFα. Fever

is beneficial to the host since it reduces viral replication and enhances adaptive immune responses. Viral replication, as well as the spread of the virus, is also blocked by type I IFNs, since type I IFNs induce an anti-viral state in the infected cell, as well as in cells surrounding the infected area. The innate immune system serves as a first line of defense against an infection as well as shapes the adaptive immune response to respond in an adequate way to the infection.

Table I. The pattern recognition receptors, their expression and function.

Name	Localization/ Expression	Examples	Function
Serum PRRs	Serum	- Complement components - Pentraxin family - LPS-binding protein	Bind to PAMPs, facilitating uptake by phagocytotic cells (MΦs) Activate the complement system
Cytoplasmic PRRs	Most cell types	- RLRs - IFN-inducible proteins - NOD-like receptors	Activate innate signaling pathways in virally infected cells of non-APC and APC origin
Helper PRRs	Innate immune cells	- CD14 - Dectin-1	Present PAMPs to other PRRs (e.g. TLR3 and 4) Promote phagocytosis by MΦs
TLRs	Innate and adaptive immune cells	- TLR 1-13	Activate innate signaling pathways

Cytoplasmic PRRs

There are several cytoplasmic PRRs that detect viral RNA, such as the retinoic acid inducible gene I (RIG-I) and the melanoma differentiation factor-5 (MDA5), members of the RIG-I-like family (RLRs). Other cytoplasmic PRRs detect DNA of pathogenic origin in the host cell cytoplasm, such as the DNA dependent activator of IRFs (DAI) (7, 8) and the absent in melanoma 2 (AIM2) (9-11). For example, some RNA viruses, such as vesicular stomatitis virus (VSV), influenza A virus and Sendai virus (SeV) are recognized by RIG-I (12), whereas positive-sense RNA viruses, such as encephalomyocarditis virus (EMCV), that generate high amounts of dsRNA during viral replication, is recognized by MDA5 (12, 13). A third member of the RLR family of receptors is the laboratory of genetics and physiology-2 (LGP-2). The role of LGP-2 has yet to be defined, since both inhibitory (14, 15) and additive (16) roles have been reported for LGP-2 in concert with RIG-I and MDA5 (Figure 1).

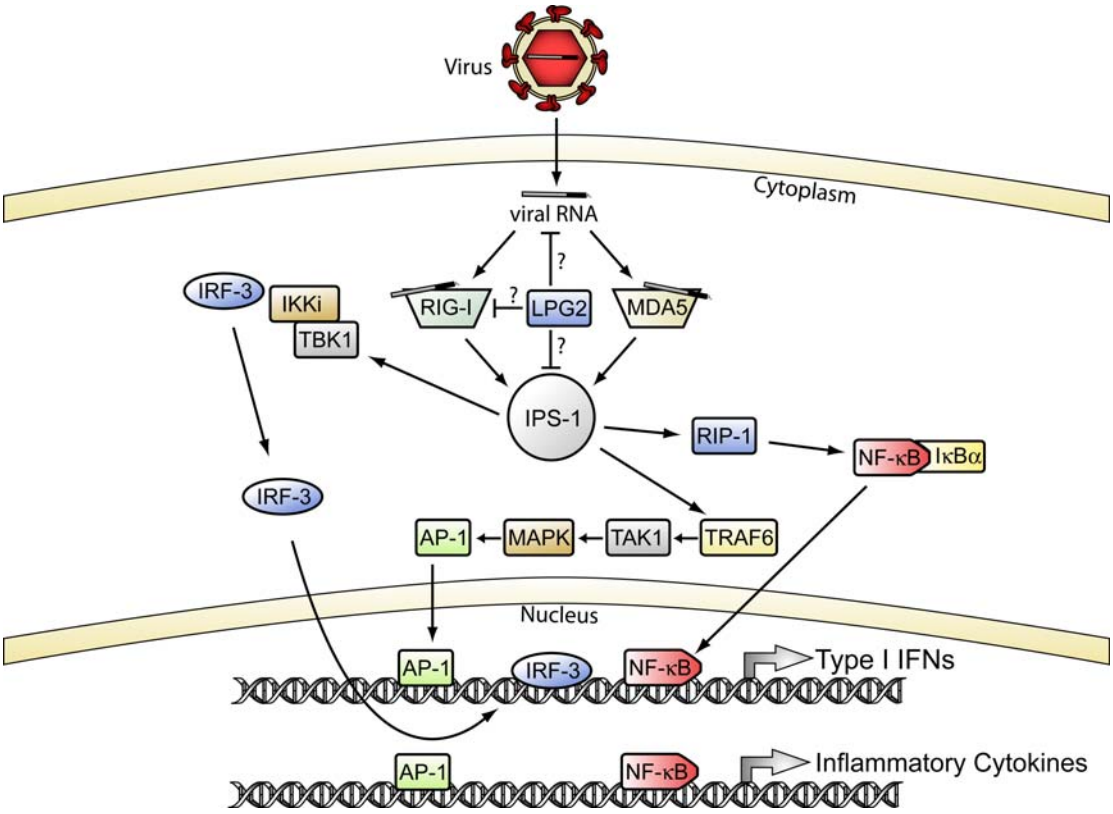

Figure 1. RIG-I and MDA5 signaling pathways. RIG-I and MDA5 are activated by viral RNA. They signal via the interferon-β promoter stimulator 1 (IPS-1), activating NF-κB, AP-1 and IRF3. This leads to transcription of pro-inflammatory cytokines and type I IFNs.

Some viruses encode proteins that block innate signaling pathways. For instance, influenza A virus (Flu) expresses NS1 protein, a potent suppressor of type I IFN production in infected cells (17-19). Since NS1 contains an RNA binding domain (20), the function of NS1 has been attributed to sequestering of dsRNA, and hence blocking the induction of cytoplasmic PRR innate signaling pathways.

The two cytoplasmic PRRs detecting dsDNA, DAI and AIM2, have recently been identified. Even though both receptors recognize dsDNA, the responses induced by DAI and AIM2 differ significantly. DAI activates IRF3 and NF-κB dependent pathways, generating type I IFNs and pro-inflammatory cytokines. In contrast, AIM2 activates the inflammasome and caspase-1 dependent pathways, generating IL-1β and pyroptosis, a form of pro-inflammatory cell death. The vastly different responses induced by these receptors indicate that DAI and AIM2 either have separate ligand specificities that are yet unknown or that they act in concert in response to cytoplasmic dsDNA.

Toll-like receptors

The toll receptor was first discovered in the fruit fly, *Drosophila melanogaster,* where it was shown to be of importance for protection against fungal infection (21). Similar receptors have been found in mammalians, where they are called toll-like receptors (TLRs). To date 13 different TLRs have been described in humans, whereas 11 TLRs are known in mice. The TLRs are mainly expressed on antigen presenting cells (APCs), such as DCs, MΦ and B cells (1, 2). They recognize different PAMPs of bacterial, viral or other pathogenic origin. For detailed

description of TLR ligands, see Table II. TLR10, 12 and 13 have yet unknown ligand specificities.

Table II. The toll-like receptors; pathogen specificity, ligands and cell localization.

TLR	Example of ligand	Pathogen	Cell localization	Reference
1/6	Lipopeptide	Bacteria	Cell-surface	(22)
4	LPS	Bacteria	Cell-surface	(23)
5	Flagellin	Bacteria	Cell-surface	(24)
11	Profilin-like molecule	*Toxoplasma gondii,* uropathogenic bacteria	Cell-surface	(25, 26)
3	dsRNA	Virus	Endosome	(27)
7/8	ssRNA	Virus	Endosome	(28-30)
2	Lipopeptide Zymosan	Bacteria, yeast and virus	Cell-surface	(31-35)
9	CpG	Virus and bacteria	Endosome	(36-40)

Adapted from Lee, M. S., and Y. J. Kim. 2007 (2).

In general, the TLRs that recognize extracellular pathogens, for example bacteria, and the TLRs that recognize intracellular pathogens, such as virus, are located in different compartments of the cell. TLRs detecting bacterial PAMPs are generally found on the cell-surface, facing outwards from the cell into the extracellular space. In contrast, the virus-specific TLRs are generally located in endosomal compartments, enabling TLR activation only subsequent to phagocytosis of virus-containing material. Exceptions to these general localization rules include TLR2, located on the cell-surface, and TLR9, found in endosomes. These receptors are capable of responding to both viral and bacterial PAMPs.

The immune responses to PAMPs differ depending on TLR localization (Figure 2). The TLRs located on the cell surface generally induce expression of pro-inflammatory cytokines, whereas the TLRs found in the endosomes induce type I IFNs, in addition to pro-inflammatory cytokines. However, TLR4, despite its cell surface localization, induces both pro-inflammatory cytokines and type I IFNs due to dual signaling pathways. After binding of their respective PAMPs, all TLRs except TLR3 recruit the myeloid differentiation factor 88 (MyD88) for subsequent intracellular signaling, resulting in cytokine production. In contrast, TLR3 recruits the toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF), whereas TLR4 recruits both MyD88 and TRIF for intracellular signaling and cytokine production.

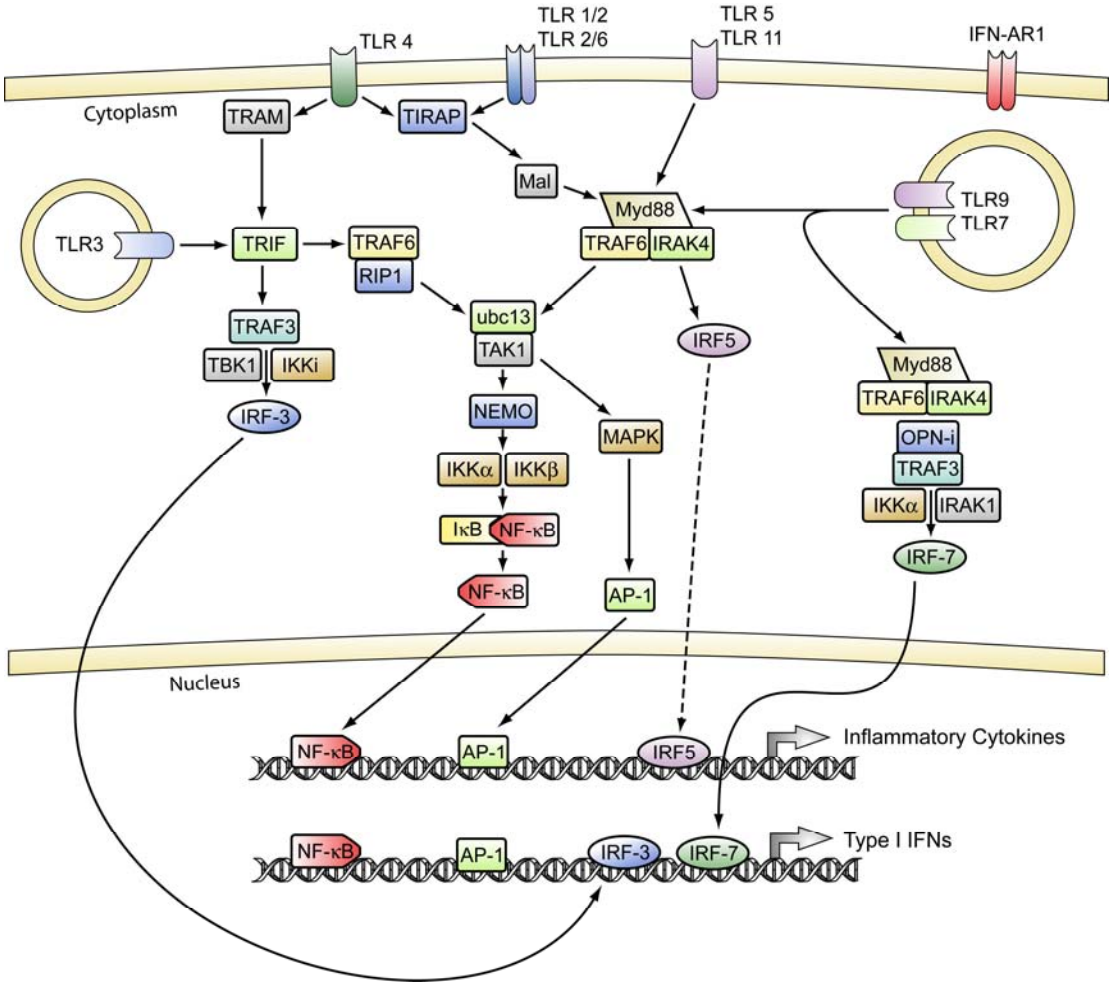

Figure 2. The TLR signaling pathways. All TLRs induce pro-inflammatory cytokines via NF-κB, AP-1 and IRF5, except TLR3, which only utilizes NF-κB and AP-1. TLR3 and TLR4 induce type I IFNs after activation via NF-κB, AP-1 and IRF3, whereas TLR7 and TLR9 induce type I IFNs via NF-κB, AP-1 and IRF7.

Suppression of TLR responses

To avoid continuous activation of the TLR signaling pathways and prolonged inflammation, the duration of the TLR responses is tightly regulated. Negative regulation occurs by degrading or blocking signaling molecules, down-regulating TLR expression or by inhibiting transcription of signaling molecules. The negative regulators are either induced by TLR signaling via negative feedback mechanisms or are constitutively expressed. One negative feedback mechanism involves the up-regulation of the Tyro3/Axl/Mer (TAM) receptors (41, 42). Type I IFNs, induced by TLR activation, signal via the type I IFN receptor (IFN-AR1) leading to activation of the ISGF3 complex, consisting of STAT1, STAT2 and IRF9 (Figure 3). The ISGF3 complex drives transcription of additional type I IFN genes, as well as the TAM receptor genes. After translation of the TAM receptors, they are transported to the cell surface where they interact with the IFN-AR1. Upon binding of one of its ligands, protein S (ProS) or growth-arrest-specific 6 (Gas6), the TAM receptor down-stream signaling re-directs the ISGF3 complex to transcribe the genes for suppressor of cytokine signaling (SOCS) 1 and 3 instead of the type I IFN genes (41-43).

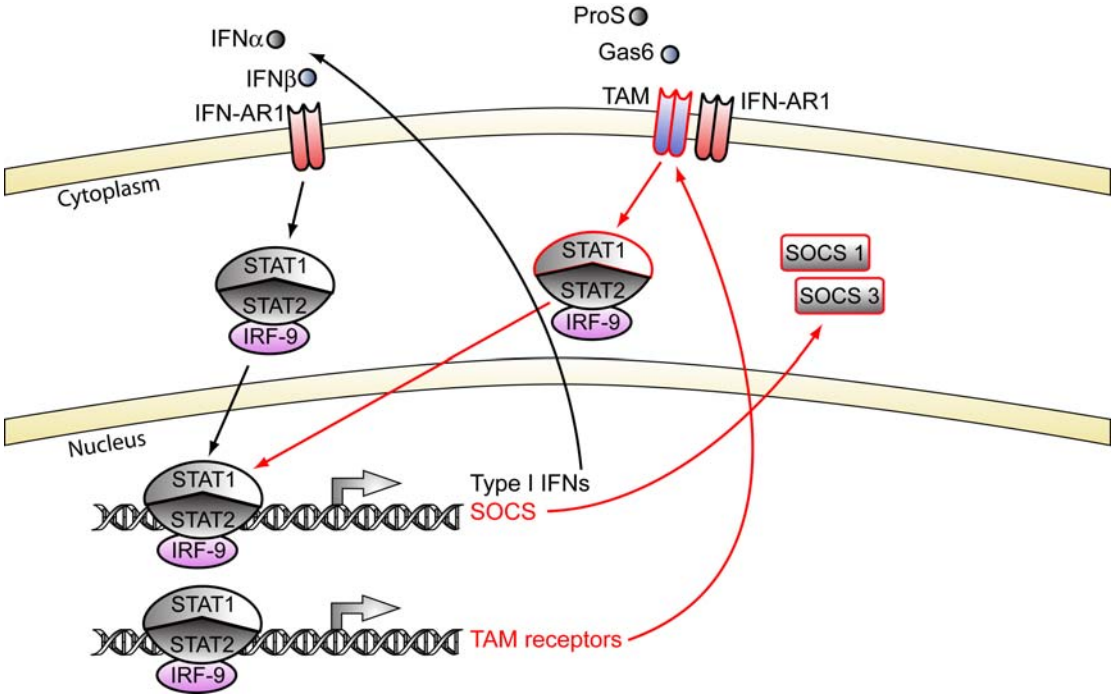

Figure 3. Up-regulation of the TAM receptors and SOCS1 and 3 proteins. IFN-AR1 signaling induces both type I IFNs and TAM receptor up-regulation via the ISGF3 complex. ProS and Gas6 activate the TAM receptors to re-direct the ISGF3 complex via STAT1 to transcribe the SOCS1 and 3 genes instead of the type I IFN genes.

The SOCS1 and 3 proteins block all TLR signaling pathways via degradation or blocking activation of several signaling molecules, such as Mal, TRAF3 and TRAF6 (Figure 4). It is currently not known from which cell type(s) the TAM ligands ProS and Gas6 originate, but regulatory T cells (Tregs) or DCs are proposed cell sources (41, 43). In addition to the TAM receptors, several other inhibitors of TLR signaling exist. For example, IRF4 (44), induced by TLR signaling, acts as an antagonist by competing with IRF5 for binding to MyD88. Triad3A (45) and IκBNS (46), both induced after TLR signaling, degrades TLR4 and 9 and interferes with NFκB activation, respectively. Pin1 (47), an example of a constitutively expressed inhibitor, binds activated IRF3 and thereby targets the IRF3 for degradation leading to suppressed IFNβ transcription. In conclusion, several mechanisms exist to keep the TLR signaling pathways under strict control.

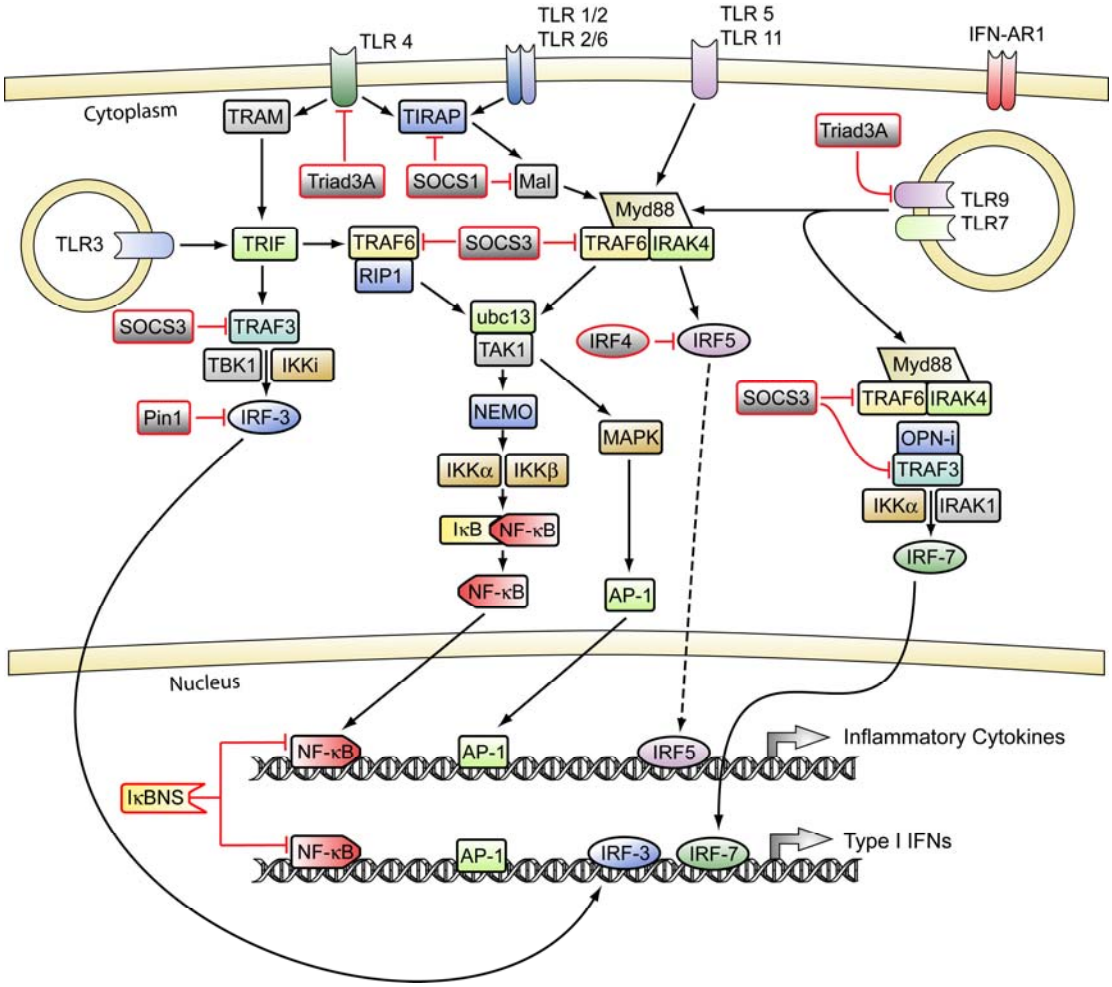

Figure 4. Inhibition of TLR signaling. SOCS1 blocks the TLR2 and 4 signaling pathways by degrading Mal. SOCS3 blocks all TLR signaling pathways, by blockage of TRAF3 and TRAF6 activation. IRF4 competes with IRF5 for MyD88 binding, and Triad3A degrades TLR4 and TLR9. IκBNS blocks NF-κB activation and the Pin1 protein targets activated IRF3 for degradation.

Type I interferons

The type I IFNs are a group of proteins consisting of one IFN-β and multiple subtypes of IFN-α (13 in humans and 14 in mice), as well as several less well characterized subtypes (ε, κ, ω, δ, τ and ζ) (5, 48). Despite having different effects *in vivo*, all type I IFNs signal through a single common receptor, the IFN-AR1 (Figure 5). All nucleated cells can both produce and respond to type I IFNs, which is an important feature during viral infection. Viral infection induces IFN-β production by activation of RIG-I, MDA5, DAI or TLR3 via NF-κB, AP-1 and IRF3. Secreted IFN-β signals via the IFN-AR1 leading to transcription of IRF7 via the ISGF3 complex. IRF7 subsequently amplifies IFN-β transcription as well as activating the IFN-α genes, creating a positive type I IFN feedback loop. IFN-AR1 signaling also leads to up-regulation of proteins involved in antiviral defense, such as protein kinase R (PKR) and 2'-5' oligoadenylate synthetase (2'-5' OAS). In addition, type I IFNs bind to IFN-AR1s on neighboring cells that leads to an anti-viral state in the cells surrounding the infected area, blocking the spread of the virus. Plasmacytoid

DCs (pDCs) constitutively express IRF7 enabling IFN-β and IFN-α transcription to occur without prior IRF3 activation. Therefore, pDCs can rapidly produce high levels of type I IFNs in response to a viral infection. Thus, type I IFNs are potent enhancers of innate immune responses. Moreover, type I IFNs are potent inducers of adaptive immune responses by direct activation of DCs, T cells and B cells (49-52).

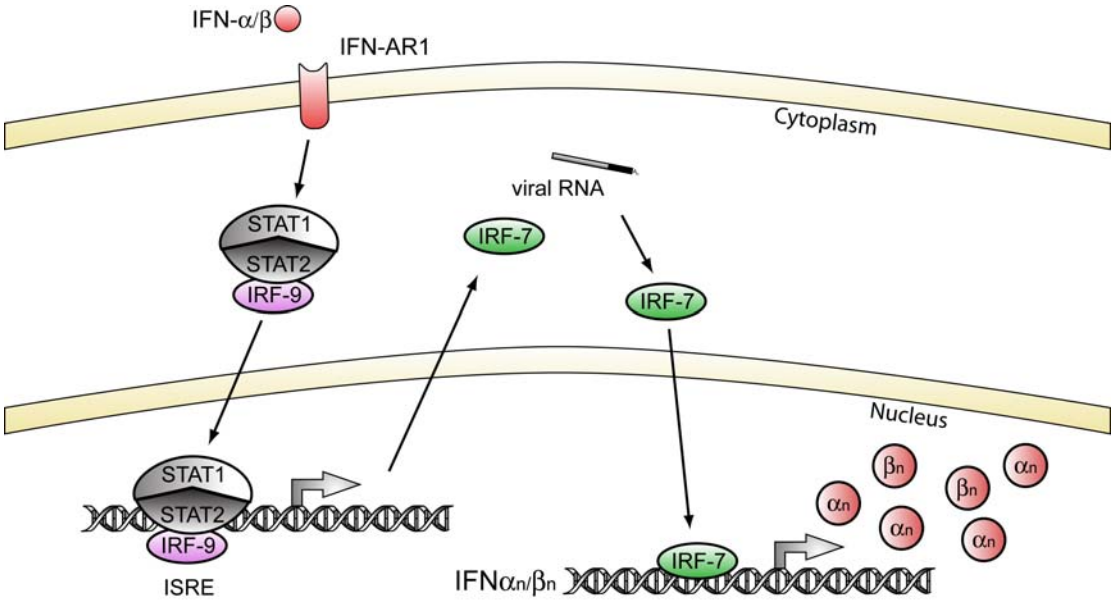

Figure 5. Down-stream signaling from IFN-AR1. IFN-AR1 signaling activates the ISGF3 complex to transcribe IRF7. In the presence of viral RNA, IRF7 is activated and translocates to the nucleus for transcription of the IFNβ and IFNα genes.

Interferon-inducible proteins

PKR and 2'-5' OAS are important proteins for the induction of an anti-viral state in the cell. PKR and 2'-5' OAS are expressed in inactive forms at low levels in uninfected cells, but their transcription is rapidly up-regulated by IFN-AR1 signaling. Both PKR and 2'-5' OAS are activated in the presence of cytoplasmic dsRNA, generated during viral replication. Activation of PKR leads to the suppression of mRNA translation by inactivation of the eukaryotic translation initiation factor 2α subunit (eIF2α), an important factor during protein synthesis (53). Activated 2'-5' OAS protein subsequently activates ribonuclease L (RNaseL), an enzyme that degrades mRNA, thus reducing viral and cellular mRNA for protein synthesis (54). These PKR- and 2'-5' OAS-triggered events ultimately result in apoptosis of the virus infected cell and hence reduce viral spread in the host.

ADAPTIVE IMMUNITY

The adaptive immune response is antigen specific in contrast to the innate immune response. As a continuous process, all nucleated cells degrade intracellular proteins and display fragments of them on the cell-surface. The proteasome complex that degrades proteins in the cytoplasm to peptides mediates this process. The generated peptides are transported by the transporter associated with antigen processing (TAP) protein into the endoplasmic reticulum (ER), where they are loaded onto the major histocompatibility complex (MHC) class I. The MHC class I molecule with its bound peptide thereafter translocates to the cell-surface. Just like the endogenous peptides, peptides derived from viral proteins will be displayed on MHC class I molecules on the surface of an infected cell. This ensures that an intracellular infection is detected by the adaptive immune system. The viral peptides are recognized by specific $CD8^+$ T cells that can kill the virus-infected cell. Professional antigen presenting cells (APCs), such as DCs, MΦ and B cells, express high levels of MHC class I, II and co-stimulatory molecules, and are capable of phagocytosis of exogenous proteins. Generated phagosomes ultimately fuse with lysosomes where the protein cargo is enzymatically degraded into peptides. During this process some of the peptides are loaded on MHC class II molecules and re-circulate to the cell surface for presentation to $CD4^+$ T cells. DCs, especially the $CD8\alpha^+$ DCs, can also re-route exogenously derived protein for loading onto MHC class I molecules, a process called cross-presentation (55, 56).

DC activation of T cells

APCs, especially DCs, are important for T and B cell activation. Immature DCs can internalize pathogens and cell-bound material, either by nibbling on viable cells (57), or by uptake of apoptotic bodies (58). PRR activation induces DC maturation by up-regulation of MHC class I and II molecules, co-stimulatory molecules (CD80 and CD86), CCR7 and cytokine production (IL-12 or IL-4, depending on the type of pathogen encountered by the DC). When the DC becomes mature, it is no longer capable of phagocytosis, and it starts to migrate towards the draining lymph node (LN) (59, 60). Stromal cells in the LN produce secondary lymphoid tissue chemokine (SLC), which attracts the DCs via interaction with CCR7 expressed on the DC. In the LN, the mature DCs start to produce SLC to attract naïve T cells that express CCR7 to the draining LNs. In the LN, T cells interact with DCs in the search for their cognate peptide that will stimulate activation and proliferation of the T cell. T cells are either $CD8^+$ T cells that recognize peptides displayed on MHC class I molecules, or $CD4^+$ T cells that recognize peptides displayed on MHC class II molecules. Initially the T cells interact with the DCs via unspecific (selectin/integrin) binding (Figure 6). Thereafter, a stronger interaction occurs via the T cell receptor (TCR) and the MHC. The co-stimulatory molecule interaction between the DC and the T cell further increases the binding strength between the two cells, which activates the T cells to produce IL-2 important for clonal proliferation of the T cell. Moreover, additional co-stimulatory cytokines are up-regulated on the T cell and DC, further potentiating the DC and T cell interaction. Thereafter, the T cell starts to proliferate and to produce effector molecules. Upon

activation, the $CD8^+$ T cells differentiate into cytotoxic T cells (CTLs) capable of killing infected cells and the $CD4^+$ T cells into helper T (Th) cells that produce cytokines that stimulate other cells.

The $CD8\alpha^+$ DCs are important for cross-priming of $CD8^+$ T cells in viral infection (61-67). The $CD8\alpha^+$ DCs is the main DC subset capable of taking up apoptotic cells (66, 67), have the highest level of TLR3 mRNA expression (68) and produce high levels of IL-12 (69) important for the induction of Th1 responses. Moreover, the $CD8\alpha^+$ DCs are capable of cross-presenting exogenously derived antigen on MHC class I. Cross-priming by $CD8\alpha^+$ DCs is dependent on danger signals from the virus-infected cell, such as type I IFNs (50). If danger signals are not present, $CD8\alpha^+$ DCs may instead promote cross-tolerance (70, 71) resulting in inactivated T cells. In addition to type I IFNs, $CD8\alpha^+$ DCs are activated by uptake of exogenously synthetic dsRNA (poly I:C) (27, 68), suggesting that dsRNA in virus-infected cells could be one signal that activates $CD8\alpha^+$ DCs for cross-priming.

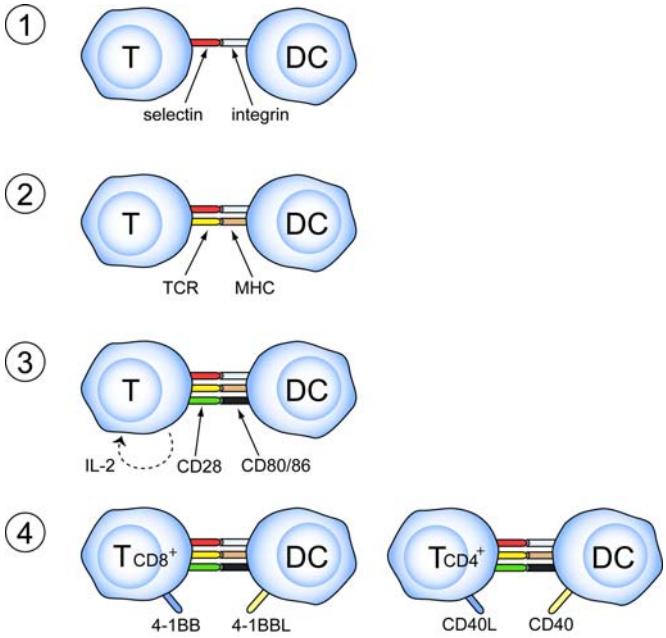

Figure 6. Sequential interaction between DCs and T cells. Initial contact between the DC and the T cell is made by unspecific binding via selectin and integrin molecules. Thereafter, a stronger interaction occurs via the TCR and the MHC molecules. Upon interaction between the co-stimulatory molecules CD80/CD86 on the DC and CD28 on the T cell, the T cell starts to produce IL-2, an important cytokine for clonal T cell expansion. Finally, additional co-stimulatory receptors are up-regulated on the $CD8^+$ T cell (4-1BB) and $CD4^+$ T cell (CD40L). The activated T cells thereafter undergo clonal expansion and acquire effector functions.

T helper cells

Depending on the cytokine environment, the Th cell further develops into a Th1 or Th2 subtype. Viruses mainly activate DCs to produce IL-12 that drives the antigen specific Th cell to develop into a Th1 subtype. The Th1 cells secrete IFNγ, which activates for example MΦs. In contrast, parasites mainly activate DCs to produce IL-4 that will stimulate the antigen specific Th cell to differentiate into a Th2 subtype. The Th2 cells also secrete IL-4, among other cytokines, that stimulate B cell proliferation, antibody production and antibody class switching.

In addition to activation of MΦ and B cells, Th cells potentiate $CD8^+$ T cell activation by stimulating upregulation of co-stimulatory molecules on the DC. This is mediated by CD40L and CD40 interaction between the Th cell and the DC. CD40

ligation initiates a signaling cascade in the DC leading to up-regulation of additional co-stimulatory molecules important for $CD8^+$ T cell activation.

Cytotoxic T cells

Activated cytotoxic T cells (CTLs) accumulate perforin and granzymes in intracellular vesicles and have a different expression pattern of certain surface molecules compared to naïve $CD8^+$ T cells. For instance, CD62L expression is down-regulated whereas very late activation antigen-4 (VLA-4) is up-regulated during activation of the cytotoxic T cells. The changed expression pattern leads to re-routing of the CTL homing capacity from LNs to inflamed tissues. After clonal expansion in the LN, the CTL exit the LN and start to circulate in the host, scanning the tissues for its cognate peptide. The CTLs interact with cells in the periphery first by unspecific selectin/integrin binding and thereafter by a stronger specific TCR and MHC class I interaction. As all nucleated cells display MHC class I on their cell surface, the CTLs can recognize and destroy cells displaying virus-derived peptides on MHC class I. Upon recognition of a virus-infected cell perforin and granzymes are released into the intracellular space between the two cells. Perforin permeabilizes the cell membrane of the target cell, through which the granzymes enter into the cytoplasm. The granzymes activate a caspase cascade leading to degradation of DNA and ultimately apoptosis of the virus-infected cell.

T cell memory

When the viral infection is cleared, the large quantities of the specific effector CTLs generated to eliminate the infection are no longer needed. Therefore, the majority of the antigen specific T cells die by apoptosis, leaving only a fraction of the specific T cells behind (72). These T cells constitute a memory pool. During a second infection with the same pathogen, the memory T cells expand faster and more robustly compared to naïve $CD8^+$ T cells during a primary infection, and hence clear the infection faster. Thus, the memory T cells are highly important to reduce morbidity after re-infection with the same pathogen and for vaccine efficacy. The memory cells are divided into two subgroups, central memory T cells (T_{CM}) and effector memory T cells (T_{EM}), based on phenotypic characterization such as expression of the surface molecules CD62L and CCR7, as well as functional features. The T_{CM} cells express CCR7 and CD62L making them capable of homing to lymphoid tissues. CCR7 expressed on the T_{CM} cells interact with SCL produced by stromal cells and DCs in the LNs, whereas CD62L bind ICAMs expressed on high endothelial venules (HEVs), through which the T cells enter the LNs (73). The T_{EM} cells lack the expression of these molecules, consequently generating cells that mainly reside in the peripheral tissue. However, even though the T_{CM} cells is the main memory subtype found in LNs, T_{EM} cells can enter LNs involved in an ongoing immune response (74). Both T_{CM} and T_{EM} cell subsets are found in blood and spleen (75-77).

In addition to differences in homing phenotype, the memory subtypes differ in function. The T_{CM} cells have a greater proliferating capacity than the T_{EM} cells and produce IL-2 upon antigen stimulation, a feature not attributed to T_{EM} cells (78). Due to the enhanced proliferative capacity of the T_{CM} subtype, it has been suggested that vaccines should strive for generation of T_{CM} cells. Indeed, the T_{CM} subtype has conferred better tumor protection than T_{EM} cells in tumor challenge models (79). In contrast, both the T_{CM} (75, 80) and T_{EM} subtypes (80) have been shown to be beneficial in protection against viral challenge, indicating that the proliferation capacity as well as the localization of the T cell are of importance. However, to investigate the role of low frequency memory subpopulations, adoptive transfer studies have been performed. When large numbers of T_{CM} or T_{EM} cell populations are injected into animals, the ratio of memory subpopulations is altered, unlike in the endogenous memory repertoire or when small numbers of cells are transferred (81, 82). Hence, the precise role of memory subpopulations might be difficult to envisage based on these non-physiological conditions.

The generation of diverse memory subtypes depends on the strength and duration of the initial T cell stimulation. Factors that influence the outcome are antigen persistence, inflammation and the ratio between DCs and T cells (75, 81-87). For example, T_{EM} cell formation is favored during conditions with low competition between the T cells for APCs, whereas the opposite appears to be the case for T_{CM} cell generation (82).

A key feature of memory cells is their long life span. Their survival is independent of antigen stimulation, but dependent on IL-7 and IL-15 signaling (88-92). IL-7 is important for survival of the memory cells, whereas IL-15 is important for the homeostatic proliferation. In addition to cytokines, Th cells are also important for the survival of memory cells. Primary and memory CTLs can be generated in the absence of Th cells, but CTL memory cells generated in the absence of Th cells fail to proliferate during a secondary expansion (93).

NUCLEIC ACID-BASED VACCINES

Conventional DNA (convDNA) vectors have gained much consideration as vaccine vectors due to promising preclinical results in mice. However, in clinical trials, low immune responses were detected in the vaccinated subjects (94). To enhance the immunogenicity of convDNA vectors, elements that activate innate signaling pathways (95) or apoptosis (96-98) have been incorporated and different models of delivery have been evaluated (99).

The recombinant viral vectors are constructed to have no or limited viral spread in the vaccinated subject. This is achieved by deletion of structural genes (100, 101) or the use of vectors that have restricted replication in human cells (102). Adenovirus vectors as well as poxvirus derived vectors, such as ALVAC, NYVAC and Modified vaccinia Ankara (MVA) virus, have been widely used in both preclinical and clinical trials (103-116). To minimize the immune response towards the viral vectors themselves, and focus the response to the inserted antigen-of-interest,

vectors have been combined in heterologous prime boost regimens (117-130). Heterologous prime boost regimens enhanced the immune responses in the phase I/II clinical trials to the antigen of interest (131-138). However, despite promising preclinical results, variable immune responses were detected in the phase I/II clinical trials. Protection against infectious disease and tumor regression in cancer patients, has so far been inconclusive. Thus, further development and evaluation of DNA and recombinant viral vectors is needed. For example, viral vectors that induce low immune responses towards the vector itself, and for which pre-existing immunity in humans is rare, should be considered. In the work of this thesis, I have investigated the potential of recombinant alphavirus-, adenovirus- and poxvirus-derived vectors with a focus on Semliki Forest virus (SFV). A detailed description of these vector systems follows below.

Semliki Forest virus

SFV is an RNA virus of the *Alphavirus* genus, *Togaviridae* family. Other members of this group includes Sindbis virus (SIN) and Venezuelan encephalitis virus (VEE). The natural reservoirs for SFV are rodents and birds, and transmission occurs mostly via mosquitoes. Wild-type SFV infection in humans causes flu-like symptoms, such as fever and severe headache, whereas the attenuated laboratory strains are considered avirulent (139). However, these attenuated strains are virulent in laboratory animals, and have been used in mice for studying acute viral encephalitis.

The SFV particle is enveloped and contains a single-strand positive sense RNA (+ssRNA) genome. The SFV genome (42S) is capped and polyadenylated and contains two open reading frames (ORFs). The 5' ORF constitutes two thirds of the genome and encodes the replicase or non-structural proteins (nsP) 1-4 (Figure 7). The second ORF, the 3'-terminal one-third of the genomic RNA, encodes the structural proteins; the capsid and the spike glycoproteins E1, E2, E3 and 6K (140). The replicase ORF is translated into an nsP1-4 polyprotein, from which the different nsPs are cleaved by proteolysis at different stages in the viral replication cycle. The early RNA polymerase (nsP1-3 polyprotein and free nsP4) generates complementary negative-strand RNA (-ssRNA) of the 42S genomic RNA (141) during the first 3 hours of infection (140, 141). Thereafter, the nsPs are cleaved into separate proteins and all four nsPs assemble into a replicase complex. The replicase complex generates positive-sense 42S genomic RNA and subgenomic 26S RNA from the negative-sense RNA strand (141).

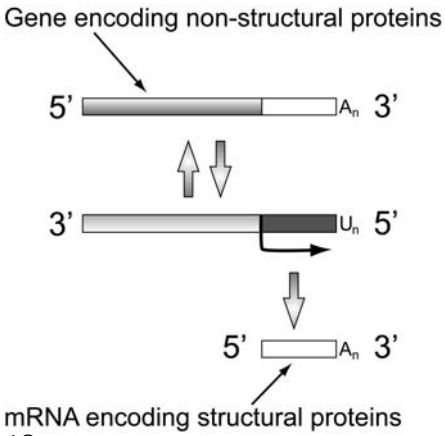

Figure 7. Replication of the SFV genome. The early RNA polymerase generate 42S negative-sense RNA. Later during the infection the replicase complex generates genomic 42S RNA and 26S RNA from the negative-sense RNA. The 26S encodes for the structural proteins, E1, E2, E3 and 6K.

Similar to the replicase RNA, the 26S RNA is translated as a polyprotein and cleaved into capsid, E1, E2, E3 and 6K in different cellular compartments. The nsP2 gene contains a packaging signal that is bound by the capsid protein and that mediates incorporation of genomic RNA into nucleocapsids (139, 141). The nucleocapsids bind to the spike glycoproteins leading to SFV particle assembly and budding from the host cell. These SFV particles infect new cells by receptor-mediated endocytosis via interaction between the E2 glycoprotein and a receptor on the host cell. After acidification of the late endosome the E1 glycoprotein undergoes a conformational change resulting in fusion between the viral envelope and the endosomal compartment. This in turn, leads to the release of the SFV genome into the cytoplasm of the host cell (142, 143).

Semliki Forest virus vector system

Liljeström and Garoff developed the SFV vector system where they exchanged the genes encoding the structural proteins for genes encoding heterologous model antigen. The structural genes were instead provided in *trans* (100). The vector system was further refined, lowering the frequency of replication competent recombinants in the preparations, by introducing the "split helper system" (144). In this system, three RNA constructs, replicon RNA (encoding the replicase, subgenomic promoter and model antigen) and the helper RNAs, spike (E1-3 and 6K) and capsid RNA, are co-electroporated into baby hamster kidney (BHK) cells (Figure 8). The replicase complex generated in these cells can act both in *cis,* replicating the replicon RNA and in *trans,* replicating the spike and capsid RNAs. Since the replicon RNA is the only RNA that contains the packaging signal, the helper RNAs are not incorporated into the recombinant viral particles. Since these viral particles do not carry RNA encoding the structural proteins, they are only capable of infecting a new cell once.

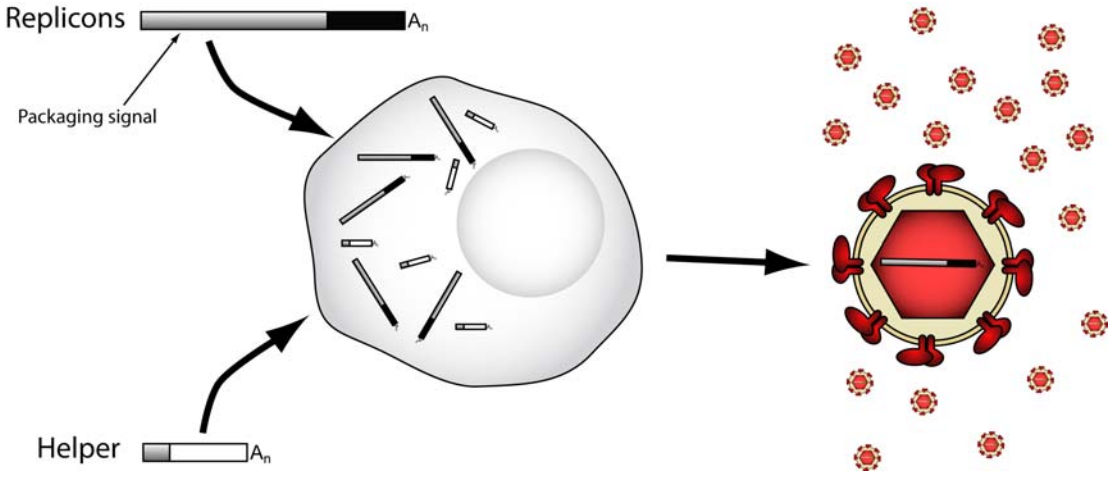

Figure 8. Generation of SFV viral particles. Co-electroporation of replicon and helper RNAs generates SFV virions carrying only the replicon RNA. Therefore, these viral particles can only infect a new cell once.

Recombinant SFV particles generate high amounts of heterologous protein in infected cells. At 3-6 hours post-infection, host cell translational shut-off down-regulates endogenous protein synthesis, generating a strong bias to the expression of the transgene in the infected cells (100, 145). Translational shut-off ultimately lead to apoptosis. It has been shown in other systems that apoptosis increase cross-priming (96-98). Therefore, it is plausible that cross-priming is also induced in alphaviral systems (146). Recombinant SFV particles induce strong T cell and B cell responses in animal models (147-152), and high levels of type I IFNs (153, 154). Type I IFNs are also induced in SFV infected cells without detectable transgene protein expression (153), indicating that the SFV viral particle itself might contain immune stimulatory components.

Alphaviral based DNA vectors

The alphaviral replicon has been inserted into convDNA vectors. In head-to-head comparisons to convDNA vectors, the alphaviral replicon-based DNA (DREP) vectors are superior in the generation of immune responses (155-166). Initially, the superior immune response induced by the alphaviral-based vectors was attributed to abundant antigen production (167, 168). However, it is now clear that these vaccine vectors activate innate immune responses, such as type I IFNs (169). Hence, the potency of the alphaviral-based vectors might depend on that they mimic the intracellular events of a viral infection.

Adenovirus

Adenovirus is a DNA virus of the *Adenoviridae* family (170). Several diverse serotypes of adenovirus exist that induce different clinical symptoms in humans. The genome consists of linear dsDNA encoding eight genes that are crucial for viral replication, the early (E1A, E1B, E2, E3 and E4), intermediate (pIX and Iva2) and late (LTU) genes, as well as genes that inactivate PKR. The viral genome is encapsidated inside a nucleoparticle, which is surrounded by a capsid. The capsid consists of a penton capsomer and fiber proteins that mediate adhesion to cellular receptors. After receptor mediated endocytosis and uncoating of the viral particle in the early endosome, the nucleoparticle is released into the cytoplasm. The nucleoparticle migrates to the nucleus and inserts the viral genome into the nucleus of the host cell. The host transcription machinery then drives the expression of the adenovirus genes.

The recombinant adenovirus subtype 5 (Ad5) vector (belonging to the serotype C group) is an attenuated laboratory strain lacking the E1 and E3 genes, thus allowing only one round of infection. For propagation of recombinant Ad5 particles, 293 cells, with a stably integrated E1 gene, are used (101). The Ad5 vector induce strong B cell and T cell responses (101, 171) and type I IFNs via TLR9 and cytoplasmic PRR innate signaling pathways (172). The Ad5 vector has been evaluated in both preclinical and clinical trials (101, 104, 106, 115, 116). Despite promising results in mice, variable outcomes were detected in the phase I/II clinical trials. Protection

against infectious disease and tumor regression in cancer patients, has so far been inconclusive. In mice, Ad5 immunization generate strong neutralizing antibodies, hampering the effect from boost immunizations (101). Since a majority of the human population have been infected by serotype C adenovirus, pre-existing immunity in humans (173) hampers the vaccination efficacy with Ad5 vector.

Poxvirus

Poxvirus is a DNA virus of the *Poxviridae* family. Several different subfamilies exist within the *Poxviridae* family, and they generate vastly different clinical symptoms in humans. For example, vaccinia virus induces mild symptoms (rash and fever), whereas smallpox is lethal in humans. The poxvirus genome consists of linear dsDNA, which replicates in the cytoplasm of the host cell. Since viral replication occurs in the cytoplasm and not in the nucleus, as is the case for other DNA viruses, the genome encodes for its own replication and transcription machinery.

Several attenuated poxvirus-based vectors have been developed, such as MVA, NYVAC and ALVAC. The poxvirus-based vectors have incomplete viral replication in human cells due to gene deletions or strain specific replication. MVA is an attenuated vaccinia strain due to loss of multiple virulence genes by extensive passage in chicken embryo fibroblasts (174-176). MVA vaccination induces multifunctional $CD8^+$ and $CD4^+$ T cell responses (177, 178) and strong type I IFN responses that are partly MyD88/TRIF dependent (179), indicating that redundant PRR signaling pathways are activated. NYVAC, another attenuated vaccinia virus vector, was generated by genetic engineered deletions of virulence and pathogenicity genes. MVA, in comparison to NYVAC, induces more type I IFNs, pro-inflammatory cytokines and exclusively up-regulate genes such as RIG-I, MDA5 and 2'-5' OAS. The enhanced immune stimulatory effect induced by MVA vectors compared to NYVAC vectors probably depends on fewer immune modulatory genes in the MVA genome (180). ALVAC (a canarypox virus), have restricted replication in human cells due to strain specific replication in birds. ALVAC infection generates $CD8^+$ and $CD4^+$ T cell responses and up-regulates genes involved in type I IFN signaling pathways, such as IRF7, STAT1, RIG-I and MDA5 (181). MVA (137, 182-191), NYVAC (114, 131, 138, 192-197) and ALVAC (104, 107-110, 112, 113, 193, 195, 198) have all been evaluated in clinical trials. These vectors induce both T cell and B cell responses in the vaccinated subjects, particularly in combination with other vectors. Similar to the adenovirus vectors, the phase I/II clinical trials with poxvirus-based vectors has so far been inconclusive.

THE P815 TUMOR MODEL

In Paper I we have used the P815 tumor model. A brief description of the model is given below.

The shared tumor-specific antigens, or cancer-germline antigens, are antigens that are expressed on several types of tumors as well as in normal tissues, such as testis

and placenta (199-202). Since both testis and placenta are immune-privileged sites, they are protected from destruction by cytotoxic T cells. The human shared tumor-specific antigens MAGE, BAGE, LAGE and NY-ESO-1 are expressed in various cancers, such as melanoma, adenocarcinomas (203), carcinomas and sarcomas (199). In mice, the tumor model P815 has been used for studying shared tumor-specific antigens. The P815 tumor cells express five known antigens (A, B, C, D and E). The P1A antigen is important for tumor rejection *in vivo* and is expressed in testis and placenta (204, 205). The P1A antigen contain one $CD8^+$ T cell epitope (P815AB) that is crucial for tumor rejection (206), hence the tumor rejection is strictly $CD8^+$ T cell dependent (205). Thus, the P815 tumor model is a relevant model for testing different vaccine regimens with human implications for the shared tumor-specific antigens.

AIMS OF THE THESIS

Distinct biological properties of different vaccine vectors influence the magnitude and quality of the elicited immune response. The aim of this thesis was to investigate innate signaling pathways induced by recombinant SFV vectors and to determine its potential as a vaccine vector compared to viral vectors already under evaluation in clinical trials.

RESULTS

Paper I: Comparative prime-boost vaccinations using SFV, adenovirus and ALVAC vectors demonstrate differences in the generation of a protective central memory CTL response against the P815 tumor

In Paper I, we compare three viral vectors, SFV-P1A, adeno-P1A$_t$ and ALVAC-P1A$_t$, in homologous and heterologous prime boost regimens for their ability to generate P1A-specific CD8$^+$ T cell responses and protection against a P815 tumor challenge.

Initially, we carefully titrated the recombinant viral vectors at different doses, immunization intervals, routes and number of immunizations. The dose and immunization schedule that generated the highest cytolytic P1A-specific T cell response for each vector was selected for the study. L1210.P1A.B7.1 cells have previously been shown to induce strong cytolytic P1A-specific T cell responses and to protect mice to a high degree against a P815 tumor challenge (205). Therefore, the L1210.P1A.B7.1 cells were included as a positive control. In general, heterologous prime boost immunizations of the viral vectors generated higher cytolytic P1A-specific T cell responses in comparison to homologous immunizations. However, the order in which the viral vectors were given influenced the magnitude of the response. SFV-P1A induced stronger responses when used as a prime, whereas adeno-P1A$_t$ generated elevated responses when used as a boost. In contrast, the potency of ALVAC-P1A$_t$ as a prime or a boost depended on the viral vector it was combined with. Among the homologous prime boost immunized groups, the strongest cytolytic P1A-specific T cell response was generated with adeno-P1A$_t$ immunizations. SFV-P1A immunization induced intermediate T cell responses, while ALVAC-P1A$_t$ immunization resulted in the lowest T cell response of them all. Since the SFV-P1A vector expressed the full-length P1A protein, in contrast to the truncated P1A protein (P1A$_t$) expressed by adeno-P1A$_t$ and ALVAC-P1A$_t$, comparisons between SFV-P1A and SFV-P1A$_t$ vectors were made. Both constructs generated similar cytolytic P1A-specific T cell responses, indicating that epitopes lacking in the P1A$_t$ protein did not increase the CD8$^+$ T cell response.

Since we measured the cytolytic P1A-specific T cell response in the blood, the mice were still alive and could be subjected to a P1A-expressing tumor challenge (P1.HTR3 tumor cells). When comparing the cytolytic P1A-specific T cell response to protection against the tumor challenge, we could not detect a correlation between these parameters, neither in the homologous nor in the heterologous immunized groups. For example, the adeno-P1A$_t$ immunized mice had a stronger cytolytic T cell response in comparison to the SFV-P1A immunized mice, but a lower protection frequency against the tumor challenge. This indicated that is was not only the level of cytolytic P1A-specific T cell activity that was of importance for protection against the tumor challenge. To investigate if the additional P1A protein

part influenced the protection capacity against a tumor challenge, SFV-P1A and SFV-P1A$_t$ immunized mice were subjected to a P1.HTR3 tumor challenge. Both SFV-P1A and SFV-P1A$_t$ immunized mice were protected against a tumor challenge to a similar frequency, indicating that epitopes lacking in the P1A$_t$ protein did not increase the protection against the tumor challenge.

To explain the differences in tumor protection induced by the adeno-P1A$_t$ and SFV-P1A vectors, we evaluated the numbers and proliferation potential of the P1A-specific CD8$^+$ T cells at the time of tumor challenge. Pre-challenge, lower numbers of P1A-specific CD8$^+$ T cells (measured by tetramer staining) were detected in the SFV-P1A immunized mice in comparison to adeno-P1A$_t$ immunized mice, both in blood and spleen. Moreover, the SFV-P1A immunized group had fewer IFNγ-secreting CD8$^+$ T cells in the spleen than the adeno-P1A$_t$ immunized group. Thus, neither the size of the P1A-specific CD8$^+$ T cell pool or the potential to secrete IFNγ at the time for tumor challenge could explain the difference in protection capacity between the two groups of mice. Next, we enumerated the P1A-specific CD8$^+$ T cells by tetramer staining at various time-points after tumor challenge in adeno-P1A$_t$ and SFV-P1A immunized mice. In respect to the P1A-specific T cell numbers pre-challenge, SFV-P1A immunization induced a larger pool of P1A-specific CD8$^+$ T cells than adeno-P1A$_t$ immunization during the peak of tumor regression, especially in the tumor-draining LN. This indicated that SFV-P1A immunization generated P1A-specific CD8$^+$ T cells that had a higher proliferation capacity in comparison to CD8$^+$ T cells generated by adeno-P1A$_t$ immunization.

Since T$_{CM}$ cells proliferate faster than T$_{EM}$ cells and preferentially localize in the LNs, we next investigated the proportion of these memory subtypes in SFV-P1A and adeno-P1A$_t$ immunized mice using CD62L expression as a marker for T$_{CM}$ cells. We detected a larger proportion of P1A-specific T$_{CM}$ cells compared to T$_{EM}$ cells in the SFV-P1A immunized mice in comparison to the adeno-P1A$_t$ immunized mice. This indicated that there was a difference in the generation of memory subtypes by the SFV-P1A and adeno-P1A$_t$ viral vectors. However, since adeno-P1A$_t$ immunization generated more P1A-specific CD8$^+$ T cells than SFV-P1A immunization, the total number of T$_{CM}$ cells did not significantly differ between the two groups of mice. Thus, the ratio between the memory subpopulations is more important than the actual number of a specific memory subpopulation.

As an overall conclusion, different viral vectors influence the ratio of T cell memory subsets. Hence, to determine the efficacy of a vaccine, the quality and not only the quantity of the antigen specific immune cells should be investigated.

Paper II: Toll-like receptor 3 promotes cross-priming to virus-infected cells

In Paper II, we investigate the specific role of dsRNA in CD8α$^+$ DC activation.

To isolate the direct effect of dsRNA on mouse derived CD8α$^+$ DCs we used xenogenic Vero cells that do not induce direct priming of T cells or produce type I IFNs. The CD8α$^+$ DCs were capable of phagocytosis of Vero cell-associated material,

an event that occurred irrespective of the presence of synthetic dsRNA (polyI:C). However, up-regulation of genes involved in innate signaling pathways (IFN-α, IFN-β, IL-6 and TNF-α) and co-stimulatory molecules (CD40 and CD86) were only detected in CD8α$^+$ DCs that had phagocytosed polyI:C loaded Vero cells. This effect was not restricted to Vero cells, since CD8α$^+$ DC activation was detected in the presence of other cell-lines, as well as syngeneic and allogenic mouse splenocytes, loaded with polyI:C. Moreover, cell-associated polyI:C was a stronger stimuli to the CD8α$^+$ DCs than exogenously added polyI:C, an effect that might depend on the CD8α$^+$ DCs' specific role in phagocytosis of cell-associated material (66, 67) or uptake of a higher concentration of polyI:C due to settling of polyI:C loaded cells.

Phagocytosis of cell-associated material was crucial for the activation of CD8α$^+$ DCs since components blocking phagocytosis, such as latrunculin B and chloroquine, rendered the CD8α$^+$ DCs refractory to IL-6 production. To investigate possible innate signaling pathways involved in polyI:C induced CD8α$^+$ DC activation, CD8α$^+$ DCs lacking candidate genes in viral recognition, such as *Pkr, MyD88* or *Tlr3* genes, were co-cultured with polyI:C loaded Vero cells. CD8α$^+$ DCs lacking *Pkr* or *MyD88* were equally well activated as wild-type CD8α$^+$ DCs. In contrast, *Tlr3* deficient CD8α$^+$ DCs were unresponsive to activation, indicating that phagocytosed polyI:C activated the CD8α$^+$ DCs via TLR3.

To investigate if virus-infected cells, similar to polyI:C loaded cells, could activate the CD8α$^+$ DCs, we used two viral vectors that did not detectably infect the DCs, the encephalomyocarditis virus (EMCV) and SFV. The CD8α$^+$ DCs were not activated, as measured by IL-6 production, in the presence of EMCV or SFV virions. However, CD8α$^+$ DCs were activated in the presence of EMCV or SFV infected Vero cells, suggesting that virus-infected cells, similar to polyI:C loaded cells, activate CD8α$^+$ DCs. It was later reported that SFV virions induce type I IFNs in bone-marrow derived DCs (BM-DCs) in the absence of detectable transgene protein production (153), a finding that was also verified by us (207). Thus, freshly isolated splenic CD8α$^+$ DCs may differ from *in vitro* cultured BM-DCs in their responsiveness to SFV, or there may be different activation thresholds for induction of IL-6 and type I IFNs by SFV.

To test the relevance of TLR3 signaling in the CD8α$^+$ DCs in the presence of virus-infected cells, wild-type CD8α$^+$ DCs and CD8α$^+$ DCs lacking the *Tlr3* gene were co-cultured with EMCV or SFV infected Vero cells. Similar to the polyI:C loaded Vero cells, CD8α$^+$ DCs lacking the *Tlr3* gene were not activated by virus-infected Vero cells. This indicated that dsRNA generated by viral RNA replication could activate the CD8α$^+$ DCs via TLR3 just like polyI:C. To investigate if virus-infected cells or polyI:C containing cells could induce cross-priming *in vivo,* wild-type mice were injected with SFV-OVA infected Vero cells or Vero cells electroporated with OVA ± polyI:C. OVA-specific T cell responses were detected in the SFV-OVA/Vero and OVA+polyI:C/Vero immunized mice, indicating that virus-infected cells or polyI:C containing cells induced cross-priming *in vivo*. To study if CD8α$^+$ DC cross-priming was dependent on TLR3 signaling *in vivo*, wild-type and TLR3 deficient mice were immunized as previously. TLR3 deficient mice did not mount an OVA-specific T cell response when immunized with OVA+polyI:C/Vero cells, indicating that TLR3

was important for cross-priming in the presence of polyI:C. However, TLR3 deficient mice could induce OVA-specific T cell responses after immunization with SFV-OVA/Vero cells, albeit to a lower degree than wild-type mice. This suggested that cross-priming of SFV-infected Vero cells was in part mediated by TLR3 signaling, but that alternative innate signaling pathways distinct from TLR3 probably compensate for TLR3 to promote cross-priming *in vivo*. It was later reported that the T cell response in TLR3 deficient mice was not decreased after immunization with SFV viral particles (154) or a Sindbis based DNA vector (208), indicating that the role of TLR3 is less important when other innate signaling pathways are present *in vivo*.

In conclusion, we could show that TLR3 ligands induce CD8α$^+$ DC cross-priming of T cells. This explains the relevance of TLR3 expression in the CD8α$^+$ DC, a DC subtype that readily phagocytoses dying cells.

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

In Paper III, we investigate the role of the cytoplasmic RNA receptor RIG-I in response to different viral vectors and how influenza A virus blocks type I IFN responses by expression of NS1 protein.

It has previously been shown that influenza A (Flu) virus blocks type I IFN responses in various cell-types by expression of NS1 protein (17-19). To investigate the role of the NS1 protein in the inhibition of type I IFN responses, we constructed a recombinant Flu virus lacking the *NS1* gene (Flu-ΔNS1). By measuring the type I IFN production in wild-type Flu and Flu-ΔNS1 infected cells we confirmed previous data that the NS1 protein suppresses type I IFN production in DCs. The type I IFN response was independent of the MyD88 adaptor molecule and hence signaling via TLR7, 8 and 9 pathways. Since the Flu NS1 protein contains an RNA-binding domain, inhibition of type I IFN responses has been linked to sequestering of dsRNA generated during viral replication. To investigate if dsRNA is generated in Flu infected cells, cells were infected with wild-type Flu or Flu-ΔNS1 virus. However, dsRNA was not detected in either wild-type Flu or in Flu-ΔNS1 virus infected cells consistent with previous reports (209).

Since SFV infection is known to generate dsRNA in infected cells (210-212) we constructed an SFV vector expressing the Flu NS1 protein (SFV-NS1), to investigate the role of the NS1 protein in the presence of abundant levels of dsRNA. We infected BM-DCs with SFV-NS1, SFV-OVA or wild-type SFV and measured type I IFNs induced by the different viral vectors. Similar levels of type I IFN were generated from the BM-DCs irrespective of the protein expressed by the SFV vector. However, since abundant levels of dsRNA are expected to be generated prior to NS1 protein expression, this was not a suitable system to investigate the effect of NS1. Therefore, we infected human embryonic kidney (HEK) 293 cells expressing the NS1 protein prior to infection with SFV or EMCV, another virus that generates abundant dsRNA during infection, and measured IFN-β gene activation. Even though the NS1 protein was present before viral infection, SFV and EMCV

induced IFN-β gene activation in the HEK293 cells. In contrast, Sendai virus (SeV) or Flu-ΔNS1, two viruses that generate small amount of dsRNA during viral infection, did not induce IFN-β gene activation. Collectively, these data suggest that Flu NS1 protein does not block type I IFN production by sequestering dsRNA.

It was previously known that both Flu virus and SeV are recognized by RIG-I while EMCV is recognized by MDA5 (12, 13). We therefore hypothesized that the NS1 protein, instead of sequestering dsRNA, may block type I IFN responses via RIG-I inactivation. To investigate if NS1 interacts with RIG-I, cells over-expressing RIG-I or MDA5 were infected with Flu virus. The cell-lysates were thereafter analyzed with Western blot. Complex-formation was detected between NS1 and RIG-I proteins, whereas no binding was seen between the NS1 and MDA5 proteins. This suggested that NS1 targets RIG-I instead of dsRNA as previously thought.

Since dsRNA was not readily detected in Flu infected cells, we speculated that RIG-I instead recognize the Flu ssRNA genome. To investigate this, genomic Flu RNA (vRNA) was extracted from Flu virions and transfected into different cell-types expressing RIG-I. Thereafter, the induction of type I IFN was investigated. Indeed, vRNA induced type I IFNs, a response that was lower in the presence of NS1 protein. Lower levels of type I IFN was also detected by inhibition of RIG-I expression prior to vRNA transfection. Hence, this suggests that RIG-I was activated by the Flu genome and that the NS1 protein could block RIG-I activation.

From a previous study it was known that *in vitro* transcribed uncapped siRNA and ssRNA with free 5' end phosphates induce type I IFNs (213). Since both EMCV and SFV have capped RNA genomes whereas Flu and vesicular stomatitis virus (VSV) have uncapped genomes, we hypothesized that RIG-I was activated by RNA containing 5'-phosphates. To investigate this, phosphates were removed by CIP enzyme treatment of vRNAs from Flu or VSV. Thereafter, vRNA ± CIP were transfected into cells and the induction of type I IFNs and pro-inflammatory cytokine was analyzed. Untreated vRNAs induced pro-inflammatory and type I IFN responses whereas the cytokine response was abolished in the presence of CIP treated vRNAs, suggesting that RNA containing 5'-phosphates activates RIG-I. Finally, to investigate if 5'-phophates contribute to RIG-I binding, RIG-I protein and Flu vRNA ± CIP treatment were co-incubated. By Western blot analyses, we detected an interaction between RIG-I and Flu vRNA that was stronger in the presence of 5' end phosphates. Moreover, co-incubation of NS1, ssRNA ± CIP and RIG-I induced complex-formation of all three components, an interaction that was lost in the presence of mutant NS1, not capable of binding to RNA, or CIP treated RNA. This suggests that RIG-I binds RNA, preferentially to RNA containing phophorylated 5' ends, and NS1 is recruited to these complexes to block RIG-I activation.

In conclusion, we show that RIG-I preferentially binds RNA containing free 5' end phosphates and that NS1 is recruited to these complexes partly by its RNA binding capacity. In accordance, Hornung *et al* (214) reported that *in vitro* transcribed RNA containing free 5' end phosphates activates RIG-I.

Paper IV: Role of innate signaling pathways in alphavirus replicon immunization

In Paper IV, we investigate innate signaling pathways induced by SFV vector immunization.

To investigate which innate signaling pathways are activated by DREP and SFV particle (VREP) inoculation, we immunized mice lacking different innate signaling molecules, including TLR3, TLR9, MyD88, IRF3 and IFN-AR1 with DREP or VREP. In accordance with other reports (154, 208), TLR3 was not crucial for the generation of an adaptive T cell response by either VREP or DREP, suggesting that TLR3 signaling is less important in the presence of other innate signaling pathways. TLR9 and MyD88 deficient DREP immunized mice showed similar levels of T cell responses as the wild-type mice, indicating that neither CpG-motifs nor ssRNA were crucial for the induction of potent T cell responses after DREP immunizations. IRF3 is a signaling molecule crucial for type I IFN production downstream of several PRRs, such as the dsRNA receptors TLR3 and the RLRs. We therefore immunized IRF3 deficient mice with VREP and DREP. In the VREP immunized mice, we detected a significant reduction in the primary T cell response in the IRF3 deficient mice compared to the wild-type mice, indicating that dsRNA is an important PAMP for activation of potent immune responses after SFV vector immunization. The responses in DREP immunized IRF3 deficient mice did not significantly differ from the wild-type mice, but followed the same trend as in the VREP immunized mice.

Type I IFNs are induced by the RLRs and some TLRs, and are known to activate DCs, T and B cells (49-52). To investigate the role of type I IFNs in the generation of T cell responses after alphaviral immunization, IFN-AR1 deficient and wild-type mice were immunized with DREP or VREP. We detected a significantly stronger T cell response in the IFN-AR1 deficient mice in comparison to wild-type mice, both after DREP and VREP immunization, suggesting that perhaps type I IFN had a suppressive effect on the adaptive immune response. To investigate if the type I IFN suppressive effect was dose-dependent, IFN-AR1 deficient and wild-type mice were immunized with increasing doses of DREP. At lower doses of DREP, similar levels of T cell responses were detected in the IFN-AR1 deficient and wild-type mice. However, at increasing doses the T cell response escalated in the IFN-AR1 deficient mice, whereas the T cell response reached a plateau in the wild-type mice. Hence, at higher DREP doses type I IFN had a suppressive effect on the T cell response. The robust T cell responses detected in the IFN-AR1 deficient mice suggest that other cytokines can compensate for the lack of type I IFNs.

We next investigated the memory T cell response in IRF3 deficient mice. In contrast to what was seen in the primary T cell response, IRF3 deficient mice had significantly stronger T cell responses than the wild-type mice after VREP immunization. The same trend was observed in the DREP immunized mice, but it did not reach statistical significance. It was known from previous studies that IRF3 is needed for IL-12 transcription (215) and that a similar T cell shift between primary and memory T cell responses was detected in *Listeria* infected IL-12

deficient mice (216). To investigate if the induction of IL-12 could explain what was detected in the IRF3 deficient mice, the IL-12 production was measured after DREP immunization in the IRF3 deficient and wild-type mice. Lower levels of IL-12 were detected in the DREP immunized IRF3 deficient mice in comparison to the wild-type mice, suggesting that IL-12 might be involved in the responses detected in the IRF3 deficient mice.

It was previously known that type I IFNs are important for maintenance of the memory cells (49, 217, 218). To investigate this, we immunized IFN-AR1 deficient and wild-type mice with DREP or VREP and measured the memory T cell responses. Similar to what was detected in the primary response, stronger T cell memory responses were detected in the IFN-AR1 deficient mice in comparison to wild-type mice. This suggests that type I IFNs are not crucial for the maintenance of the T cell memory pool after alphaviral immunization and that other cytokines can mediate this effect.

In conclusion, DREP and VREP activate multiple innate signaling pathways, such as IRF3 and IFN-AR1 dependent pathways. The results presented here add to our understanding of the relative roles of these pathways for establishing adaptive immune responses against vector-encoded antigens.

CONCLUSIONS

Four major conclusions can be drawn from the work included in this thesis.

I. Different recombinant viral vectors induce distinct immune responses, both in terms of magnitude and quality. A better understanding of these differences will inform the design of improved vaccine regimens.

II. For the immune system to detect viruses that do not infect DCs *per se* cross-priming is important. TLR3 expression on CD8α^+ DCs enables detection of dsRNA upon phagocytosis and activation of DC function.

III. RIG-I is a receptor for viral RNA with free phosphates at the 5' end. Negative-sense RNA viruses do not generate detectable levels of dsRNA during viral infection. Thus, RIG-I enables detection of these types of viruses.

IV. Alphavirus based vectors are potent activators of innate signaling pathways. The presence or absence of IRF3 and IFN-AR1 affects the T cell response induced by these vectors, while signaling via TLR3, TLR9 and MyD88 appears redundant.

DISCUSSION

Historically, evaluation of vaccine candidates has focused on the magnitude of elicited immune responses. However, it is getting increasingly clear that the quality of the response is an important factor for protection against disease. As shown in Paper I, viral vectors have different abilities to induce strong immune response and to be effective as priming or boosting agents. Moreover, the viral vectors generated different memory phenotypes of antigen-specific T cells. There are several possibilities to why the quality of the immune response is affected by the type of viral vector used, for example i) targeting of the antigen to different responder cells, ii) recognition of the viral vector by different innate pathways, iii) antigen persistence, and iv) T cell exhaustion.

The first encounter with the innate immune system shapes the adaptive immune response. Therefore, adaptive immune responses may be affected by which innate signaling pathways are activated. Different DC subpopulations express distinct subsets of TLRs, and PRR activation of a specific DC subset induces different types of T cell responses (219, 220). Thus, specific PRR activation by different viral vectors could shape the adaptive immune response into diverse directions. For example, activation of TLR2 induces Th2 responses and regulatory T cells, whereas TLR3, 4, 5, 7 and 9 activation generate Th1 responses and IL-12 cytokine production. In addition, TLR3, 4, 7 and 9 induce type I IFNs. Hence, the responder DC and/or danger signals likely play a role in the outcome of the immune response induced by the viral vectors. As detected in Paper I, injection of the SFV and adenovirus vectors at the same site, generated T cell responses of different magnitude and quality. This suggests that the different viral vectors target different DC subpopulations and/or signal through different TLRs expressed by the DCs. It is known that adenovirus infects DCs (221), whereas SFV, at least *in vitro,* is incapable of productively infecting BM-DCs (153, 207). Hence, adenovirus vectors can potentially induce both direct-priming and cross-priming by the DCs, whereas SFV only induces cross-priming.

It is known that both adenovirus and SFV vectors induce type I IFNs (153, 154, 222). However, up-stream signaling pathways differ between the viral vectors. Induction of type I IFNs by adenovirus depend on TLR9 and cytoplasmic PRR receptor signaling (172). SFV infection generates dsRNA and as suggested in Paper II, activates $CD8\alpha^+$ DCs via TLR3 signaling. However, TLR3 signaling was not crucial for the induction of potent T cell responses in the presence of other innate signaling pathways (Paper IV), from for example the SFV infected cells, indicating that redundant innate signaling pathways are induced after SFV vector immunization. Nevertheless, IRF3, a signaling molecule down-stream of TLR3, RIG-I and MDA5 signaling pathways, was needed for potent primary T cell responses after SFV viral immunization (Paper IV). This indicates that dsRNA is an important PAMP for SFV detection by the innate immune system, but that the cytoplasmic receptors detecting dsRNA override the need for TLR3 signaling for the induction of potent T cell responses *in vivo*. RIG-I and MDA5 were previously

suggested to recognize dsRNA generated during viral replication. However, SFV did not activate RIG-I, despite abundant dsRNA generated in SFV infected cells (Paper III). Instead, RIG-I was shown to recognize RNA with free 5' end phosphates and act as a receptor for negative-sense RNA viruses like influenza A virus. Since the SFV genome has a cap structure at the 5' end, RIG-I is not activated by SFV infection. However, SFV activates IRF3 dependent pathways, it is therefore plausible that dsRNA generated during SFV viral replication activates MDA5. In contrast, the viral vectors can modulate the activation of specific innate signaling pathways. For example, adenovirus blocks PKR activation in the host cell leading to enhanced viral protein production (223).

Yellow fever vaccine 17D (YF-17D) is regarded as one of the most effective live attenuated vaccines available (224). YF-17D vaccination induces both T cell and B cell responses and activates TLR2, 7, 8, and 9 signaling pathways. The activation of multiple TLRs and several subsets of DCs has been suggested to influence the generation of the diverse adaptive immune response by YF-17D vaccination (31, 225, 226). Thus, it is difficult to determine which receptors and signaling molecules that are important for the generation of a desired immune response. Moreover, since correlates of protection may be different for different diseases it is difficult to know what parameters to look for.

Antigen persistance, inflammation and strength of the TCR signaling are known to influence the quality of the memory response (75, 81-87). Hence, another possible explanation for the different T cell responses detected between SFV and adenovirus infection in Paper I could be differences in antigen dose and/or antigen persistence. SFV transgene expression *in vivo* is transient (227). In contrast, adenovirus transgene expression can be detected during extensive time-periods *in vivo* (228). It is known that by increasing the antigen load, the formation of T_{EM} cells increases (75). Thus, adenovirus immunization with a prolonged antigen expression could potentially lead to the generation of a T_{EM} phenotype.

It has been reported that sustained antigen expression induces exhausted T cells, for example during chronic viral infections in mice (229). Exhausted T cells up-regulate PD-1 expression, and by blocking PD-1 the CTL function could be restored. In accordance, adenovirus immunization has been found to induce exhausted CTLs (230). This could be an alternative explanation to the diminished protection observed against the tumor challenge in adenovirus immunized mice (Paper I).

As shown in Paper IV, the induction of T cell responses after immunization with SFV was not dependent on IFN-AR1 signaling. Type I IFNs have been implicated in stimulation and activation of both innate and adaptive immune responses, as well as being important for sustaining the memory pool (49-52, 217, 218). However, we detected that type I IFNs reduced the primary T cell response. Several explanations could account for this effect. The antigen load could vary in the wild-type and IFN-AR1 deficient mice due to prolonged transgene expression in the IFN-AR1 deficient mice. IFN-AR1 signaling activates PKR, induces apoptosis and suppresses viral replication and spread, events that are lacking in the IFN-AR1 deficient mice, thus potentially allowing a longer time-period for antigen expression in the SFV infected

cells in the IFN-AR1 deficient mice. However, recombinant SFV vectors do not generate virus progeny and it is unclear if type I IFNs reduce transgene expression in the infected cell *per se*. In addition, the IFN-AR1 deficient mice lack IFN-AR1 signaling induced negative feedback mechanisms, such as TAM receptor up-regulation. Hence, prolonged cytokine production could account for the robust primary T cell response detected in the IFN-AR1 deficient mice as well as the maintenance of the memory pool.

In this thesis, I have shown that viral vectors differ in their abilities to generate a specific immune response in both magnitude and quality. The relevance of TLR3 expression in CD8α^+ DCs and its role in cross-priming of anti-viral T cell responses have been illuminated. The distinct roles for RIG-I and MDA5 as sensors for different types of viruses have been elucidated. Furthermore, I have shown that multiple innate signaling pathways are activated after SFV immunization. In conclusion, several parameters influence the outcome of an adaptive immune response. For vaccination purposes, the generation of a functional memory response is important. Further studies are needed to understand the parameters that dictate the generation of immune responses that correlate with protection against disease.

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