Mechanisms of single-stranded RNA and type I interferon induced suppression of adaptive immune responses

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Martina Brock

aus

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Erklärung

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit persönlich, selbstständig und unter Offenlegung der erhaltenen Hilfen angefertigt habe.

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Martina Brock

Meinen Eltern

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Summary

Toll-like receptors (TLRs) belong to the pattern-recognition receptors (PRR) expressed mainly by cells of the innate immune system and recognize conserved structures derived from microbes. Stimulation of TLRs by their respective TLR ligands induces the production of proinflammatory cytokines, chemokines, and maturation of dendritic cells (DCs), which plays a crucial role in the induction of adaptive immunity. Strinkingly, we and others have shown that the systemic exposure to TLR ligands suppresses the generation of adaptive T cell immunity towards subsequent infections. Our group has recently observed that the systemic injection of single-stranded RNA (ssRNA), which is recognized by endosomal TLR7, inhibited antigen-specific cytotoxic T lymphocyte (CTL) responses in the spleen in a type I interferon (IFN) dependent manner. Whereas the immunostimulatory effects of TLR stimulation and type I IFNs are well described, it remains poorly understood how the suppression of adaptive immune responses is mediated by TLR ligands and type I IFN, respectively. In this thesis, the underlying mechanisms of ssRNA- and type I IFN-mediated inhibition of adaptive immunity were investigated.

The results presented demonstrate that the systemic administration of ssRNA leads to the suppression of CTL responses against subsequent infections with viral and bacterial pathogens, which was attributed to an impaired expansion and activation of antigen-specific CD8 T cells. Furthermore, we detected that the proliferation and cytokine production of antigen-specific CD4 T cells was impeded, which was associated with a downregulation in MHC class II molecule expression on ssRNA-preactivated splenic DCs. These results clearly indicate that a systemic injection of ssRNA inhibited the generation of CD4 T cell help, which contributed to the suppression of antigen-specific CTL responses. This interpretation was supported by the observation that ssRNA-induced CTL suppression was circumvented in the presence of effector or memory antigen-specific CD4 T helper cells. The generation of T cell immunity was not impaired if ssRNA was targeted to the cytoplasm by complexation with the carrier *in vivo*-jetPEI^{TM}, thereby avoiding the recognition by TLR7. TLR7-mediated recognition of systemic ssRNA stimulated the production of type I IFNs, which were essential in ssRNA-induced CTL suppression. We identified plasmacytoid DCs (pDCs) to respond immediately to ssRNA stimulation by releasing early type I IFNs. Based on our experiments in LysM-specific IFNAR1 deficient, STAT1 deficient, and IRF7 deficient mice, we assume that ssRNA-induced early type I IFNs are amplified via the JAK-STAT pathway in splenic macrophages. The resulting secretion of substantial amounts of IFNα affected splenic DCs, which were impaired in their ability to cross-prime antigen-specific CD8 T cells, thus contributing to the lack of antigen-specific cytotoxicity.

A clinical relevance of RNA-mediated immune suppression was shown in a combinatorial anti-tumor therapy model, mimicking small-interfering RNA (siRNA) application and

induction of tumor-specific CTL responses. We could demonstrate that, depending on the carrier used, systemic *in vivo* siRNA application interfered with an adenoviral-based melanoma vaccination due to the lack of a tumor-specific CTL response.

Moreover, we revealed that CTL suppression was not restricted to the application of synthetic RNA oligoribonucleotides. Infection with an Influenza virus impeded the generation of the CTL response against a subsequent adenovirus infection, which was ascribed to the production of type I IFNs. This observation implies that type I IFN-triggering viruses in general might inhibit adaptive immune responses in a similar fashion as we observed with systemic ssRNA.

Taken together, our results point out that ssRNA- and virus-induced type I IFNs cannot only stimulate, but also inhibit antigen-specific T cell responses in the spleen. Furthermore, we indicate that a therapeutic siRNA application *in vivo*, e.g. in tumor therapies, might lead to undesired off-target effects, such as immune suppression, due to the recognition of siRNA by nucleic acid receptors such as TLR7.

Zusammenfassung

Toll-like Rezeptoren (TLRs) gehören zu den Muster-Erkennungsrezeptoren (Pattern-recognition receptors, PRR), welche hauptsächlich auf den Zellen des innaten Immunsystems exprimiert werden. Sie dienen der Erkennung von bestimmten konservierten Strukturen, die ausschließlich auf oder in Pathogenen vorkommen. Die Stimulation von TLRs durch Bindung ihrer spezifischen TLR Liganden induziert die Produktion von proinflammatorischen Zytokinen, Chemokinen und Reifung von dendritischen Zellen (DCs). Dieser Prozess spielt eine entscheidende Rolle bei der Aktivierung der adaptiven Immunität. Jedoch haben wir und andere Gruppen gezeigt, dass die systemische Gabe von TLR Liganden nachfolgende T Zell Antworten supprimieren kann. Wir haben kürzlich beobachtet, dass die systemische Applikation von einzelsträngiger RNA (ssRNA), welche vom endosomalen TLR7 erkannt wird, antigen-spezifische zytotoxische T Lymphozyten (ZTL) Antworten in der Milz inhibieren. Diese Hemmung der ZTL Antwort war von der Induktion von Typ I Interferonen (IFN) abhängig. Während die immunstimulatorischen Eigenschaften von TLR Liganden und Typ I IFN gut beschrieben sind, ist es noch unzureichend verstanden, wie TLR Liganden beziehungsweise Typ I IFN die Suppression von adaptiven Immunantworten vermitteln. Ziel dieser Arbeit war es, die zugrunde liegenden Mechanismen der ssRNA- und Typ I IFN-induzierten Hemmung der adaptiven Immunität zu untersuchen.

Die Ergebnisse dieser Arbeit zeigen, dass eine systemische Gabe von ssRNA zu einer Suppression der ZTL Antworten führt, die gegen nachfolgende Infektionen mit viralen und bakteriellen Pathogenen gerichtet sind. Die fehlende antigen-spezifische zytotoxische Aktivität konnte auf eine gestörte Expansion und Aktivierung von antigen-spezifischen CD8 T Zellen zurückgeführt werden. Des Weiteren konnten wir aufzeigen, dass die antigen-spezifischen CD4 T Zellen hinsichtlich ihrer Proliferation und Zytokinproduktion beeinträchtigt waren, was mit einer verringerten Expression von MHC Klasse II Molekülen auf ssRNA-aktivierten Milz-DCs verbunden war. Diese Daten weisen deutlich darauf hin, dass die vorherige Gabe von ssRNA die Generierung der CD4 T Zell Hilfe verhindert, was zur Suppression einer antigen-spezifischen ZTL Antwort beiträgt. Die T Zell Antworten waren nicht beeinträchtigt, wenn die ssRNA durch Komplexierung in *in vivo*-jetPEI™ in das Zytoplasma gelenkt wurde, wodurch die Erkennung durch TLR7 umgangen wurde. Die TLR7-vermittelte Erkennung führte zur Produktion von Typ I IFN, die eine essentielle Rolle in der ssRNA-induzierten Suppression von ZTL Antworten spielten. Wir zeigten, dass plasmazytoide dendritische Zellen (pDCs) unmittelbar auf die Stimulation mit ssRNA reagierten, indem sie „frühes" Typ I IFN ausschütteten. Basierend auf unseren Experimenten in IFNAR1 defizienten, STAT1 defizienten und IRF7 defizienten Mäusen nehmen wir an, dass das ssRNA-induzierte „frühe" Typ I IFN durch den JAK-STAT Signalweg in Milz-Makrophagen amplifiziert wird. Die daraus resultieren Mengen an

sezerniertem IFNα beeinträchtigen Milz-DCs in ihrer Funktion, antigen-spezifische CD8 T Zellen zu aktivieren (cross-priming), was letztendlich zum Fehlen einer antigen-spezifischen ZTL Antwort beiträgt.

Eine klinische Bedeutung der RNA-vermittelten Immunsuppression wurde anhand eines Modells zur kombinatorischen Anti-Tumor Therapie gezeigt, in dem wir die Verwendung von „small-interfering RNA" (siRNA) und die Induktion einer tumor-spezifischen ZTL Antwort nachahmten. Wir konnten zeigen, dass die systemische *in vivo* Applikation von siRNA, abhängig vom verwendeten Komplexierungsreagenz, eine Adenovirus-basierte Tumorvakzinierung beeinträchtigte, indem die Induktion einer tumor-spezifischen ZTL Antwort verhindert wurde.

Des Weiteren konnten wir aufzeigen, dass die Suppression einer ZTL Antwort nicht nur durch die systemische Gabe von synthetischen RNA Oligonukleotiden erfolgt. Vielmehr führte auch eine Infektion mit Influenza Virus dazu, dass die Generierung einer ZTL Antwort gegen eine nachfolgende Adenovirusinfektion aufgrund der Produktion von Typ I IFN verhindert wurde. Diese Beobachtung weist darauf hin, dass Typ I IFN-induzierende Viren eine adaptive Immunantwort in einer ähnlichen Art und Weise, wie wir sie für systemische ssRNA beobachtet haben, unterdrücken können.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass ssRNA- als auch Virus-induziertes Typ I IFN nicht nur antigen-spezifische T Zellantworten in der Milz stimulieren, sondern auch hemmen kann. Darüber hinaus weisen wir darauf hin, dass eine systemische *in vivo* Behandlung mit siRNA, z.B. in Tumortherapien, zu ungewünschten „Off-target" Effekten, wie Immunsuppression, führen kann. Aufgrund dieser Daten ist es unerlässlich, die therapeutische Anwendung von siRNA sorgfältig zu überdenken, um unerwünschte Nebenwirkungen zu vermeiden.

1   Introduction

1.1   The immune system

Vertebrates are constantly threatened by the invasion of microorganisms, such as viruses, bacteria, or parasites and have evolved multiple mechanisms of immune defense, which ensure the elimination of infective pathogens in the body. The mammalian immune system is comprised of two branches:

(1) The innate immune system is the first line of host defense against intruding pathogens.

(2) The aquired (adaptive) immune system, which is characterized by high specificity, is involved in the elimination of pathogens in the late phase of infection and generation of immunological memory.

1.1.1   The innate immune system

The innate immune system is an evolutionary conserved (Hoffmann et al., 1999) and ancient form of host defense that serves to limit infection in the first minutes to hours after exposure to microorganisms. The major components of the innate immune system include:

   (1) Physical epithelial barriers, such as the skin as well as the mucosal epithelia of the respiratory, gastrointestinal, and reproductive tracts, prevent the intrusion of microbes into the organism.

   (2) Phagocytes, such as macrophages and neutrophils, ingest and eliminate microbes from the blood or tissue.

   (3) Soluble molecules, such as antimicrobial peptides (e.g. defensins), complement, acute-phase proteins, chemokines, and cytokines are released by a variety of cells and contribute to innate immune protection as well as initiation of adaptive immunity.

Central to host protection – according to the hypothesis postulated by Charles Janeway in 1989 - is the ability to discriminate between "noninfectious self" and "infectious nonself" in order to initiate appropriate defense responses (Janeway, 1989, 1992; Medzhitov and Janeway, 2002). The innate immune system recognizes microorganisms via a limited number of germline-encoded, nonclonal, and broadly expressed pattern-recognition receptors (PRRs). PRRs sense conserved microbial pathogen-associated molecular patterns

(PAMPs), which are unique to microbes and absent from the host. Furthermore, PAMPs are often essential for the survival and pathogenicity of the microorganism, thus limiting the ability of the microorganism to evade innate immune recognition through mutation of these molecules (Akira et al., 2006; Janeway and Medzhitov, 2002; Medzhitov, 2007; Medzhitov and Janeway, 2000; Medzhitov and Janeway, 1997, 2002; Palm and Medzhitov, 2009). Stimulation of PRRs, mainly expressed on professional antigen-presenting cells (APCs), results in APC activation, which subsequently leads to T cell priming and initiation of acquired immunity. Critical mediators of this process are dendritic cells (DCs), which are specialized in their function to translate innate recognition into adaptive immunity (Banchereau and Steinman, 1998; Iwasaki and Medzhitov, 2004; Reis e Sousa, 2001, 2004). Thus, the innate and adaptive immune systems do not act independently, but these two branches interact at several points to ensure an optimal protection of the host.

1.1.2   The adaptive immune system

The adaptive immune response is divided into a B lymphocyte-mediated humoral and a T lymphocyte-mediated cellular immune response. The main distinction between the innate and adaptive immune system lies in the mechanisms and receptors used for immune recognition. The T cell receptor (TCR) and the B cell receptor (BCR) are not encoded in the germline but are generated somatically during the development of T and B cells. This process, which is called somatic recombination, is characterized by the rearrangement of genes encoding for the receptor, which finally endows every cell with a structurally unique receptor. In that way, an extremely diverse repertoire of receptors is provided, which allows the recognition of a nearly infinite number of antigens (Goldrath and Bevan, 1999; Medzhitov and Janeway, 2000; Thompson, 1995). Extracellular pathogens and their toxins as well as soluble proteins are targets of the humoral immune response mediated by antibodies, the secreted form of the BCR. In contrast, infections with intracellular pathogens are cleared by T cells, which are the mediators of the cell-mediated immune response. Different from the BCR, which recognizes foreign antigens as such, the TCR only recognizes processed antigenic peptides that are presented on major histocompatibility molecules (MHC) expressed by APCs. Upon activation, B and T lymphocytes proliferate and differentiate into effector cells, which clear the present infection. Importantly, the adaptive immune response is involved in the generation

of long-lived immunological memory, which ensures an immediate and strong antigen-specific immune response upon re-encounter with the pathogen.

However, cells of the adaptive immune system cannot reliably discriminate between infectious microorganisms and innocuous environmental antigens or self-antigens due to their random receptor specificities. Activation of the adaptive immune system can be deleterious to the host when antigens are self or environmental antigens, leading to autoimmune diseases and allergies. Therefore, adaptive immune responses must be educated and tightly controlled by the innate immune system, which is able to sense intruding microbes via the detection of PAMPs by PRRs expressed on APCs. In that way, an adaptive immune response is only initiated in case of a pathogenic infection (Medzhitov and Janeway, 2000; Medzhitov and Janeway, 1998; Palm and Medzhitov, 2009).

1.2 Pathogen recognition via pattern-recognition receptors

The innate immune system uses a variety of pattern-recognition receptors (PRRs), which are specialized to detect conserved structures of pathogens. PRR-expressing cells are primarily professional APCs, such as DCs, macrophages, and B cells. Upon stimulation of the respective PRR, APCs mature and immediately perform their effector functions, namely secretion of pro-inflammatory cytokines and chemokines, increased expression of surface molecules as well as activation of adaptive immune response. PRRs are expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. Secreted PRRs function as opsonins by binding microbial cell walls and flagging them for recognition by the complement system and phagocytes (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2000). The best-characterized secreted form of PRRs is the mannan-binding lectin, which binds microbial carbohydrates to initiate the lectin-pathway of the complement cascade (Epstein et al., 1996; Fraser et al., 1998). Endocytic PRRs expressed on the cell surface are involved in the uptake and delivery of the pathogen into the lysosome. Examples of endocytic PRRs are the mannose receptor and the scavenger receptor, both expressed by macrophages, which are essential for the clearance of bacteria from the circulation (Fraser et al., 1998; Pearson, 1996; Thomas et al., 2000). Intracellular PRRs are present in endosomal compartments and the cytoplasm. Examples of intracellular PRRs are the retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated gene 5 (MDA5). Both are expressed in the cytoplasm

and are specialized for the recognition of double-stranded RNA viruses (Kato et al., 2006; Yoneyama et al., 2004). Intracellular bacteria can be detected by the nucleotide-binding oligomerization domains (Nod1, Nod2), which sense bacterial cell wall components (Fritz et al., 2007; Kaparakis et al., 2007; van Beelen et al., 2007). The most familiar PRRs are the family of Toll-like receptors (TLRs), which play an essential role in the activation of host immunity.

1.2.1   Toll-like receptors

TLRs are evolutionary conserved from the worm *Caenorhabditis elegans* to mammals (Akira and Takeda, 2004; Akira et al., 2006; Janeway and Medzhitov, 2002). *Toll*, the founding member of the TLR familiy, was initially identified as a component of a signaling pathway that controls dorsoventral polarity in *Drosophila* embryos (Hashimoto et al., 1988). Later studies performed in *Toll*-deficient mutants demonstrated that *Toll* is critically involed in antifungal responses of flies (Lemaitre et al., 1996). TLRs are type I membrane glycoproteins with a trimodular structure, which are characterized by an extracellular domain containing varying numbers of leucine-rich-repeat motifs that are responsible for the detection of PAMPs and a cytoplasmic domain homologous to the cytoplasmic region of the IL-1 receptor, known as the Toll/IL-1 receptor (TIR) domain. This intracellular domain is required for the interaction and recruitment of various adaptor molecules to activate the downstream signaling pathway (Kawai and Akira, 2007; Kumar et al., 2009). TLRs are expressed on various immune cells, including DCs, macrophages, and B cells. But expression can vary depending on the activation status or the cell subset. TLR expression has been even identified on non-immune cells, such as fibroblasts and epithelial cells (Akira et al., 2006; Iwasaki and Medzhitov, 2004). To date, 11 TLRs in humans and 13 TLRs in mice have been identified, with each receptor recognizing distinct PAMPs derived from various pathogens, including bacteria, viruses, protozoa, and fungi. TLRs are expressed in distinct cellular compartments: TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 (only found in mice) are expressed on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are located in the endosome (Figure 1.1).

1.2.2 Toll-like receptor ligands

TLRs can be classified into several groups based on the types of PAMPs - also known as TLR ligands - they recognize (Figure 1.1).

TLRs sense mainly components of the bacterial cell wall and nucleic acids expressed by microbes. They dimerize to homo- or heterodimers upon ligation. TLR4, together with its coreceptors MD-2 and CD14, recognizes lipopolysaccharide (LPS) from gram-negative bacteria (Lien et al., 2000; Poltorak et al., 1998). TLR2 forms heterodimers with TLR1, TLR6, and non-TLRs such as CD36 to discriminate a wide variety of TLR ligands, including peptidoglycan, lipopeptides, and lipoproteins of gram-positive bacteria, mycoplasma lipopeptides and fungal zymosan. In particular, TLR1/2 and TLR2/6 are able to discriminate triacyl- and diacyl-lipopeptide, respectively (Akira et al., 2006). Flagellin from flagellated bacteria is recognized by TLR5 (Hayashi et al., 2001), whereas mouse TLR11 senses yet unknown structures of uropathogenic bacteria (Zhang et al., 2004) and profilin-like protein of the protozoan parasite *Toxoplasma gondii* (Yarovinsky et al., 2005).
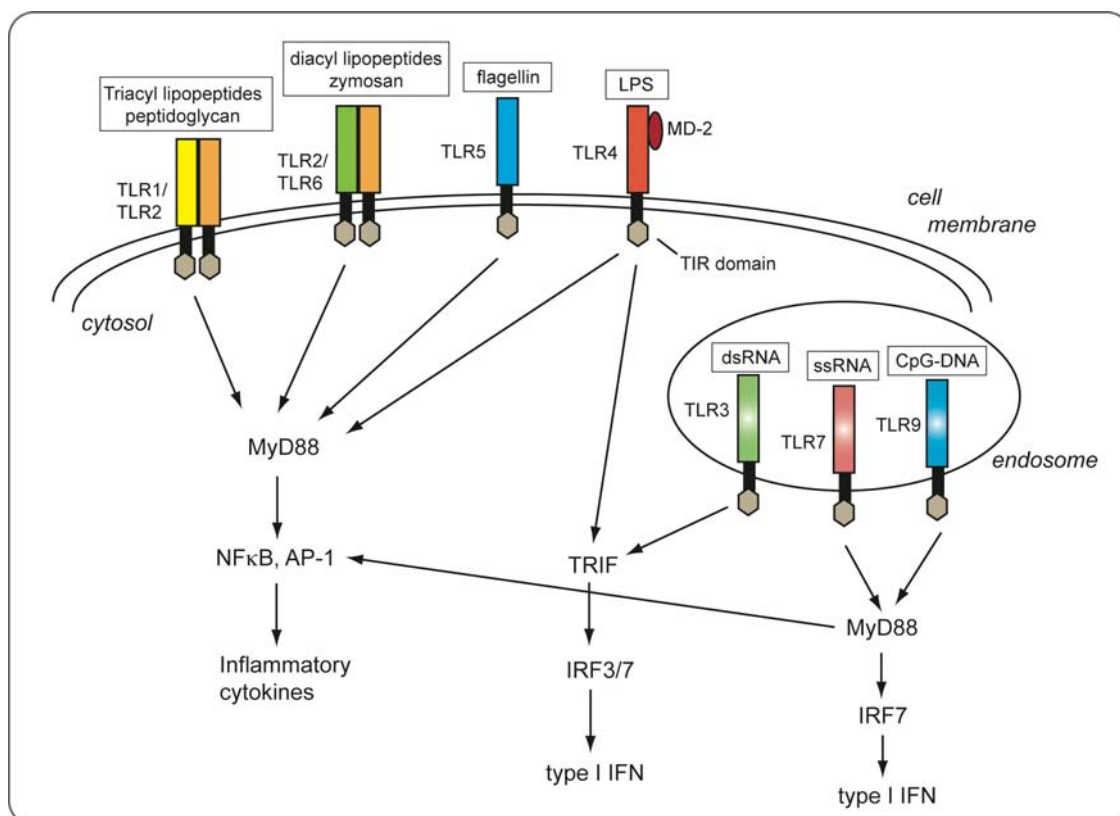
Figure 1.1   Toll-like receptors and their ligands.
Toll-like receptors (TLRs) are expressed on the cell surface or in intracellular compartments, such as the endosome. TLRs are specialized to recognize distinct conserved pathogen-associated molecular patterns (PAMPs), which are also known as TLR ligands. Upon TLR stimulation, intracellular adaptor molecules are recruited and intracellular signaling pathways are initiated that activate NFκB and IRF dependent target genes resulting in the expression of proinflammatory cytokines and type I interferons (IFN).

Intracellular TLRs, expressed in the endosome, are involved in the recognition of bacterial- and viral-derived nucleic acids. TLR3 recognizes double-stranded RNA (dsRNA), which is generated during replication of many viruses. PolyI:C is a synthtetic ligand of TLR3 (Alexopoulou et al., 2001). TLR7 senses synthetic imidazoquinoline-like molecules, guanosine analogues such as loxoribine, single-stranded RNA (ssRNA), and small interfering RNA (siRNA) (Diebold et al., 2004; Heil et al., 2004; Hornung et al., 2005). Human TLR8, with highest homology to TLR7, participates in the detection of imidazoquinolines and ssRNA, whereas in mice the function and ligands of TLR8 remain elusive. TLR9 is responsible for the recognition of CpG-DNA motifs present in bacterial and viral genomes (Hemmi et al., 2000). Furthermore, it was shown to sense non-nucleic acid structures, such as hemozoin from the malaria parasite (Coban et al., 2005).

TLRs as PRRs are critically involved in the discrimination between "self" and "non-self". However, it has been shown that TLRs, especially TLR4, TLR7/8, and TLR9 are able to sense endogenous ligands. TLR4 recognizes heat shock protein 60 (HSP60) and fibronectin (Ohashi et al., 2000; Okamura et al., 2001), whereas immunocomplexed "self-RNA" or "self-DNA" can act as agonists for TLR7/8 or TLR9, respectively (Barrat et al., 2005). These findings support the "danger" model postulated by Polly Matzinger (Barrat et al., 2005; Matzinger, 1994, 2002). According to her theory, the immune system does not solely tend to discriminate between "self" and "foreign", but is rather activated by "danger" signals derived from damaged and stressed tissue. Thus, stimulation of TLRs by endogenous ligands may activate the immune system under certain conditions, e.g. stress, and promote the development of autoimmiune diseases.

1.2.3   TLR signaling

Upon binding of PAMPs, TLRs undergo conformational changes and a set of intracellular TIR-domain-containing adaptors, including MyD88, TIRAP (also known as MAL), TRIF (also known as TICAM1), and TRAM (also known as TICAM2), are recruited via TIR-TIR interactions. MyD88 is a universal adaptor that is shared by all TLRs except for TLR3 (Akira and Takeda, 2004). TLR1, TLR2, TLR4, and TLR6 recruit TIRAP, which serves as a linker molecule between the TIR domain of TLRs and MyD88. TRIF is recruited to TLR3 and TLR4, where TLR4 uses TRAM as a linker molecule to bind TRIF.

Stimulation of the MyD88-dependent pathway used by all TLRs except for TLR3 leads to the recruitment of MyD88 and IRAK familiy of protein kinases, which results in the activation of TRAF6. TRAF6 induces the activation of TAK1, which in turn results in the activation of NFκB and AP-1 through the IKK complex and the MAP kinase pathway (Figure 1.1, simplified illustration).

The TRIF-dependent pathway, which is initiated by TLR3 and TLR4, activates NFκB via two independent pathways. The N-terminal domain of TRIF interacts with TRAF6, whereas the C-terminal domain interacts with RIP1. Activated TRAF6 and RIP1 subsequently activate NFκB and MAP kinases via TAK1. Type I interferon (IFN) production is induced by interaction of TRIF with TRAF3, which in turn activates the IKK-related kinase, TBK1 and IKKi. In consequence, the transcription factors IRF3 and IRF7 are activated, translocate into the nucleus, and induce the transcription of type I IFNs (Figure 1.1, simplified illustration).

Stimulation of TLR7 and TLR9 are able to activate NFκB and MAP kinases via the MyD88-dependent pathway resulting in the production of inflammatory cytokines. To trigger the production of type I IFNs, MyD88 associates with the IRAK family of proteins, where IRAK1 and IKKα activate IRF7. IRAK1 also binds to TRAF3 leading to the activation of IRF7 (Figure 1.1, simplified illustration) (Akira and Takeda, 2004; Kawai and Akira, 2007; Kumar et al., 2009).

Taken together, stimulation of TLRs by their respective TLR ligand results in the activation of intracellular signaling pathways that trigger the induction of proinflammatory cytokines, such as TNFα, IL-6, IL-12, and IL-1β. Furthermore, several TLRs are capable to induce type I IFNs. TLR signaling also initiates the upregulation of costimulatory molecules on APCs, an essential step for the induction of adaptive immune responses.

1.3   T cell activation by APCs

All T cells originate from hematopoietic stem cells in the bone marrow. Progenitors derived from hematopoietic stem cells populate the thymus and develop into immature thymocytes. In the thymus, thymocytes undergo developmental processes known as positive and negative selection. Only those T cells leave the thymus, which are able to recognize "self-MHC molecules" (positive selection) and which do not react with "self-peptides" presented on MHC (negative selection). About 98% of thymocytes die during the

development processes in the thymus by failing either positive selection or negative selection, whereas only 2% survive and leave the thymus as naive CD4 or CD8 T cells (Schwarz and Bhandoola, 2006; von Boehmer and Kisielow, 1990).

Naive T cells populate the T cell areas of secondary lymphoid organs, such as the lymph nodes or the spleen, until they are activated by mature APCs presenting the TCR-specific peptide. The most potent APCs are DCs, which are sparsely but widely distributed cells specialized in antigen capture, transport, processing, and presentation. Immature DCs act as sentinel cells in peripheral tissues, continuously sampling the environment. Upon encounter with a pathogenic stimulus, e.g. recognition of PAMPs by PRRs, DCs undergo a maturation process that is characterized by phenotypical and functional changes of the DC. Following activation, DCs loose their ability to phagocytose and process antigen from the environment, while they upregulate the surface expression of MHC molecules and the costimulatory molecules CD80, CD86, as well as CD40, important signals required to activate T cells (Banchereau and Steinman, 1998). In addition, DCs migrate towards secondary lymphoid organs. DC migration is mediated by the downregulation of chemokine receptors (CCRs) involved in the homing to peripheral tissues, whereas CCR7 is upregulated facilitating the migration and entry into T cell areas of the secondary lymphoid tissues, where the CCR7 ligands CC-chemokine ligand 19 (CCL19) and CCL21 are expressed (Mebius and Kraal, 2005; Sallusto et al., 1998; Sozzani et al., 1998). Intimate contact between antigen-presenting DCs and naive T cells in the T cell areas of the spleen and lymph nodes plays a central role in the induction of adaptive immunity.

1.3.1   Antigen presentation on MHC molecules

T cells expressing the αβ TCR are incapable to recognize foreign antigens as such. Instead T cells only recognize their antigen, if it is processed and presented as antigenic peptides on MHC molecules on the cell surface. There exist two different classes of MHC molecules involved in antigen presentation, namely MHC class I and MHC class II (Germain, 1994).

MHC class I molecules are expressed on all nucleated cells and function mostly in the presentation of endogenous peptides to antigen-specific cytotoxic T cells expressing the CD8 co-receptor. Intracellular cytosolic proteins are degraded into peptides by the proteasome present in all cells. Recognition of MHC class I-presented peptides by

cytotoxic CD8 T cells is a crucial mechanism in viral defense, as viral-derived peptides are presented on MHC class I molecules upon intracellular virus replication. In this way, activated antigen-specific cytotoxic CD8 T cells are able to lyse and eliminate infected cells (Wong and Pamer, 2003).

In contrast, MHC class II molecules are exclusively expressed on professional APCs, such as DCs, macrophages, and B cells, and present exogenous derived antigenic peptides to CD4 positive T cells. Exogenous antigens are internalized by APCs and shuttled into endosomes or phagosomes, respectively. Upon fusion with lysosomes, uptaken antigen is enzymatically degraded and loaded onto MHC class II molecules, which are recruited to the lysosome from the endoplasmatic reticulum (ER). Peptide-loaded MHC class II molecules translocate to the cell surface, where they present the antigenic peptide to antigen-specific CD4 T cells (Wolf and Ploegh, 1995).

Activation of T cells can only be achieved by APCs. However, APCs are not always infected themselves. Therefore presentation of exogenous antigens on MHC class I molecules, a process called cross-presentation, is important for mounting antigen-specific CD8 T cell immunity. APCs acquire antigen for cross-presentation by taking up soluble antigens or cell-associated antigens derived from apoptotic or virally infected cells. Internalized antigens are shuttled into the cytoplasm, processed via the proteasome, loaded onto MHC class I molecules, and presented to cytotoxic CD8 T cells (Bevan, 1976, 2006; Heath et al., 2004; Heath and Carbone, 2001). Moreover, cross-presentation of exogenous self-antigens contributes to the elimination of autoreactive CD8 T cells, a process known as cross-tolerance (Kurts et al., 1997).

1.3.2 T cell activation and differentiation into effector cells

T cell activation follows a so-called "two signal" model provided by mature APCs (Janeway and Bottomly, 1994). According to this theory, signal 1 is delivered by the TCR recognizing the cognate antigen presented on MHC molecules. Signal 1 is not sufficient to induce the development into effector T cells. Naive T cells receiving signal 1 only become anergic, meaning they are inactive and unresponsive (Schwartz, 1996, 1997). T cells require a second signal, which is provided by mature APCs, mainly DCs, expressing the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) (Collins et al., 2005). CD80/86 expression is upregulated upon DC maturation and binds to CD28 expressed by naive

T cells. Stimulation of the TCR together with CD28 induces an intracellular signaling cascade that leads to clonal expansion and differentiation of T cells (Acuto and Michel, 2003; Shaw and Dustin, 1997; Viola and Lanzavecchia, 1996). In addition, signaling via CD28 induces the production of proinflammatory cytokines, such as IL-2, and expression of CD25, the α-chain of the IL-2 receptor (Acuto and Michel, 2003). IL-2 acts in an autocrine manner and promotes the proliferation of T cells (Malek, 2008). Furthermore, costimulation via CD28 augments T cell survival through the induction of Bcl-X_L (Acuto and Michel, 2003; Boise et al., 1995).

Full activation of CD8 T cells and development of effector functions requires a third signal in addition to TCR engagement (signal 1) and costimulation (signal 2). Signal 3 is provided by IL-12 or type I IFNs (Cousens et al., 1999; Curtsinger et al., 2003b; Curtsinger et al., 1999; Curtsinger et al., 2005), which is produced by DCs upon TLR stimulation or CD40 ligation. CD40 expressed by matured DCs interacts with CD40L, which is expressed on activated CD4 T helper cells. In that way, CD4 T cell help licenses the DC to stimulate an efficient and long-lasting cytotoxic CD8 T cell response (Bennett et al., 1998; Schoenberger et al., 1998; Toes et al., 1998). Moreover, CD4 T cell help is crucial for the generation of long-lived and functional CD8 T cell memory (Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003; Williams and Bevan, 2007).

T cells differentiate into CD4 or CD8 T effector cells upon priming by mature APCs. CD4 T cells develop into T helper cells (T_H), which are subdivided into T_H1, T_H2, and T_H17 cells (Bettelli et al., 2008; Moser and Murphy, 2000). T_H1 cells produce mainly IFNγ upon IL-12 secretion by DCs and are essential for the protection against a variety of intracellular infections. T_H2 responses are characterized by the production of IL-4 and are directed against certain extracellular pathogens (Moser and Murphy, 2000). Recently identified T_H17 cells synthesize large quantities of IL-17 and play a role in the clearance of extracellular pathogens, which have not been efficiently eliminated by T_H1 or T_H2 cells. Furthermore, they are potent inducers of autoimmunity and tissue inflammation (Bettelli et al., 2008). As already mentioned before, CD4 T helper cells are critically involved in providing CD4 T cell help to DCs (Bevan, 2004; Castellino and Germain, 2006; Williams and Bevan, 2007).

CD8 T cells differentiate into cytotoxic T lymphocytes (CTL), which recognize their specific peptide presented on MHC class I molecules of infected cells in the tissue. CTLs induce apoptosis of infected cells by three distinct pathways, namely (1) the granule-dependent exocytosis pathway, (2) the Fas-FasL intercellular linkage-mediated pathway,

and (3) cross-linking of TNF and TNFR type I. In the granule-dependent exocytosis pathway granules or lysosomes are formed within the CTL. Upon contact with the infected target cell, stored lytic molecules such as perforin, granzymes, and granulysin are released. Perforins penetrate the target cell membrane leading to the formation of pores, through which granzymes and granulysin enter the cell to induce apoptosis. In addition, CTLs express Fas ligand (FasL; CD178), whereas target cells express Fas receptor (CD95; Apo-1). Binding of Fas to FasL induces trimerization and the formation of the death-inducing signaling complex (DISC), which transduces a downstream signaling cascade resulting in apoptosis. Binding of TNFα, secreted by activated cells, to the TNF receptor type I results in receptor multimerization and formation of DISC, which in consequence activates the apoptosis cascade (Chavez-Galan et al., 2009). CTLs secrete additionally effector cytokines such as IFNγ, which contribute to the elimination of pathogens.

1.3.3   T cell memory

The adaptive immune system is able to generate a long-lived immunological memory. Memory T cells mediate rapid and strong recall responses upon re-encounter with a given pathogen, which provides an enhanced protection of the host (Sprent and Surh, 2001, 2002).

Upon clearance of the pathogen during the effector phase (comprising 1-2 weeks post infection), the majority (90-95%) of effector cells die. During this contraction phase, a pool of memory T cells is left behind, which are stably maintained in the absence of antigen for a long time. One can differentiate between two different subtypes of memory T cells: Central memory (T_{CM}) and effector memory (T_{EM}) T cells, which can be distinguished by their phenotype and function. Generally, T_{CM} cells reside in the lymphoid organs, do not exert immediate effector functions, express high levels of CD62L and are positive for CCR7. In contrast, T_{EM} cells are found in non-lymphoid tissues, display lytic activity, express low levels of CD62L, and are CCR7 negative (Williams and Bevan, 2007). It is controversially discussed whether T_{EM} and T_{CM} cells represent interconnected or distinct lineages. Three models of differentiation have been proposed, with the first being that T_{CM} cells provide a continuous source of T_{EM} cells (Lanzavecchia and Sallusto, 2000; Sallusto et al., 1999). The second model proposes that T_{CM} and T_{EM} cells represent mostly separate lineages (Baron et al., 2003). In contrast, an alternative model indicates that T_{EM} cells

convert into T_{CM} cells over time (Wherry et al., 2003). In a study by Marzo et al it has been reported, that the commitment to a particular memory cell lineage was governed by the initial T cell precursor frequency and clonal competition (Marzo et al., 2005).

1.4   Type I interferons

Type I interferon (IFN) was first discovered by Isaacs and Lindenmann in 1957 and derives its name from a function to "interfere" in viral replication (Isaacs and Lindenmann, 1957). In mammals the type I IFN genes form a large multigene family comprising the species α, β, κ, ω, and τ, with IFNα and IFNβ being the best studied ones. In mice, there exist at least 12 different isoforms of IFNα and one IFNβ (Kelley and Pitha, 1985a, b). IFNα/β signal via the type I IFN receptor complex, which consists of at least two subunits, IFNAR1 and IFNAR2 (Stark et al., 1998). Type I IFN production is triggered upon recognition of viral and non-viral nucleic acids by certain PRRs, such as TLR7 and TLR3 as well as MDA-5 and RIG-I (Uematsu and Akira, 2007; Yoneyama et al., 2005). In addition to their key role in antiviral immunity, type I IFNs enhance DC maturation and promote the activation as well as differentiation of CD8 T cells by providing signal 3 (Curtsinger et al., 2005; Le Bon et al., 2003; Luft et al., 1998; Montoya et al., 2002). Furthermore, type I IFNs can elicit direct effects on CD8 T cells by promoting their survival and IFNγ production during viral infections (Cousens et al., 1999; Marrack et al., 1999; Nguyen et al., 2002). Thus, type I IFNs are crucially involved in linking the innate and adaptive immune response (Uematsu and Akira, 2007). However, it has also been shown that type I IFNs can inhibit the production of IL-12 by DCs (Cousens et al., 1997) and interfere with T_H1 cell differentiation (McRae et al., 1998).

Almost all cells are capable to produce type I IFN upon viral infection. PRR stimulation results in the secretion of immediate-early type I IFN, especially IFNβ and IFNα4, whose transcription is promoted by interferon regulatory factor 3 (IRF-3), which is constitutively expressed in most cell types. Rapid but low-level synthesis of IFNβ and IFNα4 initiates a positive feedback loop via paracrine or autocrine stimulation of the JAK-STAT pathway through the type I IFN receptor. The IFNAR1 and IFNAR2 are permanently associated with members of the Janus protein tyrosine kinase family, specifically with tyrosine kinase 2 (Tyk2) and Jak1, respectively. Ligand binding stimulates the activation of the Janus kinases and the phosphorylation of the receptor complex. This creates the

phosphotyrosine-based motif, which is required for the recruitment of STAT1 and STAT2. Phosphorylation of STAT1 and STAT2 by the Janus kinases, results in their heterodimerization. The STAT1-STAT2 heterdimer translocates into the nucleus and associates with IRF9 to form a transcription factor complex, known as IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to the IFN-stimulated response element (ISRE) promoter sequence and induces the transcription of IRF7. In turn, IRF7 binds to the IRF-binding site (IRFBS) and stimulates the delayed production of different IFNα subtypes. Thus, IRF7 is a key factor in the positive feedback regulation of IFNα production, thereby boosting IFN secretion (Figure 1.2) (Decker et al., 2005; Levy et al., 2003; Marie et al., 1998; Sato et al., 1998; Taniguchi and Takaoka, 2001).
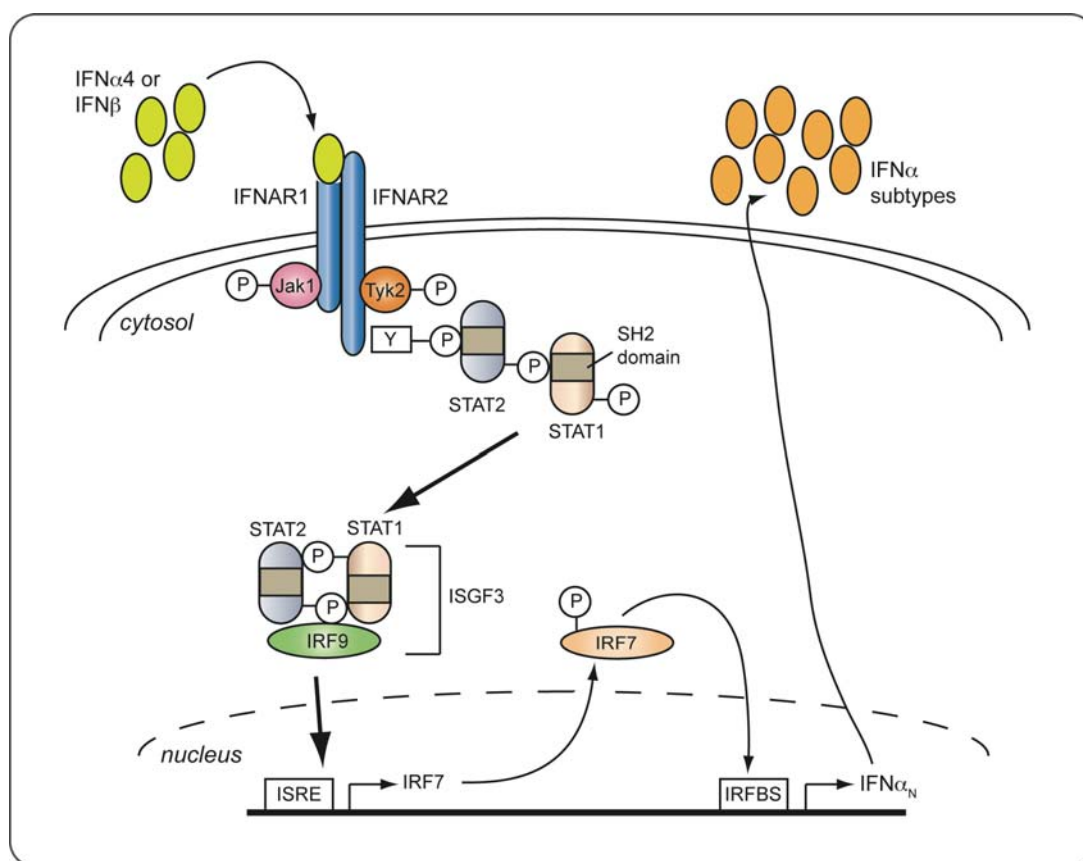
Figure 1.2   Amplification of the type I interferon response via the type I interferon receptor and the JAK-STAT pathway.
(adapted from Taniguchi and Takaoka, 2001)

In contrast, plasmacytoid DCs (pDCs) are capable to immediately secrete vast amounts of type I IFN in response to TLR stimulation (especially TLR7 and 9 stimulation) independent of the positive amplification loop (Asselin-Paturel et al., 2001; Asselin-Paturel and Trinchieri, 2005; Barchet et al., 2005a; Decker et al., 2005). Rapid IFNα production by pDCs is ascribed to a constitutive expression of the key transcription factor IRF7

(Coccia et al., 2004; Dai et al., 2004; Honda et al., 2005; Kawai et al., 2004). Mouse pDCs are characterized by CD11c intermediate, PDCA-1 positive, B220 positive, and CD11b negative expression.

1.5   The spleen

The spleen is a secondary lymphoid organ that is characterized by highly organized and specialized structures. It functions as a blood filter that removes senescent erythrocytes from the circulation and it is essential for the induction of innate and adaptive immune responses against blood-borne pathogens. The unique architecture of the spleen comprises two functionally and morphologically distinct compartments, the red pulp and the white pulp, which are spatially separated by the marginal zone (Mebius and Kraal, 2005).

A functional spleen is important as splenectomized patients are impaired in their ability to cope with several infections and most often are highly susceptible to bacterial infections with *Streptococcus pneumoniae, Neisseria meningitides*, or *Haemophilus influenzae*. Furthermore, a diminished responsiveness to certain vaccines has been observed (Jia and Pamer, 2009; Zandvoort and Timens, 2002).

1.5.1   The red pulp

The red pulp functions as a blood filter that removes cellular debris and senescent red blood cells. It is also a storage site for iron, erythrocytes, and platelets. The red pulp is composed of a three dimensional meshwork of splenic cords and venous sinuses allowing the spleen to efficiently perform its filter function. Arterial blood flows into the splenic cords, which consist of reticular fibers, fibroblasts, and associated macrophages. Red pulp macrophages are actively phagocytic and take up aging and damaged erythrocytes, which are incapable to pass from the cords into the sinuses due to their stiffening membranes. Following erythrophagocytosis, erythrocytes are degraded and iron is recycled (Cesta, 2006; Mebius and Kraal, 2005).

1.5.2   The white pulp

The white pulp constitutes the lymphoid compartment of the spleen, which presumably contains one-fourth of the body's lymphocytes, and is thus involved in the induction of adaptive immunity (Cesta, 2006). The white pulp is organized by lymphoid sheaths, with T- and B-cell areas, also known as periarteriolar lymphoid sheaths (PALS) or follicles, respectively. The T- and B-cell areas are found around the branching arterial vessels and resemble the structure of a lymph node. The correct architecture and maintainance of the white pulp is controlled by specific chemokines that attract T and B cells to their respective sites, therby establishing distinct zones within the white pulp (Mebius and Kraal, 2005). The CXC-chemokine ligand 13 (CXCL13) is required for B cells to migrate to B cell follicles (Ansel et al., 2000; Cyster et al., 1999), whereas CCL19 and CCL21 contribute to the recruitment of T cells and DCs to the T cell zones (Forster et al., 1999; Gunn et al., 1999). In the T cell zone, T cells interact with DCs and passing B cells, while clonal expansion of activated B cells occurs in the B cell follicles, which leads to isotype switching and somatic hypermutation (Mebius and Kraal, 2005). All cells that enter the white pulp have to pass the marginal zone, which surrounds the white pulp and separates it from the red pulp. As no erythrocytes and few granulocytes are present in the white pulp, it is believed that the entry into the white pulp is an active process, which involves signaling through G-protein coupled receptors (Cyster and Goodnow, 1995), chemokines and cell-cell interactions (Johnston and Butcher, 2002; Kraal and Mebius, 2006).

1.5.3   The marginal zone

The marginal zone is involved in the induction of an innate and adaptive immune response. In addition to being a transit area, the marginal zone harbours many different cell types of lymphoid and myeloid origin. These cells not only have unique properties but also seem to depend on each other for their localization, thereby establishing and maintaing the integrity of the marginal zone (Mebius and Kraal, 2005). The thicker outer ring of the marginal zone (adjacent to the red pulp) is composed of marginal zone macrophages, reticular fibroblasts, DCs, and marginal zone B cells. The marginal sinus, which is lined by endothelial cells, separates the outer ring from the inner ring of the marginal zone. The inner ring consists of marginal zone metallophilic macrophages surrounding the white pulp. Marginal zone macrophages and marginal zone metallophilic macrophages can be distinguished by the

expression of different surface molecules. Metallophilic macrophages are characterized by the expression of sialic acid-binding immunoglobulin-like lectin-1 (Siglec-1, CD169, sialoadhesin) (Crocker and Gordon, 1989; Kraal and Janse, 1986), which serves a phagocytic receptor for sialylated pathogens such as *Neisseria meningitides* (Jones et al., 2003). Marginal zone macrophages can be identified by the expression of the C-type lectin SIGNR1 mediating binding to polysaccharides on pathogens such as *Streptococcus pneumoniae* (Geijtenbeek et al., 2002; Kang et al., 2004; Lanoue et al., 2004). The type I scavenger receptor MARCO (Kraal et al., 2000), which is expressed on both subsets, recognizes a broad range of antigens, like acetylated LDL, apoptotic cells, and bacteria, such as *Staphylococcus aureus* or *Neisseria meningitides* (Pluddemann et al., 2006; Pluddemann et al., 2007; van der Laan et al., 1999). Due to the expression of these receptors, macrophages in the marginal zone function in trapping and elimination of blood-borne microbes and cellular debris. Recently, it has been shown that marginal zone metallophilic macrophages are involved in the transfer of antigen to CD8 positive splenic DCs, thereby contributing to the initiation of adaptive immunity (Backer et al., in press).

1.5.4   Dendritic cells in the spleen

Most DCs of the spleen are derived from lymphoid origin and arise from local precursors without a monocytic intermediate (Varol et al., 2007). In contrast to macrophages, DCs are specialized APCs that hold a dominating role in inducing T cell responses. Murine splenic DCs can be divided into different subsets, based on their expression of phenotypic markers, localization, and function (Heath et al., 2004). CD8 positive DCs, characterized by the expression of DEC205, are found in the T cell zone and outer marginal zone (Idoyaga et al., 2009), whereas CD8 negative DCIR2 positive DCs are mainly localized in the marginal zone next to marginal zone B cells and marginal zone macrophages. CD8 positive DCs are specialized in cross-presentation of antigens and in the activation as well as tolerization of cytotoxic T lymphocytes (Belz et al., 2005; den Haan et al., 2000; Dudziak et al., 2007; Schnorrer et al., 2006). Moreover, they are important for the generation of anti-tumor specific immune responses and elimination of tumors *in vivo* (Hildner et al., 2008). In contrast, CD8 negative DCs preferentially activate CD4 T cells via MHC class II presentation (Dudziak et al., 2007). Upon activation, all DC (subsets)

migrate into the T cell zone, where they interact with antigen-specific T cells (De Smedt et al., 1996).

1.6 Virus-induced suppression of immune responses

It has been known for a long time that certain viral infections result in a temporary lack in cell-mediated immune responses, which is frequently ascribed to the effects of virus-induced type I IFNs (De Maeyer et al., 1975; De Maeyer-Guignard et al., 1975). Viral infections can elicit a negative impact on DCs. For instance, measles virus and lymphocytic choriomeningitis virus (LCMV) cause a profound immunosuppression by interfering with DC differentiation and expansion, which is dependent on type I IFN signaling (Hahm+Oldstone, Immunity, 2005). Furthermore, it has been reported that Herpes simplex virus (HSV) infection inhibits DC maturation, whereas a functional impairment of DCs is observed upon murine cytomegalovirus (MCMV) infection (Salio+Lanzavecchia, EJI, 1999; Andrews, NI, 2001). Moreover, apoptosis is induced upon Epstein-Barr virus (EBV) contact and DCs downregulate costimulatory molecules after LCMV infection (Li et al., 2002; Sevilla et al., 2004). However, not only DCs are the targets of virus-induced immunosuppression, as virus-induced type I IFNs are able to induce apoptosis in T cells and T cell proliferation is inhibited upon measles virus infection (Bahl et al., 2006; Hahm et al., 2003; Jiang et al., 2005; McNally et al., 2001; Sun et al., 1998). In these ways, viruses are able to interfere with the host's immune response, thereby enhancing their survival.

2 Aim of the thesis

Toll-like receptors (TLRs) are important members of germ-line encoded pattern-recognition receptors (PRRs) and recognize conserved microbial components, known as pathogen-associated molecular patterns (PAMPs). TLR stimulation triggers the production of proinflammatory cytokines, chemokines, and maturation of DCs that generally leads to initiation of adaptive immunity. Thus, TLRs have an immune stimulatory effect and are a crucial link between innate and adaptive immunity.

However, it has been reported by our group that a systemic application of CpG, a TLR9 ligand, inhibited subsequent antigen-specific T cell responses. In her PhD thesis, Beatrix Schumak showed that the suppression of cytotoxic T cell responses was not restricted to CpG, but rather also other systemically applied TLR ligands were capable to impede antigen-specific cytotoxic T cell responses. She revealed that the systemic injection of single-stranded RNA (ssRNA) suppressed antigen-specific cytotoxic T cell responses in a TLR7 and type I interferon dependent manner.

Based on these key findings, we aimed to further elucidate the underlying mechanisms of ssRNA- and type I interferon-induced inhibition of adaptive immune responses.

In this thesis, we were particularly interested in the following questions:

- How does systemic ssRNA and type I interferon influence antigen-specific cytotoxic T cell responses in the spleen?

- Does systemic ssRNA impair the expansion, activation, and cytokine production of CD4 and CD8 T cells?

- Is there a possibility to avoid ssRNA-induced immune suppression of CD4 and CD8 T cell responses?

- Which are the type I interferon producing and which are the responding cell types? Which signaling molecules downstream of the type I interferon receptor are involved?

- What is the influence of ssRNA stimulation on phenotypic and functional maturation of DCs? Are ssRNA pre-activated DCs impaired in cross-priming of antigen-specific CD8 T cells?

3 Material and Methods

3.1 Materials

3.1.1 General laboratory equipment

Autoclave	Belimed, Köln
AutoMACS	Miltenyi Biotec, Bergisch Gladbach
Balances	U4100-OD2.2 and MC BA 100 (Sartorius, Göttingen)
Beakers	5 ml, 10 ml, 25 ml, 50 ml, 100 ml, 150 ml, 250 ml, 500 ml (Schott, Mainz)
Centrifuges	Multifuge3 S-R, Biofuge fresco, Biofuge pico (Heraeus, Braunschweig)
Counting Chamber	Neubauer (Brand, Wertheim)
Digital camera	CCD-1300 (Vosskuehler, Osnabrück)
ELISA Reader	SpectraMax 250 (Molecular Devices, Sunnyvale, USA)
Erlenmeyer flasks	50 ml, 100 ml, 250 ml, 500 ml (Schott, Mainz)
Flow cytometers	FACSCalibur, CantoII, LSRII (BD Biosciences, Heidelberg)
Freezers (-20°C)	Liebherr, Biberach
Freezers (-80°C)	Hera freeze (Heraeus, Braunschweig)
Freezing container	Nalge Nunc Cryo (Nunc, Wiesbaden)
Heating block	ThermoStat plus (Eppendorf, Hamburg)
Ice machine	Icematic (Scotsman®, Frimont Bettolinc, Pogliano, Italy)
Incubators	HERAcell (Heraeus, Braunschweig)
IVIS®200	Xenogen, San Francisco, USA
Magnet stirrer	IKA® Laboratory Equipment, Staufen
Measuring cylinders	50 ml, 100 ml, 250 ml, 500 ml, 1l (Schott, Mainz)
Microscopes	IX71 and CKX31 (Olympus, Hamburg)
	Epifluorescence microscope Edipse TE 2000 (Nikon, Düsseldorf)
Microtiter plate washing - device	Nunc-Immuno^{TM} Wash12 (Nalge Nunc International, Thermo Fisher Scientific, Wiesbaden)
Microwave	Panasonic, Wiesbaden

pH-meter	pH 523 (Wissenschaftlich-Technische Werkstätten, Weilheim)
Pipette-Boy	Pipetus (Hirschmann Labortechnik, Eberstadt)
Pipettes	Gilson, Heidelberg and Eppendorf, Hamburg
Preparation Instruments	Labotec, Göttingen
Refrigerators (+4°C)	Bosch, Stuttgart and Liebherr, Biberach
Sieves, steel	University Bonn, Department "Feinmechanik"
Sonificator	UW2070/Sonoplus (Bandeln electronic, Berlin)
Spectrophotometers	Ultrospec 3100 UV/VIS (Amersham Pharmacia, Freiburg); NanoDrop™ ND 1000 (NanoDrop Products, Wilmington, USA)
Threaded bottles	100 ml, 250 ml, 500 ml, 1l, 2l (Schott, Mainz)
Ultra-pure water system	NANOpure Diamond, Barnstead (Werner, Reinstwassersysteme, Leverkusen)
Waterbath	TW8 (Julabo, Seelbach)
Workbench, sterile	HERAsafe (Heraeus, Hanau)

3.1.2   Software

Endnote X1	Thomson ISI ResearchSoft, USA
FACS Diva V6.1.1	BD Biosciences, Heidelberg
Flowjo V8.8.4	Tree star, Inc., USA
Illustrator CS4	Adobe, USA
Living Image 2.50 (IVIS)	Xenogen, USA
Microsoft Office 2008	Microsoft, USA
Photoshop CS4	Adobe, USA
Prism 4 for Macintosh	GraphPad Software, USA
SPF ELISA software	Molecular Devices, USA

3.1.3 Consumables

Cover slides	21x46mm (Marienfeld, Lauda-Königshofen)
Cryo vials	VWR International, Darmstadt
Cryomolds	VWR International, Darmstadt
ELISA plates	Microlon, 96-well, flat-bottom, high-binding (Greiner bio-one, Solingen)
FACS tubes	polystyrene, 12/75 mm (Sarstedt, Nümbrecht)
FlexiPERM	silicone cell culture chamber (Greiner bio-one, Solingen)
Injection needles	27G (grey), 25G (orange), 20G (yellow) (BD Microlance, Heidelberg)
Microtiter plates	96-well, round and flat bottom (Greiner bio-one, Solingen)
Nylon wool	Kisker GbR (by Labomedic GmbH, Bonn)
Parafilm	Parafilm "M"® (American National Can TM, Greenwich, USA)
Pasteur pipettes	150 mm and 230 mm (Roth, Karlsruhe)
Petri dishes	10 cm (Greiner bio-one, Solingen)
Pipette tips	10 µl, 200 µl, 1000 µl (Greiner bio-one, Solingen)
Plastic Pipettes	5 ml, 10 ml, 25 ml (Sarstedt, Nümbrecht)
Polyamide tissue	"Gaze" (Labomedic, Bonn)
Polypropylene tubes	sterile, 15 ml and 50 ml (Greiner bio-one, Solingen)
Reaction tubes	0.5 ml, 1.5 ml, 2 ml (Eppendorf, Hamburg)
Scalpel	Feather (Osaka, Japan)
Sterile filter	0.2 µm (Schleicher & Schuell)
Syringes	2 ml, 5 ml, 10 ml, 20 ml BD Discardit^{TM} (BD Bioscience, Heidelberg)
Tissue culture flasks	25 cm^2, 75 cm^2, 150 cm^2 (TPP, St.Louis, USA or Sarstedt, Nümbrecht)
Tissue culture plates	6-, 12-, 24-, 48-, 96-well (TPP, St. Louis, USA or Sarstedt, Nümbrecht)
Tuberculin syringes	Omnifix®-F (Braun, Melsungen)

3.1.4 Chemicals and reagents

Ammonium chloride (NH_4Cl)	Merck, Darmstadt
Biotin/Avidin blocking kit	Vector Laboratories, Burlingame, USA
Bovine serum albumin (BSA)	Roth, Karlsruhe
Brain Heart Infusion Broth	Roth, Karlsruhe
CFSE	Molecular Probes, Leiden, Netherlandes
Collagenase A	Roche, Mannheim
DEPC treated water (RNase free)	Invitrogen, Karlsruhe
Dimethylsulfoxide (DMSO)	Merck, Darmstadt
Disodium ethylene diamine tetraacetic acid (Na_2EDTA)	Merck, Darmstadt
Disodium hydrogen phosphate (Na_2HPO_4)	Merck, Darmstadt
DMEM medium	Sigma Aldrich, München
DOTAP®	Roth, Karsruhe
Ethanol, absolute	Merck, Darmstadt
ethylene diamine tetraacetic acid (EDTA)	Sigma Aldrich, München
Far Red	Molecular Probes, Leiden, Netherlands
Fetal calf serum (FCS)	PAA, Cölbe
G418 sulfate	PAA, Cölbe
Golgi Plug/Stop	BD Biosciences, Heidelberg
Heparin	Ratiopharm, Ulm
Hoechst 33258	Sigma Aldrich, München
Hoechst 33342	Molecular Probes, Leiden, Netherlands
Hydrogen peroxide (H_2O_2)	Merck, Darmstadt
IMDM medium	Lonza, Köln
in vivo-jetPEI^{TM}	Polyplus Transfection, Illkirch, France
Isoflurane	DeltaSelect, Pfullingen
L-Glutamine (200 mM)	PAA, Cölbe
Liquid nitrogen	Linde, Wiesbaden
Luciferin	Caliper Lifesciences, Rüsselsheim
Lymphocyte separation medium	PAA, Cölbe
Natural silk 5.0	Catgut, Markneukirchen
Neutralized streptavidin-peroxidase	Uptima, Interchim, France

Normal donkey serum	Jackson ImmunoResearch, West Grove, USA
Normal goat serum	Jackson ImmunoResearch, West Grove, USA
o-Phenylenediamine dihydrochloride (OPD)	Sigma Aldrich, München
Optimem medium	Gibco, BRL, Karlsruhe
Ovalbumin (OVA)	Serva, Heidelberg
Ovalbumin (OVA), grade V	Sigma Aldrich, München
Paraformaldehyde (PFA)	Fluca, Buchs
Penicillin/Streptomycin (100x)	PAA, Cölbe
Phosphate buffered saline (PBS)	Biochrom, Berlin
Phosphate buffered saline (PBS) solution	Sigma Aldrich, München
Potassium bicarbonate ($KHCO_3$)	Merck, Darmstadt
RPMI 1640 medium	Sigma Aldrich, München
Saponin	Sigma Aldrich, München
Sodium azide	Sigma Aldrich, München
Sodium bicarbonate (Na_2HCO_3)	Sigma Aldrich, München
Sodium chloride, 0.9%	B. Braun, Melsungen
Sodium hydroxide (NaOH)	Merck, Darmstadt
Sucrose	Roth, Karlsruhe
Sulfuric acid (H_2SO_4), 96%	Merck, Darmstadt
Tacosil	Nycomed, Konstanz
Tissue-Tek® OCT	Sakura, Netherlands
TMB substrate	Uptima, Interchim, France
Trisodium citrate ($Na_3C_6H_5O_7$)	Sigma Aldrich, München
Trypane blue (0.4%)	Lonza, Köln
Trypsin/EDTA	Lonza, Köln
TSA^{TM} Fluorescein System	PerkinElmer, Rodgau-Jügesheim
Tween20	Roth, Karlsruhe
Vectashield mounting medium with DAPI	Vector Laboratories, Burlingame, USA
β-mercaptoethanol	Sigma Aldrich, München
2-Methylbutane	Merck, Darmstadt

3.1.5 Buffers, media and solutions

DC and T cell culture medium

RPMI 1640 medium supplemented with 10% (v/v) FCS, 2mM L-glutamine, 1% (v/v) penicillin/streptomycin, 50 µM β-mercaptoethanol. Kept under sterile conditions at 4°C.

BMDC medium

IMDM medium supplemented with 10% (v/v) FCS, 2mM L-glutamine, 1% (v/v) penicillin/streptomycin, 50 µM β-mercaptoethanol, 30% (v/v) GM-CSF supernatant. Kept under sterile conditions at 4°C.

B16-luc melanoma cell medium

DMEM medium supplemented with 5% (v/v) FCS, 2mM L-glutamine, 1% (v/v) penicillin/streptomycin. Kept under sterile conditions at 4°C.

Ag8653 medium

IMDM medium supplemented with 10% (v/v) FCS, 2mM L-glutamine, 1% (v/v) penicillin/streptomycin, 50 µM β-mercaptoethanol. Kept under sterile conditions at 4°C.

FCS (fetal calf serum)

FCS was heat-inactivated at 56°C for 30 min and stored à 50 ml aliquots at -20°C.

PBS (phosphate buffered saline)

1xPBS was adjusted to pH 7.4, aliquoted à 500 ml, autoclaved and stored at 4°C.

0.5 M EDTA

186.1g EDTA and 20g NaOH were dissolved in 1000 ml ultra-pure water and the pH was adjusted to 7.8 – 8.0. The solution was autoclaved and stored at room temperature.

5 mM β-mercaptoethanol

178 µl of 14.3 M β-mercaptoethanol was added to 500 ml PBS and kept under sterile conditions at 4°C.

FACS buffer

1x PBS containing 1% (v/v) FCS and 0.1% (v/v) NaN_3. Stored at room temperature.

MACS buffer

1x PBS containing 1% (v/v) FCS and 2mM EDTA. Stored under sterile conditions at 4°C.

Saponin/FACS buffer

FACS buffer supplemented with 0.5% (w/v) Saponin, prepared freshly.

ACK lysis buffer

16.58 g NH_4Cl, 2 g $KHCO_3$, 74.4 mg Na_2EDTA was dissolved in 2000 ml ultra-pure water and the pH was adjusted to 7.2 - 7.4. Stored under sterile conditions at room temperature.

ELISA coating buffer

0.1 M $NaHCO_3$ in ultra-pure water, pH 8.2, autoclaved and stored at 4°C.

ELISA blocking buffer

1% BSA (w/v) in 1xPBS, prepared freshly.

OPD substrate buffer

15.6 g NaH_2PO_4 x $2H_2O$ and 14.7 g $Na_3C_6H_5O_7$ x $2H_2O$ were dissolved in 500 ml ultra-pure water and the pH was adjusted to 5.0. The buffer was stored at room temperature.

1M H_2SO_4 OPD stopping solution

26.5 ml 96% H_2SO_4 was added to 500 ml ultra-pure water and kept at room temperature.

0.18 M H_2SO_4 TMB stopping solution

5 ml 96% H_2SO_4 was added to 500 ml ultra-pure water and kept at room temperature.

ELISA washing buffer

1x PBS containing 0.01% (v/v) Tween20

4% (w/v) PFA solution

8 g PFA was dissolved in 200 ml 1xPBS by gradual heating. The pH was adjusted to 7.4 and aliquots were stored at -20°C.

30% (w/v) sucrose solution

15 g sucrose were dissolved in 50 ml 1xPBS, freshly prepared.

50 mM luciferin solution

1 vial of luciferin (1g) was dissolved in 70 ml sterile PBS to obtain a 50 mM stock solution (14mg/ml). Aliquots á 2 ml were stored at -20°C.

3.1.6   Synthetic RNA oligoribonucleotides and TLR ligands

Synthetic RNA oligoribonucleotides (ORN) were ordered from Biomers, Dharmacon, or Qiagen. Lyophilized RNA was reconstituted in sterile 0.9% NaCl or Qiagen siRNA suspension buffer (1 µg/µl). Aliquots were stored at -80°C.

ORN	Sequence	Target	Reference
9.2 (dsRNA)	5'-AGC UUA ACC UGU CCU UCA A dTdT-3' (sense) 5'-UUG AAG GAC AGG UUA AGC U dTdT-3' (antisense)	human TLR9	(Hornung et al., 2005)
9.2s (ssRNA)	5'- AGC UUA ACC UGU CCU UCA A-3' (sense)		(Hornung et al., 2005)
siVEGF	5'-AUG UGA AUG CAG ACC AAA GAA dTdT-3' (sense) 5'-UUC UUU GGU CUG CAU UCA CAU dTdT-3' (antisense)	murine VEGF-A	(Filleur et al., 2003; Kornek et al., 2008)
RNA-Al647	5'-GAA CUU CAG GGU CAG CUU GCC G-3'		commercial RNA (Qiagen)

CpG-rich oligonucleotides (ODN) were purchased from TIB MolBiol, Berlin. Lyophilized CpG ODN was dissolved in sterile PBS at a concentration of 1nmol/µl (= 6.4 µg/µl) by incubation at 37°C for 30 min. Dissolved CpG was stored at 4°C.

ODN	Sequence	Type	Reference
CpG-1668	TCC ATG ACG TTC CTG ATG CT	CpG-B	(Krieg et al., 1995)

polyI:C was ordered from Sigma-Aldrich, München, and dissolved in 0.9% NaCl at a concentration of 1 µg/µl. Aliquots were stored at -20°C.

Substance	Description	Reference
polyI:C	$(C_{10}H_{10}N_4NaO_7P)_x \cdot (C_9H_{11}N_3NaO_7P)_x$	(Ranjith-Kumar et al., 2007)

3.1.7 Peptides

Peptides were obtained from Pineda, Invitrogen, or Sigma-Aldrich. They were dissolved as 20 mM stock solutions in DMSO and stored at -20°C.

Peptides	MHC haplotype	Amino acid sequence
$OVA_{254-267}$ (S8L)	H-2k^b	SIINFEKL
$OVA_{265-279}$ (OT3p)	I-A^b	EKLTEWTSSNVMEER
mTRP2$_{180-188}$	H-2k^b	SVYDFFVWL
Influenza peptides	H-2k^b	ASNENMETM and SSLENFRAYV
HSV peptide	H-2k^b	SSIEFARL

3.1.8 Recombinant cytokines

Recombinant mouse cytokines were kept at -80°C for long-term storage. Working aliquots of 5 µg/ml were stored at -20°C.

Cytokine	Company	Application
IL-2	eBioscience, USA	ELISA standard
IL-6	eBioscience, USA	ELISA standard
IL-12p70	eBioscience, USA	ELISA standard
IL-12p70	provided by Edgar Schmitt, Mainz	DC/T cell co-culture
IFNγ	eBioscience, USA	ELISA standard
IFNαA	PBL Interferon Source, USA	*in vivo*
IFNβ	PBL Intereron Source, USA	*in vivo*

3.1.9 Antibodies

Antibodies used in cytokine ELISA

All antibodies used in cytokine ELISAs were purchased from eBioscience or BD Pharmingen. Matching antibody pairs included a primary unlabeled capture antibody and a biotinylated detection antibody. Each antibody was applied in previously titrated amounts.

Cytokine	Clone (capture antibody)	Clone (detection antibody)
IL-2	JES6-1A12	JES6-5H4
IL-12p70	C18.2	C17.8
IFNγ	XMG1.2	RA-6A2

Antibodies used for detection of antibody responses

OVA-specific IgG antibodies were detected using a biotinylated goat anti-mouse IgG (Dianova, Hamburg).

Antibodies coupled to magnetic beads (MACS® Beads)

Murine anti-CD8a, anti-CD4, and anti-CD11c antibodies conjugated to magnetic beads (MACS® Beads) were obtained from Miltenyi Biotech, Bergisch Gladbach.

Immunohistology antibodies

The following antibodies were used in immunohistology of spleens derived from IFNβ/YFP reporter mice.

Antibody	Company
biotinylated donkey anti-rabbit	Jackson Immuno Research, West Grove, USA
goat anti-rat Cy3	Jackson Immuno Research, West Grove, USA
monoclonal rat anti MOMA-1	BMA Biomedicals, Augst, Switzerland
polyclonal rabbit anti-GFP	Abcam, Cambridge, UK

Fluorochrome conjugated antibodies used in FACS

The following antibodies were purchased from eBioscience or BD Biosciences (if not otherwise stated) for flow cytometric analysis of murine molecules expressed at the cell surface or intracellularly. All antibodies were labeled with a fluorochrome (FITC, PE, PE-

Cy7, PerCP-Cy5.5, APC, Al647, APC-Cy7, Pacific Blue, Al405) and employed at previously determined concentrations.

Antigen	Isotype	Clone	Annotation
B220	rat IgG2a, κ	RA3-6B2	= CD45R
CD11b	rat IgG2b, κ	M1/70	= Mac-1, integrin αM chain
CD11c	hamster IgG	N418	= integrin αX chain
CD16/32	rat IgG2b, κ	2.4G2	= anti FcγR III + II
CD19	rat IgG2a, κ	eBio1D3	
CD3e	hamster IgG	145-2C11	
CD4	rat IgG2b, κ	GK1.5	
CD40	rat IgG2a, κ	1C10	= TNFRSF5
CD62L	rat IgG2a, κ	MEL-14	= L-selectin, LECAM-1, Ly-22
CD69	hamster IgG	H1.2F3	
CD80	hamster IgG	16-10A1	= B7.1
CD86	rat IgG2b, κ	P03.1	= B7.2
CD8a	rat IgG2a, κ	53-6.7	= Ly-2
F4/80	rat IgG2a, κ	BM8	
IFNγ	rat IgG1, κ	XMG1.2	
IL-12p40/p70	rat IgG1	C15.6	
IL-2	rat IgG2b, κ	JES6-5H4	
PDCA-1	rat IgG2b	JF05-1C2.4.1	purchased from Miltenyi Biotech
S8L/H-2K^b Pentamers			purchased from Proimmune
Vα2 TCR	rat IgG2a	B20.1	= OT-I TCR
Vβ5 TCR	mouse IgG1, κ	MR9-4	= OT-I TCR

3.1.10 Cell lines

B16-luc melanoma

B16-luc is a C57BL/6-specific murine melanoma cell line expressing luciferase, OVA, and the green fluorescence protein (GFP). This cell line was obtained from Gunther Hämmerling, DKFZ, Heidelberg.

Ag8653

Ag8653 is a hybridoma cell line that is used to produce GM-CSF containing supernatant (Karasuyama and Melchers, 1988; Stockinger et al., 1996).

3.1.11 Viruses and bacteria

Viruses and bacteria used in this study were kindly provided by other scientists.

Pathogen	Description	Source (Reference)
AdOVA	E1 and E3 deleted recombinant adenoviral vector expressing the full length OVA protein under the CMV promoter	Dr. Andreas Untergasser; Prof. Dr. Ulrike Protzer
AdLOG	E1 and E3 deleted recombinant adenoviral vector expressing luciferase, OVA-derived peptide SIINFEKL, and GFP	Prof. Dr. Thomas Tüting (Schweichel et al., 2006)
AdmTRP2	E1 and E3 deleted recombinant adenoviral vector expressing the mTRP2-derived peptide SVYDFFVWL and GFP	Prof. Dr. Thomas Tüting (Steitz et al., 2002; Tuting et al., 1999)
HSV-1	Wild type Herpes simplex virus type 1	Dr. Christoph Coch; Nico Busch
Influenza virus	Wild type Influenza A PR8/H1N1 virus	Dr. Christoph Coch; Nico Busch
LmOVA	Recombinant *Listeria monocytogenes* expressing the full length OVA protein	Prof. Dr. Klaus Pfeffer; Dr. Stefanie Scheu (Pope et al., 2001)

3.1.12 Mouse strains

C57BL/6(N) or C57BL/6(J) wild type strains ($H-2K^b$) were purchased from Charles River, Sulzfeld, or Janvier, France. Mice were bred under pathogen free conditions and in accordance to institutional animal guidelines in the animal facility (HET, House of Experimental Therapy) of the University of Bonn. Following knock out (ko) or transgenic animals backcrossed on C57BL/6 were used:

Strain	Description	Source (Reference)
CD11c-Cre x IFNAR1$^{fl/fl}$	DC-specific type I IFN receptor ko	Prof. Dr. Ulrich Kalinke (Cervantes-Barragan et al., 2009)
CD19-Cre x IFNAR1$^{fl/fl}$	B cell-specific type I IFN receptor ko	Prof. Dr. Ulrich Kalinke (Prinz et al., 2008)
CD4-Cre x IFNAR1$^{fl/fl}$	T cell-specific type I IFN receptor ko	Prof. Dr. Ulrich Kalinke (Prinz et al., 2008)
CD4/CD19-Cre x IFNAR1$^{fl/fl}$	T cell/B cell-specific type I IFN receptor ko	Prof. Dr. Ulrich Kalinke (Prinz et al., 2008)
CD4 ko	deficient in CD4 T cells	(Rahemtulla et al., 1991)
I-A^b ko	deficient in MHC class II and CD4 T cells	(Cosgrove et al., 1991; Grusby et al., 1991)
IFNAR1 ko	deficient in the type I IFN receptor	(van den Broek et al., 1995b)
IFNβ ko	deficient in IFNβ	Prof. Dr. Thomas Decker; Prof. Dr. Mathias Müller (Erlandsson et al., 1998)
IL-10 ko	deficient in IL-10	Prof. Dr. Achim Hörauf (Kuhn et al., 1993)
IRF3 ko	deficient in IRF3	Prof. Dr. Thomas Decker; Prof. Dr. Mathias Müller (Sato et al., 2000)
IRF7 ko	deficient in IRF7	Prof. Dr. Thomas Decker; Prof. Dr. Mathias Müller (Honda et al., 2005)
LysM-Cre x IFNAR1$^{fl/fl}$	macrophage-specific type I IFN receptor ko	Prof. Dr. Ulrich Kalinke (Prinz et al., 2008)
MDA5 ko	deficient in MDA5	Dr. Winfried Barchet (Gitlin et al., 2006)
MOB	IFNβ/YFP reporter mice	Dr. Stefanie Scheu (Scheu et al., 2008)

Strain	Description	Source (Reference)
OT-I/RAG	transgenic CD8 T cell line carrying the OVA$_{257-264}$ specific TCR Vα2Vβ5; lacks CD4 T cells and B cells	(Hogquist et al., 1994)
OT-I/Thy1.1	OT-I transgenic T cell line expressing the congenic marker Thy1.1	
OT-II	transgenic CD4 T cell line carrying the OVA$_{323-339}$ specific TCR	(Barnden et al., 1998)
STAT1 ko	deficient in STAT1	Prof. Dr. Thomas Decker; Prof. Dr. Mathias Müller (Durbin et al., 1996)
TLR3 ko	deficient in TLR3	(Alexopoulou et al., 2001)
TLR7 ko	deficient in TLR7	(Hemmi et al., 2002)
Tyk2 ko	deficient in Tyk2	Prof. Dr. Thomas Decker; Prof. Dr. Mathias Müller (Karaghiosoff et al., 2000)

3.2   Methods

3.2.1 Experimental treatment of mice

Pathogens, cells, and other reagents were adjusted in 0.9% NaCl or PBS for experimental injection of mice. Intravenous (i.v.) and intraperitoneal (i.p.) injections were performed with a volume of 250 µl, subcutaneous (s.c.) injections with a volume of 100 µl.

3.2.1.1 Infection with viruses

Mice were infected i.v. with 5×10^9 virus particles of different recombinant adenoviruses (see 1.1.11).

Mice were infected i.v. with 1×10^4 PFU Herpes simplex virus type 1 and 1×10^5 or 1×10^6 PFU Influenza A PR8/H1N1 virus.

3.2.1.2 Infection with recombinant *Listeria monocytogenes*

Recombinant *Listeria monocytogenes* expressing OVA (*LmOVA*) were cultured overnight in Brain Heart Infusion Broth shaking at 37°C. Colony forming units (CFU) of *LmOVA* were quantified by OD measurement at 600 nm. Mice were infected i.v. with $5x10^3$ CFU bacteria.

3.2.1.3 B16-luc tumor implantation

Mice were anesthesized by inhalation of 2% isoflurane and shaved at the surgical site. An incision was made at the abdomen and the duodenum was carefully removed from peritoneum using cotton swabs to expose the portal vein. $5x10^4$ B16-luc melanoma cells were injected intraportally (i.po.) in 50 μl volume using a tuberculin syringe with a 29G injection needle. The injection site was closed with a $2mm^2$ piece of Tacosil. The peritoneal cavity and skin was closed using natural silk.

3.2.1.4 Activation of OVA-specific CD4 T cells *in vivo*

Endogenous OVA-specific CD4 T cells were activated *in vivo* with 50 μg of $OVA_{265-279}$ peptide (OT3p) (Boscardin et al., 2006) mixed with 5 μg of CpG-1668 and 5 μg of pI:C by s.c. injection into the flank. C57BL/6 mice were immunized six days before AdOVA infection to generate effector CD4 T cells and four weeks before AdOVA infection to generate OVA-specific memory CD4 T cells.

3.2.1.5 CD40 stimulation *in vivo*

Mice were injected i.p. with 100 μg of anti-CD40 antibody (FGK-45) on the day of adenoviral infection. Stimulation was repeated by an additional i.v. administration of 100 μg one day after adenovirus immunization.

3.2.1.6 Depletion of plasmacytoid DCs

Plasmacytoid DCs were depleted using an anti-PDCA-1 antibody, which was given twice twelve hours apart. Mice were injected i.v. with 200 μg each. Depletion of splenic pDCs was determined by flow cytometric analysis 24 hours later.

ssRNA was injected 24 hours and AdOVA 48 hours after the last anti-PDCA-1 antibody administration.

3.2.1.7   Injection of recombinant type I interferons

Mice were injected i.v. with 1000 U of either mouse recombinant IFNαA, IFNβ, or IFNαA and IFNβ one day prior to adenovirus infection.

3.2.2   *In vivo* RNA oligoribonucleotide application

3.2.2.1   Complexation of RNA with DOTAP

RNA complexation using DOTAP was performed according to manufacturer's instruction with some modifications for *in vivo* application. 10 μg of single-stranded RNA or 20 μg of double-stranded RNA was incubated with 30 μl DOTAP and incubated for 20 minutes at room temperature under sterile and RNase-free conditions. The volume was adjusted to 250 μl with 0.9% NaCl for i.v. injection.

3.2.2.2   Complexation of RNA with *in vivo*-jetPEI^{TM}

RNA complexation with *in vivo*-jetPEI^{TM} was conducted according to the manufacturer's guide. Briefly, 10 μg of single-stranded RNA was diluted in 40 μl of 5% glucose solution (20 μg of double-stranded RNA in 80 μl of 5% glucose solution). 2 μl of *in vivo*-jetPEI^{TM} was mixed with 40 μl of 5% glucose solution at a N/P ratio of 10 (4 μl of *in vivo*-jetPEI^{TM} was added to 80 μl 5% glucose solution). The *in vivo*-jetPEI^{TM} solution was added to the RNA solution, mixed thoroughly, and incubated for 20 minutes at room temperature under sterile and RNase-free conditions (Important: Do not mix the solutions in the reverse order). The volume was adjusted to 250 μl with 5% glucose solution for i.v. injection.

3.2.3   *Ex vivo* analysis of splenic DCs

Splenic DCs were enriched by collagenase digestion. Spleens were perfused *ex vivo* using a syringe with 0.04% collagenase A, cut into small pieces, and incubated for 30 minutes at

37°C in a waterbath. Afterwards, digestion was stopped by adding MACS buffer and spleens were homogenized by passing through a metal sieve (mesh size 250 µm). Splenocytes were immediately used for antibody staining of surface molecules to determine the maturation status by flow cytometry. For intracellular IL-12p70 staining, splenocytes were resuspended in DC/T cell medium. 5×10^6 splenocytes were plated in 100 µl per well of a 48-well plate and Golgi-Plug/Stop was added in 200 µl medium with a final concentration of 1 µl/ml. Cells were collected after four hours of incubation at 37°C and applied to an intracellular cytokine staining.

3.2.4   Peptide-specific restimulation of T cells *ex vivo*

Splenocytes were isolated five days post AdOVA or ssRNA/AdOVA immunization and resuspended in DC/T cell medium. 2×10^6 splenocytes were plated in 100 µl per well of a 96-well plate. OVA-specific CD8 T cells were restimulated by addition of 1 µM S8L peptide, whereas OVA-specific CD4 T cells were restimulated using 100 µg/ml OT3p peptide (each added in 100 µl). IFNγ and IL-2 production by T cells was determined after overnight incubation at 37°C by flow cytometric analysis of intracellular cytokines or in supernatants by ELISA.

3.2.5   *Ex vivo* cross-priming assay

C57BL/6 wild type and IFNAR1 deficient mice were immunized with AdOVA or ssRNA/AdOVA and splenic DCs were purified using the AutoMACS based separation.
20 hours after adenovirus injection, mice were sacrificed and spleens were digested by *ex vivo* perfusion with 0.04% collagenase A, cut into small pieces, and incubated for 30 minutes at 37°C in a water bath. Upon digestion, spleens were homogenized by passing through a metal sieve. Cells were washed once and the cell suspension was filtered. After another centrifugation step for 10 minutes at 486 x g, splenocytes were resuspended in cold MACS buffer (0.5 ml per spleen) and anti-CD11c MACS beads were added (30 µl per spleen). Cells were incubated for 15 minutes at 4°C, washed once in MACS buffer, and CD11c positive DCs were purified by positive selection (Possel) using the AutoMACS cell

separation system. Subsequently, CD11c positive cells were adjusted to 3×10^6 cells per ml in DC/T cell medium and 3×10^5 DCs were plated in 100 µl per well of a 96-well plate.

OVA-specific CD8 T cells were prepared from spleens and lymph nodes of naive OT-I/RAG mice using nylon wool. Organs were homogenized by passing through a metal sieve and the cell suspension was centrifuged for 10 minutes at 486 x g. Erythrocytes were lysed by addition of 1 ml ACK lysis buffer and incubation for 2 minutes at room temperature. Tubes were filled with PBS to stop lysis reaction and cells were centrifuged again for 10 minutes at 486 x g. Afterwards, the cell pellet was resuspended in DC/T cell medium and applied to a sterile 10 ml syringe filled with 0.6 g nylon wool (The nylon wool containing syringe had been previously blocked with 2% (v/v) FCS/PBS at 37°C for at least 45 minutes). Cells were incubated for 60 minutes at 37°C to allow macrophages and DCs to adhere to the nylon wool. Afterwards, T cells were washed off by carefully adding 20 ml of warm DC/T cell medium. Eluted T cells were counted and 1×10^5 OVA-specific CD8 T cells were added in 100 µl to the pre-plated splenic DCs. Co-culture supernatants were collected after 24 hours. IFNγ and IL-2 production by T cells was determined by ELISA or flow cytometric analysis of intracellular cytokines. In addition, CD69 expression was analyzed by antibody staining and subsequent flow cytometry.

3.2.6   Collection of serum for cytokine analysis

Blood was collected from the portal vein using a heparinized syringe and allowed to coagulate. Blood samples were centrifuged for 15 minutes at 3835 x g and serum was transferred to a clean tube and stored at -20°C until cytokine analysis.

3.2.7   Expansion and activation of transgenic OT-I T cells *in vivo*

The spleen was taken from one NK/NKT cell-depleted OT-I/Thy1.1 transgenic mouse and homogenized using a metal sieve. The homogenate was centrifuged for 10 minutes at 486 x g and the cell pellet was resuspended in 10 ml MACS buffer for filtration. After another centrifugation, cells were resuspended in 0.5 ml cold MACS buffer and 30 µl of anti-CD8a MACS beads were added. Cells were incubated with MACS beads for 15 minutes at 4°C, washed once, and purified by positive selection (Possel) using the

AutoMACS cell separation system. Purified OT-I T cells were counted and 5×10^4 OT-I T cells were injected i.v. into recipient mice. One day upon transfer, mice were either left untreated or injected i.v. with complexed ssRNA. 24 hours later, mice were infected with AdOVA. The expansion and activation of transgenic OT-I T cells was determined in spleens five days post infection by flow cytometric analysis for CD8a, Thy1.1, and CD62L. In addition, an intracellular cytokine staining was performed in order to analyze IFNγ production by OT-I T cells upon overnight S8L-specific restimulation.

3.2.8 CFSE proliferation assay of CD4 T cells *in vivo*

OVA-specific CD4 T cells were isolated from spleens of OT-II transgenic donor mice. Organs were homogenized by passing them through a metal sieve and homogenates were centrifuged for 10 minutes at 486 x g. The cell pellet was resuspended in 10 ml cold MACS buffer and filtered to remove residual tissue and debris. After another centrifugation, cells were resuspended in cold MACS buffer (0.5 ml per spleen) and anti-CD4 MACS beads (30 µl per spleen) were added. Cells were incubated with MACS beads for 15 minutes at 4°C, washed once in MACS buffer, and CD4 T cells were isolated by positive selection (Possel) using the AutoMACS cell separation system. Purity of OT-II T cells was assessed by flow cytometric analysis of CD4, Vβ5, and Vα2. Afterwards, CD4 T cells were labeled with 2 µM CFSE in PBS by incubation at 37°C for 20 minutes. The reaction was stopped by adding ice cold PBS and cells were washed extensively. Finally, purified CD4 T cells were counted and 2×10^6 CFSE-labeled CD4 T cells were injected i.v. per recipient mouse. Two days upon adoptive transfer, mice were immunized with AdOVA alone or were previously injected i.v. with DOTAP- or *in vivo*-jetPEI[TM]- complexed ssRNA one day prior to AdOVA infection. Proliferation of splenic OT-II T cells was assessed three days after AdOVA immunization by a CFSE dilution assay using flow cytometry.

3.2.9 Flow cytometric analysis (FACS)

Flow cytometry (fluorescence activated cell sorting, FACS) was used to investigate the expression of different surface molecules, to detect intracellular cytokines, and to

determine the generation of endogenous OVA-specific CD8 T cells by S8L/H-2K^b pentamer staining. Data were acquired using a FACS Calibur, LSR II, or Canto II and subsequently analyzed using the FlowJo software.

3.2.9.1 Surface staining of molecules

Cells were transferred into a 96-well round bottom plate for antibody staining for flow cytometric analysis (approximately $1x10^6$ cells/well) and washed once in FACS buffer. The staining was performed in a volume of 50 µl per sample. A mastermix was prepared containing the desired antibodies and anti-CD16/CD32 (blocking of FcγR, "Fc block"). Upon centrifugation for 3 minutes at 486 x g, cells were resuspended in 50 µl of the mastermix and incubated for 20 minutes on ice protected from light. Cells were washed twice in FACS buffer and afterwards analyzed by flow cytometry. Hoechst-33258 (1 µg/ml) was added to cells shortly before acquisition to exclude dead cells from analysis.

3.2.9.2 Intracellular staining of cytokines

To prevent secretion of cytokines, cells were incubated in the presence of 1 µl/ml Golgi-Plug/Stop for four to five hours at 37°C. Afterwards, cells were harvested and transferred into a 96-well round bottom plate. At first, a surface staining was performed as described in 1.2.9.1. Afterwards, stained cells were fixed for ten minutes at room temperature by incubation in 2% (v/v) PFA/FACS buffer. PFA was immediately washed off by centrifugation at 486 x g for 3 minutes. Cells were permeabilized by adding 100 µl of Saponin/FACS buffer and incubated for 20 minutes at room temperature (protected from light). Plates were centrifuged for 3 minutes at 486 x g and a mastermix containing the cytokine antibodies (1:100 dilution) and anti-CD16/CD32 was prepared in Saponin/FACS buffer. Cells were resuspended in 50 µl of the antibody mastermix and incubated protected from light for 30 minutes at room temperature (after 20 minutes another 100 µl of Saponin/FACS buffer was added to allow unbound antibody to diffuse out of the cell). Afterwards, cells were washed twice in FACS buffer and applied to flow cytometry.

3.2.9.3 S8L/H-2K^b specific pentamer staining

The generation of endogenous OVA-specific CD8 T cells was determined in spleens of mice five days post AdOVA or AdLOG infection using S8L/H2-K^b pentamers. The staining was conducted on a 96-well round bottom plate. Isolated splenocytes were washed once in FACS buffer and centrifuged for 3 minutes at 486 x g. Cells were resuspended in 30 µl FACS buffer containing 5 µl S8L/H-2K^b pentamers as well as anti-CD16/CD32 and were incubated for 20 minutes at room temperature (light-protected). Afterwards, 20 µl of a CD8a antibody was directly added (without centrifugation), which had been diluted in FACS buffer. Cells were incubated for further 20 minutes on ice and washed twice in FACS buffer before flow cytometric analysis. Hoechst-33258 (1 µg/ml) was added to the cells immediately before data acquisition to discriminate between viable and dead cells.

3.2.10 Analysis of antigen-specific cytotoxic CD8 T cells (*in vivo* cytotoxicity assay)

Cytotoxic activity of endogenous antigen-specific CD8 T cells was measured in spleens five days post pathogen infection by performing an *in vivo* cytotoxicity assay (Feuerer et al., 2003). Briefly, splenocytes from syngeneic donor mice were divided into two fractions. One fraction was pulsed with 1 µM of the specific H-2K^b peptides SIINFEKL (OVA), SVYDFFVWL (mTRP2), ASNENMETM/SSLENFRAYV (Influenza virus), or SSIEFARL (HSV-1 virus) for 20 minutes at 37°C. Subsequently, peptide-loaded cells were labeled with 1 µM of CFSE or Far Red by incubation for 15 minutes at 37°C (CFSE^{high} or Far Red, target cells). The second fraction was not pulsed with peptide and labeled with 0.1 µM CFSE (CFSE^{low}, reference cells). Upon labeling, cells were washed extensively and counted. Both populations were mixed in a 1:1 ratio (CFSE^{high}/Far Red:CFSE^{low}) and 1×10^7 cells were injected i.v. into recipient mice. Unless otherwise stated, spleens were removed four hours later and homogenized by passing through a metal sieve. After centrifugation for 10 minutes at 486 x g, splenocytes were resuspended in 5 ml MACS buffer. Lysis of peptide-loaded cells was measured by flow cytometric analysis. Unimmunized naive mice served as a control. The percentage of specific lysis was calculated using the following formula:

% specific cytotoxicity = $100 - [(\text{CFSE}^{high}/\text{CFSE}^{low})_{sample} / (\text{CFSE}^{high}/\text{CFSE}^{low})_{control}] \times 100$

3.2.11 Cytokine ELISA (enzyme linked immunosorbent assay)

ELISA microtiter plates were coated with 50 µl per well of the primary capture antibody, which was diluted to 1 µg/ml in ELISA coating buffer, and incubated overnight at 4°C. Unbound antibody was removed by extensive washing with ELISA washing buffer. To prevent unspecific binding, the ELISA plate was blocked by addition of 100 µl blocking buffer and incubated for two hours at 37°C. After washing the plate thoroughly, 50 µl of each sample (supernatants or sera in triplicates) and the recombinant cytokine standard (1 to 3 dilution series starting at 80 ng/ml, 8 steps) was added and incubated overnight at 4°C. The plate was washed extensively and 50 µl of the biotinylated detection antibody was added, which had been diluted to 0.5 µg/ml in blocking buffer. The plates were incubated for two hours at room temperature and afterwards washed thoroughly again. The streptavidin-conjugated peroxidase was diluted to 1 µg/ml in blocking buffer and 50 µl were added per well. After incubation for 30 minutes at 4°C and extensive washing, 50 µl of TMB substrate was applied to each well and the absorbance was immediately measured at 650 nm in a ELISA plate reader. Afterwards, the enzymatic reaction was stopped by addition of 50 µl 0.18M H_2SO_4 stopping solution. The absorbance was measured at 450 nm again. The standard curve was plotted as a sigmoidal curve, as peroxidase is an allosteric enzyme.

3.2.12 OVA IgG ELISA

Mice were injected with AdOVA, with or without previous ssRNA injection. Serum titers of OVA-specific IgG were quantified 25 days post infection by ELISA. ELISA plates were coated with 100 µl of 50 µg/ml OVA (grade V), which was diluted in ELISA coating buffer. Plates were incubated overnight at 4°C and afterwards washed extensively in washing buffer to remove any unbound OVA. After blocking of ELISA plates (150 µl blocking buffer, two hours at 37°C), sera were applied in a volume of 100 µl per well. Sera were consecutively diluted 1:1 in blocking buffer on the plate (5 dilution steps) and incubated overnight at 4°C. After a thorough washing step, 100 µl of biotinylated goat anti-mouse IgG antibody was added (1:10000 dilution in blocking buffer) and the plate was incubated for one hour at room temperature. Unbound detection antibody was removed by extensive washing before 100 µl of streptavidin-conjugated peroxidase was

added at a concentration of 1 µg/ml and incubated for one hour at room temperature. In the meantime, the OPD substrate solution was freshly prepared. 5 mg OPD was dissolved in 5 ml OPD substrate buffer and supplemented with 5 µl of 30% H_2O_2. After washing off the peroxidase, 50 µl of the OPD substrate solution was applied per well and incubated until a color change to yellow was clearly visible. The enzymatic reaction was stopped by addition of 50 µl 1M H_2SO_4. The absorbance was measured at 490 nm using the ELISA reader. OVA-specific IgG titers were quantified by plotting the serum dilution (y-axis) against the measured OD values (x-axis) to obtain a linear equation. The serum dilution was read at a fixed OD value for all samples (e.g. 0.5) and the titer corresponded to the reciprocal value of the serum dilution.

3.2.13 Type I interferon bioassay

Type I interferon (IFN) production was assayed in sera and splenocyte supernatants with the help of Prof. Dr. Rainer Zawatzky, DKFZ, Heidelberg.

Biological activity of type I IFN was measured in a standard cytopathic effect inhibition assay using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test, which was described previously (Nickolaus et al., 1998). Briefly, monolayers of murine L-929 cells (15000 cells in 100µl per well) were incubated overnight with twofold dilutions of the test samples and then infected with mouse encephalomyocarditis virus at a multiplicity of infection of 0.1. After 20 hours, methylthiazolyldiphenyl-tetrazoliumbromide was added for four hours at a final concentration of 1 mg/ml. The virus-induced cytopathic effect was quantified by ELISA. A twofold dilution of an internal laboratory standard preparation of mouse IFN based on the NIH mouse reference IFNα/β preparation was included in each test. One laboratory unit corresponded to one international unit (IU) defined as the concentration of type I IFN resulting in a 50% protection against viral lysis. Colorimetric analysis of the alcohol-soluble dye was performed at 570 nm using an ELISA reader. IFNγ activity was excluded by using an IFNγ blocking antibody.

3.2.14 Immunohistology of IFNβ expression

IFNβ expression was investigated *in vivo* in spleens upon ssRNA stimulation using IFNβ knockin reporter mice, which express the yellow fluorescence protein (YFP) under the IFNβ promoter (Scheu et al., 2008). Immunohistological analysis was performed with the help of Philipp Dresing and Dr. Stefanie Scheu, Uni Düsseldorf.

Organs were fixed in 4% PFA at room temperature for two hours, incubated in 30% sucrose solution overnight at 4°C, then embedded in OCT-TissueTek, and frozen in methylbutane on dry-ice. After blocking of endogenous peroxidases and biotin, 7-µm sections were stained by using a rabbit anti-GFP antibody (cross-reacts with YFP) followed by a biotinylated donkey anti-rabbit antibody. After a repeated biotin and peroxidase blocking, sections were counterstained with a rat anti-MOMA-1 antibody followed by a goat anti-rat-Cy3 antibody. Signal amplification with TSA fluorescein or biotin kits was performed according to manufacturer's instruction. Sections were mounted with Vectashield containg DAPI. Images were captured on an epifluorescence microscope with a digital camera and overlaid by using Photoshop software.

3.2.15 *In vivo* imaging of bioluminescence

Mice were analyzed at indicated time points after injection of recombinant AdLOG or B16-luc melanoma cells. Immediately before measurement of bioluminescence using the real-time IVIS Imaging System, mice were injected i.p. with 200 µl luciferin solution. Analysis was performed under inhalational anesthesia with isoflurane. Data were aquired using the Living Image 2.50 software.

3.2.16 Production of GM-CSF supernatant

The GM-CSF producing cell line Ag8653 was thawed, added to 10 ml cold IMDM medium, and centrifuged at 486 x g for 10 minutes in order to remove DMSO. Afterwards, cells were resuspended in 10 ml warm IMDM medium and cultured in a T25 flask for 24 hours at 37°. 1 mg/ml of G418 sullfate was added to select for GM-CSF producing cells and the cells were incubated for another 48 hours. Cells were splitted into three T75 flasks

containing 30 ml medium supplemented with 1 mg/ml G418 sulfate and grown until confluency. Then, cells were passaged into two T175 flasks containing 40-50 ml medium supplemented with 1 mg/ml G418 sulfate and incubated until they were confluent and the medium had turned orange. Ag8653 cells were harvested, counted and plated with 1.5×10^6 cells per dish (150x20 mm culture dish) in 30 ml medium without G418 sulfate. After three days of incubation, supernatants were collected in 50 ml tubes and centrifuged at 486 x g for 10 minutes and for further 20 minutes at 2683 x g. Supernatants were pooled in a sterile Erlenmeyer flask. Aliquots à 45 ml were stored at -20°C.

3.2.17 Generation of bone-marrow derived DCs (BMDCs)

Hind legs of C57BL/6 mice were cut at the pelvis and put into sterile PBS. Feet, skin, and muscle tissue were removed and blank bones were shortly dipped into ethanol. The femoral and the shank bones were separated and cut at the ends with a scissor. The bone marrow was eluted into a petri dish using a syringe filled with PBS. Afterwards, the bone marrow was homogenized by pipetting up and down several times. The cell suspension was centrifuged at 486 x g for 10 minutes and resuspended in IMDM medium supplemented with 30% GM-CSF supernatant. Cells were plated in 20 ml medium onto three petri dishes (10 cm) and incubated at 37°. After three to four days in culture, cells were passaged onto six petri dishes in fresh IMDM medium containing 30% GM-CSF supernatant and incubated for three further days. Differentiated BMDCs were used to identify the intracellular localization of DOTAP- or *in vivo*-jetPEI™- complexed RNA (see 1.2.18).

3.2.18 Intracellular localization of RNA in BMDCs

1×10^5 BMDCs were plated on microscopy glass slides using flexiPERM cell culture chambers and incubated for 30 to 45 minutes at 37°C to let BMDCs become attached. In the meantime, RNA-Al647 was complexed with either DOTAP or *in vivo*-jetPEI™. 500 ng RNA-Al647 was dissolved in 25 µl of Optimem medium and 2 µl DOTAP was mixed with 25 µl Optimem medium. The DOTAP solution was added to the RNA solution, mixed, and incubated for 20 minutes at room temperature. For complexation with *in vivo-*

jetPEI^{TM}, 500 ng RNA-Al648 was added to 25 µl of Optimem medium. A 1:10 pre-dilution of *in vivo*-jetPEI^{TM} was prepared and 0.5 µl of the dilution was added to 25 µl Optimem medium. The *in vivo*-jetPEI^{TM} Optimem solution was mixed with the RNA solution and incubated for 20 minutes at room temperature. Upon incubation, further 200 µl of Optimem medium was added and the RNA complexes were given onto the BMDCs after the medium had been removed. To identify endosomal compartments, OVA-FITC was added simultaneously at a concentration of 20 µg/ml. Fluorescence microscopy was performed 20 minutes after RNA addition. Hoechst 33342 was added to cells shortly before microscopical analysis in order to identify cell nuclei.

3.2.19 Determination of cell number

The cell suspension was diluted 1:10 or 1:100 in 0.04% trypane blue to discriminate between live and dead cells. 10 µl of this suspension was applied to a Neubauer counting chamber and living cells were counted in all four large squares. The cell count was calculated using the following formula:

Cell number/ml = counted viable cells/4 x dilution x 10^4

3.2.20 Statistics

Results are depicted as mean +/- standard error of the mean (SEM). Statistical significance was calculated by an unpaired two-tailed Student's t test using the Prism software.

p values of 0.01 to 0.05 were considered as significant (*), *p* values of 0.001 to 0.01 as highly significant (**) and *p* of <0.001 as extremely significant (***).

4 Results

4.1 Suppression of adaptive immune responses by the TLR7 Ligand single-stranded RNA (ssRNA)

4.1.1 ssRNA suppresses antigen-specific CTL responses in a TLR7-dependent manner

Recognition of Toll-like receptor (TLR) ligands by their respective TLRs triggers the activation of the vertebrate's immune system and generally results in immunity (Iwasaki and Medzhitov, 2004; Janeway, 1992; Kawai and Akira, 2005; Medzhitov and Janeway, 2002). It is well established, that the local application of synthetic CpG oligonucleotides, a TLR9 ligand, enhances the immunogenicity of co-administered antigens (Klinman et al., 2004; Krieg, 2006). However, we and other groups have shown that the systemic injection induces immunoregulatory mechanisms that cause the suppression of subsequent antigen-specific adaptive immune responses (Mellor et al., 2005; Wilson et al., 2006; Wingender et al., 2006).

In the present study, we investigated how a systemic administration of a TLR7 ligand affected antigen-specific T cell responses in the spleen against subsequent infections by viral or bacterial pathogens. As a synthetic TLR7 ligand we used a RNA oligoribonucleotide (ORN), termed 9.2, which is complementary to the human TLR9 mRNA and recognized by murine TLR7 (Hornung et al., 2005). To study antigen-specific CTL responses, we infected mice i.v. with recombinant adenoviruses expressing OVA only (AdOVA) or luciferase, OVA, and GFP (AdLOG), respectively. The OVA-derived MHC class I peptide SIINFEKL (S8L) is presented on H-2K^b and recognized by peptide-specific T cells, which results in the expansion and activation of S8L-specific CTLs. Here, mice were immunized i.v. with 5×10^9 virus particles of AdLOG alone or they received 20 µg of 9.2 (duplex RNA) or 10 µg of the single-stranded RNA (ssRNA) 9.2s (sense strand of 9.2) i.v. one day prior to AdLOG administration. OVA-specific cytotoxicity was analyzed in spleens of mice five days post AdLOG infection using an *in vivo* cytotoxicity assay. CTL activity was completely inhibited in 9.2-injected mice, while AdLOG-infected mice generated a strong CTL response. Furthermore, 9.2-mediated suppression of the CTL response was dependent on the recognition of the single-strand RNA motif, in this case the sense strand of 9.2 (Figure 4.1 A, key finding by Beatrix Schumak). An influence of the antisense strand on the CTL response could be excluded, as it has been already reported by

Hornung et al. that the sense strand and not the antisense strand of 9.2 is recognized by TLR7 (Hornung et al., 2005).
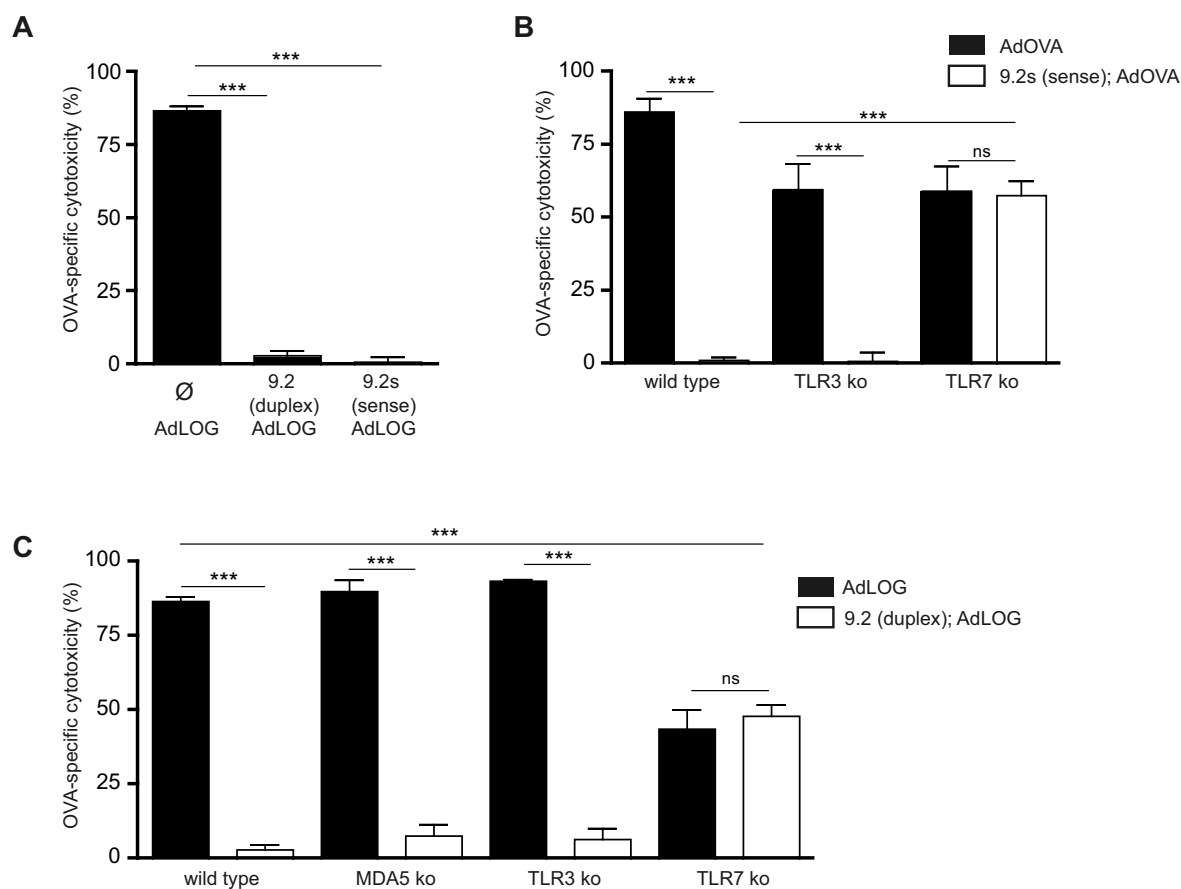

Figure 4.1   RNA oligoribonucleotides suppressed adeno-specific CTL responses in a TLR7 dependent manner.
OVA-specific cytotoxic CD8 T cell responses were determined in spleens of mice five days post AdLOG infection. (A) Inhibition of the CTL response was dependent on the recognition of single-strand RNA motifs. C57BL/6 wild type mice were injected i.v. with 5×10^9 virus particles AdLOG alone or received 20 µg of 9.2 (duplex RNA) or 10 µg 9.2s (ssRNA, sense strand of 9.2) i.v. one day prior to AdLOG immunization. (B, C) RNA oligoribonucleotides suppressed CTL responses in a TLR7 dependent manner. C57BL/6 wild type, TLR3 ko, TLR7 ko, or MDA5 ko were immunized i.v. with AdLOG alone or were previously injected with 9.2s (sense, B) or 9.2 (duplex, C). OVA-specific cytotoxicity is shown as mean +/- SEM (n=3 mice per group). Data of one representative experiment out of at least two independent experiments are depicted.

Single-stranded RNA has been identified as a physiological ligand of TLR7 (Diebold et al., 2004; Heil et al., 2004), whereas double-stranded RNA is recognized by endosomal TLR3 (Alexopoulou et al., 2001) or by the cytoplasmic receptors MDA5 or RIG-I (Gitlin et al., 2006; Hornung et al., 2006; Schlee et al., 2009; Yoneyama et al., 2005). In order to confirm, that 9.2-mediated CTL suppression required the recognition by TLR7 and not other nucleic acid receptors, we performed immunization experiments in TLR3 ko, TLR7

ko, and MDA5 ko mice. 9.2 and 9.2s ORN injection still impeded the generation of AdLOG-specific CTL responses in TLR3 and MDA5 ko mice, respectively. However, the OVA-specific cytotoxic activity was not impaired in TLR7 ko mice, proving that RNA-mediated CTL inhibition depended on the recognition by endosomal TLR7 (Figure 4.1 B and C; Figure 4.1 C was kindly provided by Beatrix Schumak). All following experiments were performed with the single-stranded RNA oligoribonucleotide 9.2s if not otherwise stated.

To identify the mechanisms underlying the CTL suppression, we first examined the possibility that innate immune stimulation by 9.2s triggered antiviral effects that led to a fast elimination of the virus, which might have resulted in a substantial reduction in antigen load in the organism. We infected C57BL/6 wild type mice either with AdLOG alone or injected mice i.v. with 10 μg of 9.2s either one day before, simultaneously with, or one day after AdLOG administration. We assessed the adenoviral burden by *in vivo* imaging of bioluminescent luciferase expression two days post AdLOG immunization. In addition, OVA-specific lysis was quantified in spleens of mice five days post AdLOG injection.
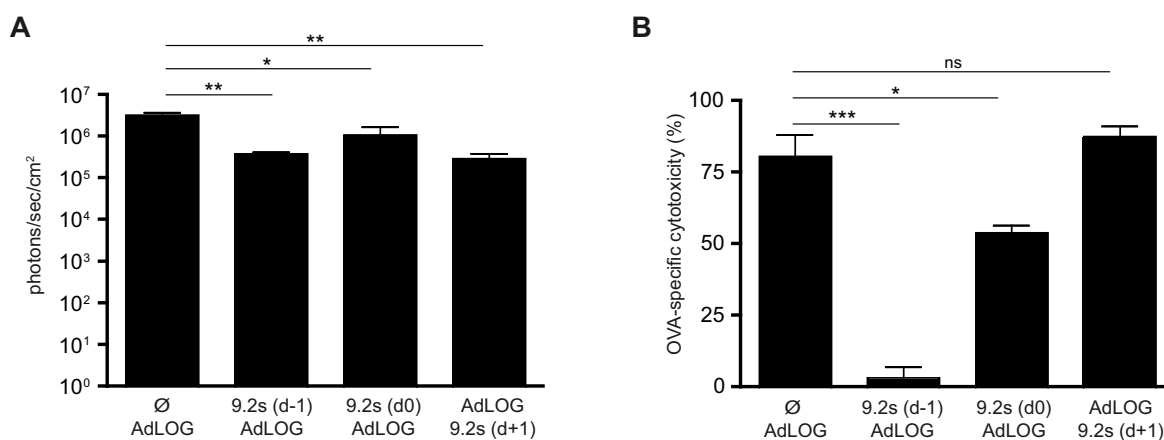

Figure 4.2   ssRNA-induced CTL suppression was not due to a reduced adenoviral burden.
C57BL/6 wild type mice were infected with 5×10^9 virus particles of AdLOG alone or they received i.v. 10 μg of 9.2s either one day before, simultaneously with, or one day after AdLOG injection. (A) Adenoviral burden was quantified by *in vivo* imaging of bioluminescent luciferase expression two days post AdLOG immunization. (B) OVA-specific cytotoxicity was determined in spleens five days post AdLOG administration. Representative data are shown as mean +/- SEM (n=3 mice per group) of two independent experiments.

In all 9.2s injected groups, an approximately tenfold reduction in luciferase expression was observed in comparison to mice injected with AdLOG alone, which indicated a diminished viral load due to innate immune stimulation by 9.2s (Figure 4.2 A). Nevertheless, the

OVA-specific CTL response was only compromised if 9.2s was given before AdLOG immunization. A simultaneous administration had only a minor influence on CTL activity, whereas a subsequent 9.2s injection did not impair the CTL response against AdLOG (Figure 4.2 B). In conclusion, these results demonstrated that the suppressive effect of 9.2s was not a consequence of a reduced viral burden but rather involved a regulatory mechanism.

In further experiments we examined how a repetitive systemic administration of the ORN 9.2s affected subsequent adenoviral-specific CTL responses. Here, three consecutive i.v. injections of 9.2s (d-3; d-2; d-1) prior to AdLOG infection also clearly inhibited the generation of an efficient CTL response (Figure 4.3 A). Even a second administration one day after AdLOG immunization did not rescue the CTL response (Figure 4.3 A). Moreover, we analyzed how long a previous injection with 9.2s interfered with the establishment of an AdLOG-specific CTL response. To this end, mice were injected i.v. with 10 µg of 9.2s one day (d-1), three days (d-3) or five days (d-5) before AdLOG infection. OVA-specific CTL activity was suppressed regardless of whether 9.2s was given one day or three days before adenoviral infection. If mice received 9.2s already five days in advance, an antigen-specific CTL response was established, although it was less efficient (circa 50% reduction) in comparison to mice that were immunized with AdLOG alone (Figure 4.3 B). Furthermore, 9.2s-mediated suppression of CTL activity was dose-dependent, as only the highest dose of 10 µg clearly impeded an antigen-specific CTL response against a subsequent AdOVA infection. 1 µg of 9.2s induced only a partial suppression of the CTL response (approximately 23% reduction), whereas a low dose of 0.1 µg 9.2s rather enhanced the cytotoxic activity (Figure 4.3 C). In conclusion, these results showed that ssRNA suppressed CTL responses in a time- and dose-dependent manner.
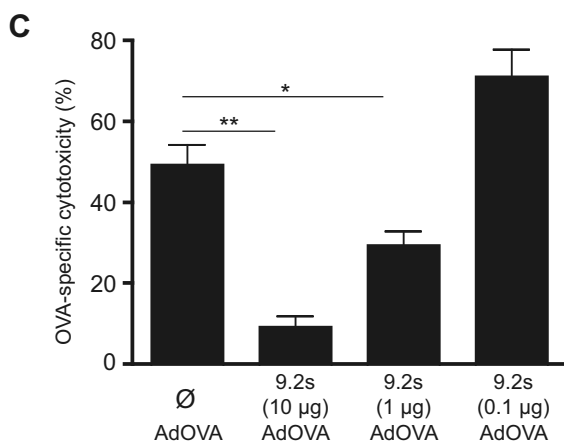
C
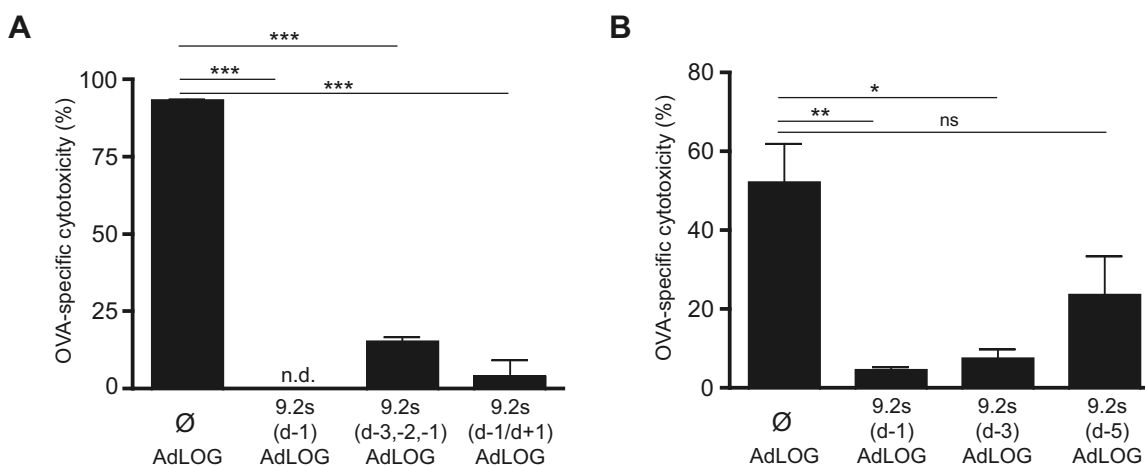
Figure 4.3 Time kinetics and dose dependency of ssRNA-mediated CTL suppression.
Analysis of OVA-specific CTL responses in spleens of C57BL/6 wild type mice five days post AdLOG or AdOVA immunization. (A, B) 10 µg 9.2s was administered i.v. at different time points with regard to AdLOG infection. (C) Wild type mice were injected i.v. with varying amounts of 9.2s (0.1; 1, or 10 µg) one day before infection with AdOVA. OVA-specific lysis is shown as mean +/- SEM with n=3 mice per group.

To investigate if the observed suppressive impact induced by systemic 9.2s was restricted to adenoviral vectors, we performed experiments, in which C57BL/6 wild type mice were infected with other viral or bacterial pathogens. Mice were infected with 1×10^4 PFU Herpes simplex virus type I (HSV-1), 1×10^5 PFU Influenza A PR8/H1N1 virus, or 5×10^3 CFU OVA-expressing *Listeria monocytogenes* (*LmOVA*). Indicated groups received 10 µg of 9.2s i.v. one day prior to infection. Antigen-specific CTL responses were determined in spleens of mice five days post infection by performing *in vivo* cytotoxicity assays. We detected potent CTL responses against HSV-1 (Figure 4.4 A), Influenza virus (Figure 4.4 B), and *LmOVA* (Figure 4.4 C), which were significantly compromised when mice were previously injected with 9.2s. Thus, these results clearly showed that a systemic injection of ssRNA not only abrogated a CTL response against adenoviral vectors, but rather caused a general immune suppression against subsequent infections with various pathogens.
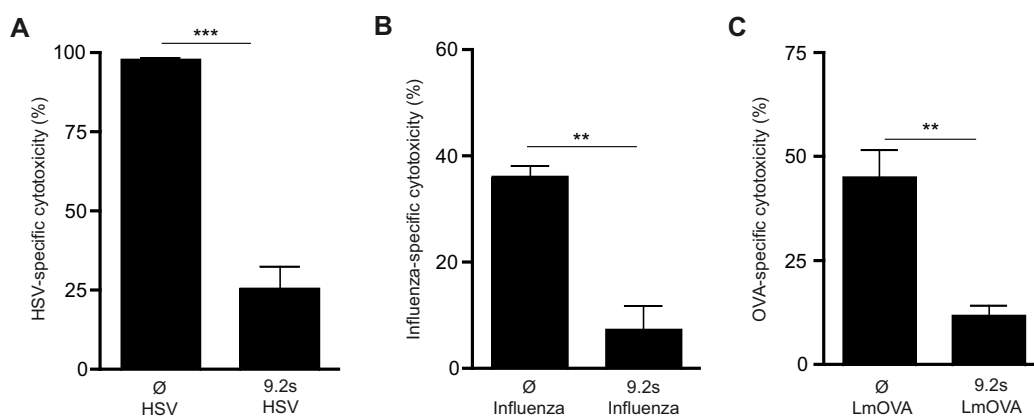
Figure 4.4 ssRNA impeded antigen-specific CTL responses against various pathogens.
C57BL/6 wild type mice were infected with $1x10^4$ PFU HSV-1 (A), $1x10^5$ PFU Influenza
A PR8/H1N1 virus (B), or $5x10^3$ CFU *LmOVA* (C). Indicated groups received 10 µg of 9.2s i.v.
one day prior to infection. Antigen-specific CTL responses were determined in spleens five days
post infection and are illustrated as mean +/- SEM (n=3 mice per group). Representative data of
two independent experiments are shown.

4.1.2 Cytoplasmic targeting of ssRNA prevents suppression of antigen-specific CTL responses

Unlike immunostimulatory DNA sequences, naked RNA molecules are highly susceptible
to degradation by RNases. Thus, a complexation with different carriers, like cationic
liposomes or polyethylene imines is necessary (Diebold et al., 2004; Heil et al., 2004;
Hornung et al., 2005). Data that have been presented in the previous chapter were obtained
with 9.2s that had been stabilized with cationic liposomes (DOTAP). DOTAP not only
protects RNA from degradation but also increases the uptake into cells and targets RNA to
the endosomal compartment containing TLR7 (Almofti et al., 2003; Yasuda et al., 2005).
A different complexing agent termed *in vivo*-jetPEI™, which is a linear polyethylene
imine, facilitates the delivery into the cytoplasm after osmotic rupture of the endosome and
enhances the entry into the nucleus (Boussif et al., 1995; Brunner et al., 2002; Kichler et
al., 1995). Therefore, we analyzed whether CTL suppression was associated with the
subcellular distribution of RNA oligoribonucleotides and whether inhibition of subsequent
antigen-specific immune responses could be prevented when ssRNA was complexed with
the carrier *in vivo*-jetPEI™, thereby circumventing endosomal recognition by TLR7. To
this end, we infected C57BL/6 wild type mice i.v. with AdLOG, HSV-1, Influenza A
PR8/H1N1 virus, or *LmOVA*. Indicated groups were previously injected with 10 µg 9.2s,
which had been complexed with either DOTAP (9.2s/DOTAP) or *in vivo*-jetPEI™
(9.2s/PEI). Antigen-specific CTL responses were quantified in spleens of mice five days
post infection. Only 9.2s/DOTAP caused suppression of antigen-specific CTL responses
against AdLOG (Figure 4.5 A), HSV-1 (Figure 4.5 B), Influenza virus (Figure 4.5 C), and
LmOVA (Figure 4.5 D) as already shown in Figure 4.4. However, if 9.2s was stabilized by
in vivo-jetPEI™, antigen-specific CTL responses were not impeded but were comparable
to mice that had been exposed to the pathogen alone (Figure 4.5). Thus, ssRNA-induced
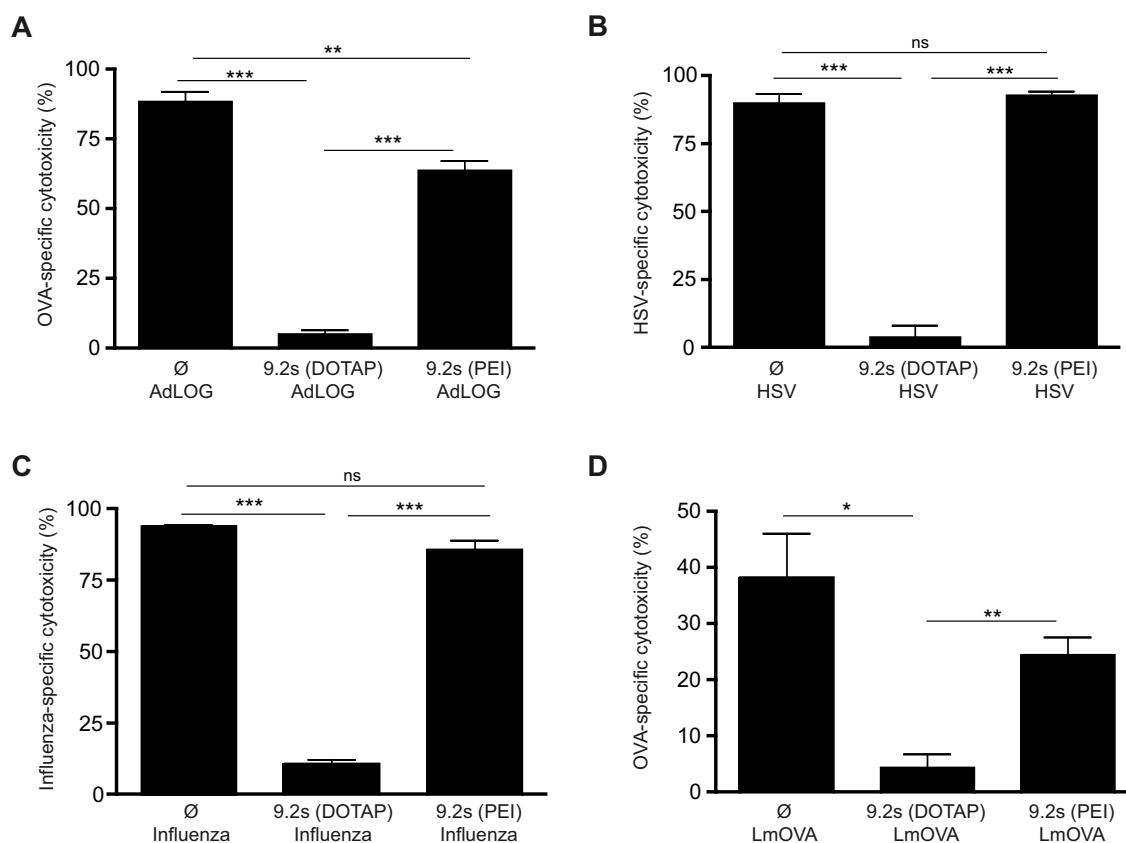immune suppression was avoided by complexation with the carrier *in vivo*-jetPEI™.

Figure 4.5   ssRNA delivery into the cytoplasm abolished suppression of antigen-specific CTL responses.
C57BL/6 wild type mice were infected with $5x10^9$ virus particles of AdLOG (A), $1x10^4$ PFU HSV-1 (B), $1x10^5$ PFU Influenza A PR8/H1N1 virus (C), or $5x10^3$ CFU *LmOVA* (D). Indicated groups were injected i.v. with 10 µg 9.2s, which had been complexed with either DOTAP (9.2s/DOTAP) or *in vivo*-jetPEI^{TM} (9.2s/PEI), one day prior to pathogen infection. Antigen-specific CTL responses were quantified in spleens five days post infection and are displayed as mean +/- SEM with n=3 mice per group. Representative data of at least two independent experiments are shown.

Furthermore, we aimed to confirm that RNA complexation with *in vivo*-jetPEI^{TM} circumvented the suppression of CTL responses due to a distinct intracellular localization and not due to a difference in the *in vivo* distribution of ssRNA. Therefore, we made use of Alexa647-labeled RNA that was complexed with either the carrier DOTAP or *in vivo*-jetPEI^{TM}. Regardless of whether this RNA was stabilized with DOTAP or *in vivo*-jetPEI^{TM}, an overall *in vivo* distribution was seen upon systemic injection (Figure 4.6 A). To study the intracellular localization of DOTAP-versus *in vivo*-jetPEI^{TM}- complexed RNA, we incubated bone marrow-derived DCs (BMDCs) with 500 ng of RNA-Alexa647 that had been stabilized with either DOTAP or *in vivo*-jetPEI^{TM}. Endosomes were identified by coincubation with 20 µg/ml FITC-labeled OVA known to be directed into endosomes upon receptor-mediated uptake (Burgdorf et al., 2007). Fluorescence microscopic analysis was

performed 20 minutes after RNA addition. In accordance to other studies, we detected an endosomal localization of DOTAP-complexed RNA, while RNA was distributed in the cytoplasm and nucleus when stabilized with *in vivo*-jetPEI^{TM} (Figure 4.6 B).
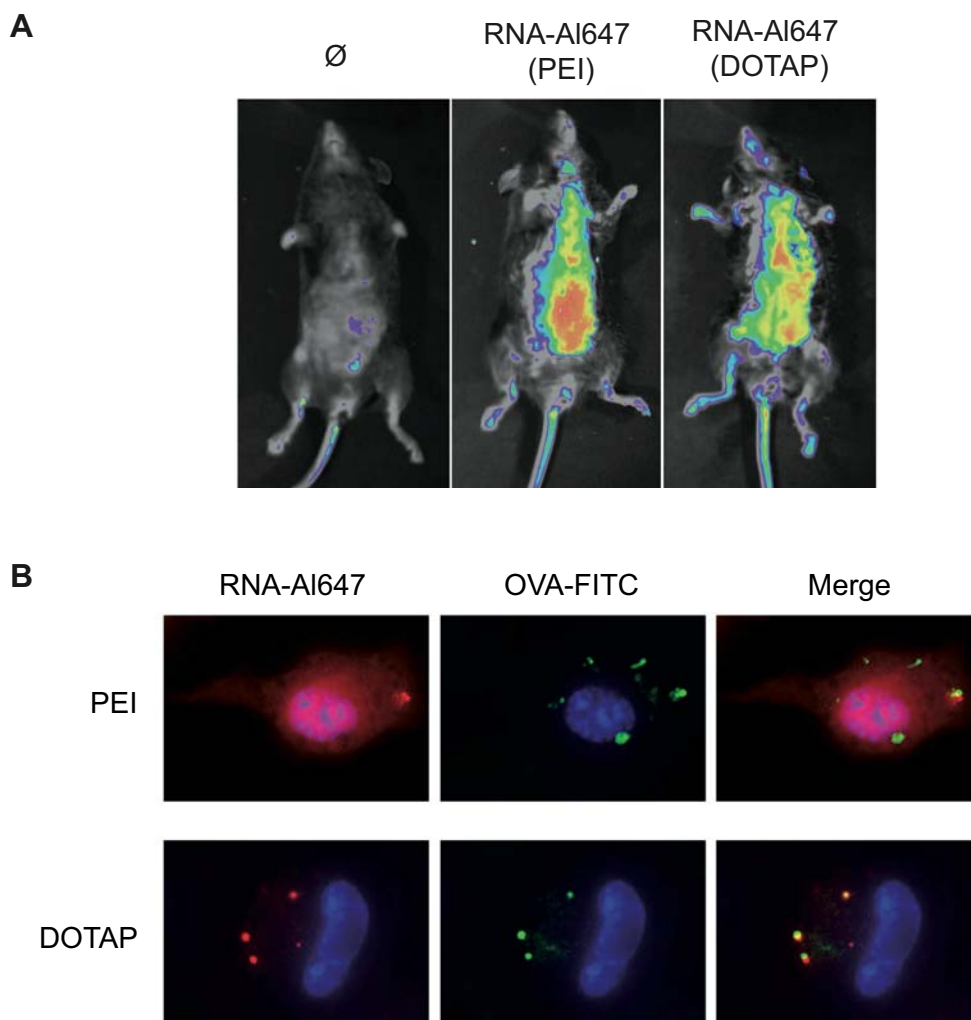

Figure 4.6   Complexation of RNA with DOTAP or *in vivo*-jetPEI^{TM} targeted RNA to distinct intracellular compartments.
(A) *In vivo* distribution of Alexa647-labeled RNA, complexed with either the carrier DOTAP or *in vivo*-jetPEI^{TM}, was analyzed upon i.v. injection using the NightOWL Scanner applying a Cy5 filter. One representative mouse out of two is depicted. (B) Intracellular localization of RNA was investigated in BMDCs by fluorescence microscopy. BMDCs were incubated with 500 ng of RNA-Alexa647, which had been complexed to either DOTAP or *in vivo*-jetPEI^{TM}. Endosomes were identified by coincubation with 20 µg/ml FITC-labeled OVA. Fluorescence microscopic analysis was performed 20 minutes after RNA addition. Representative pictures of at least three independent experiments are shown.

In conclusion, our data revealed that ssRNA-induced immune suppression was avoided by complexation with the carrier *in vivo*-jetPEI^{TM}, which promoted the delivery of RNA to the cytoplasm thereby circumventing endosomal TLR7 recognition.

4.1.3 Differential regulation of AdOVA-specific effector CD8 T cell responses by distinct intracellular targeting of ssRNA

Based on our findings that systemic ssRNA induced a TLR7 dependent suppression of subsequent CTL responses, we wondered whether the lack of cytotoxic activity was due to tolerization (Steinman et al., 2003) or rather due to an absent generation of antigen-specific effector CD8 T cells. To address this question, mice were either left untreated or injected i.v. with 10 µg of 9.2s/DOTAP or 9.2s/PEI one day prior to AdOVA infection. The generation of endogenous OVA-specific CD8 T cells was assayed five days post AdOVA infection in spleens by flow cytometric analysis of S8L/H-2K^b specific pentamers and the total number of OVA-specific CD8 T cells per spleen was calculated. A massive expansion of pentamer-positive OVA-specific CD8 T cells was detected in mice that had been immunized with AdOVA alone, whereas the previous injection of DOTAP-complexed 9.2s avoided the generation of OVA-specific CD8 T cells and the number of pentamer-positive CD8 T cells was comparable to those observed in naive mice. In contrast, a significant increase in endogenous OVA-specific CD8 T cells was also noticed if 9.2s was stabilized using the carrier *in vivo*-jetPEI^{TM} (Figure 4.7 A and B).

Moreover, we monitored the activation status of OVA-specific CD8 T cells by staining for CD62L on S8L/H-2K^b pentamer-positive cells. CD62L expression on naive T cells is required for their efficient homing to lymph nodes. It is rapidly downregulated upon T cell activation, which then gain access to peripheral tissues (Butcher and Picker, 1996; Mascarell and Truffa-Bachi, 2004). In AdOVA immunized mice, most OVA-specific CD8 T cells (74.0% ± 1.8) showed an activated phenotype, as evidenced by a low expression level of CD62L. If mice received a pre-injection of 9.2s/DOTAP, only 37% ± 1.8 of OVA-specific CD8 T cells exhibited a low expression of CD62L, whereas 68.3% ± 2.9 of pentamer-positive CD8 T cells downregulated CD62L, if 9.2s was stabilized with *in vivo*-jetPEI^{TM} (Figure 4.7 D). Furthermore, just like in naive mice, almost no activated OVA-specific CD8 T cells were present in spleens after AdOVA immunization, if mice had been pretreated with 9.2s/DOTAP. Comparable total numbers of activated pentamer-positive CD8 T cells were present in mice, which had been infected with AdOVA alone or received 9.2s/PEI prior to AdOVA administration (Figure 4.7 C).

In addition to activation and cytotoxic activity of OVA-specific CD8 T cells, we determined how effector cytokine production was affected by a preceding administration of 9.2s in response to AdOVA immunization. Splenocytes were isolated from AdOVA or 9.2s/AdOVA injected mice and restimulated for 24 hours with the OVA-derived peptide

S8L. IFNγ production was measured by intracellular cytokine staining and flow cytometric analysis or in cell culture supernatants by ELISA. IFNγ was produced by 2.7% ± 0.3 of CD8 T cells in response to AdOVA infection. However, IFNγ production was almost absent if mice had been injected with 9.2s/DOTAP before (0.2% ± 0.1). In contrast, effector cytokine production was rescued, if 9.2s was directed into the cytoplasm by stabilization with *in vivo*-jetPEI^{TM} (1.9% ± 0.3) (Figure 4.7 E and F). The same result was obtained, when IFNγ secretion was quantified in culture supernatants of S8L-restimulated splenocytes (Figure 4.7 G). In summary, suppression of antigen-specific CTL responses by DOTAP-complexed ssRNA was accompanied by an impaired expansion, activation, and IFNγ production of OVA-specific CD8 T cells.
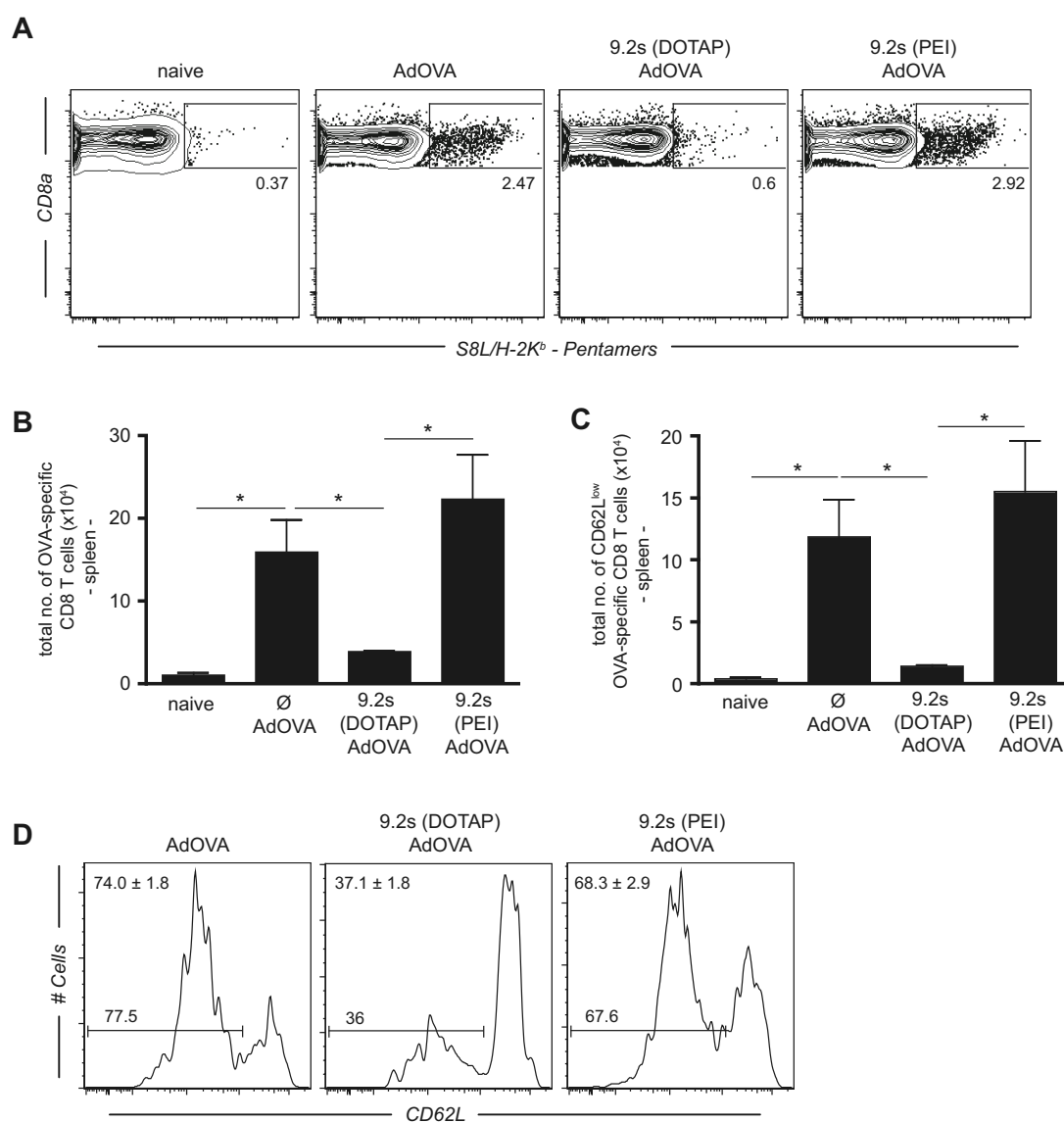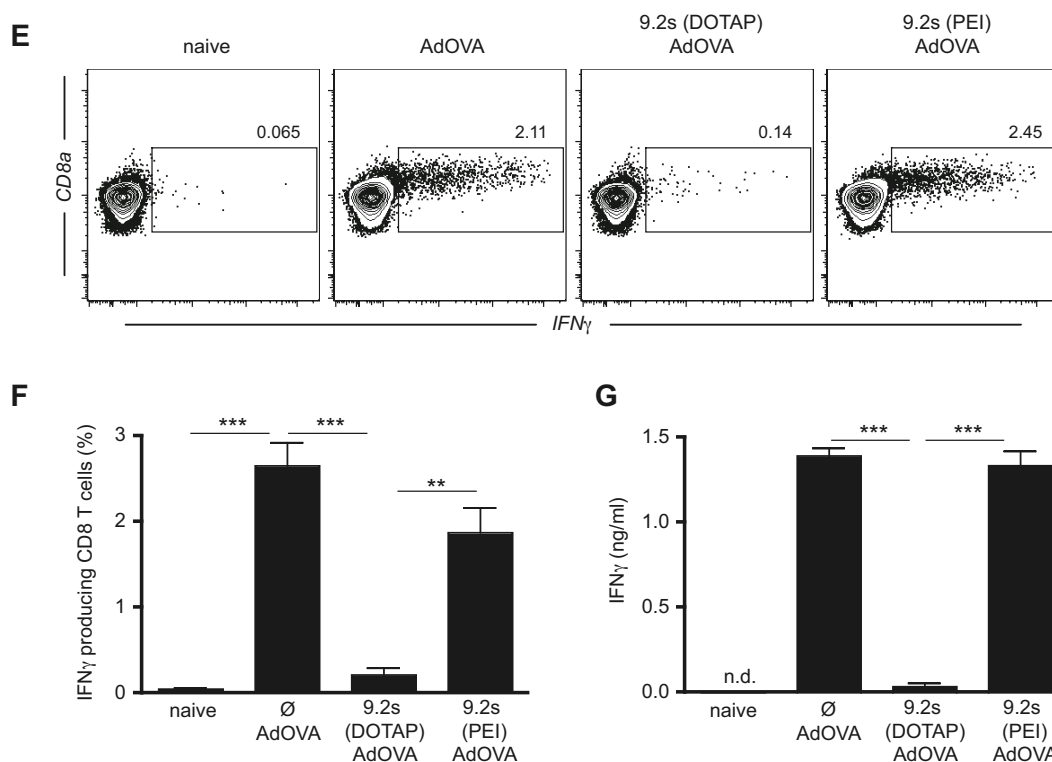
Figure 4.7 DOTAP-stabilized ssRNA interfered with the expansion, activation, and cytokine secretion of OVA-specific CD8 T cells.

C57BL/6 wild type mice were immunized i.v. with 5×10^9 virus particles of AdOVA alone or mice received i.v. 10 µg of 9.2s/DOTAP or 9.2s/PEI one day prior to infection. (A) The generation of endogenous OVA-specific CD8 T cells was assayed by flow cytometry five days post AdOVA infection by S8L/H-2K^b pentamer staining of splenocytes and (B) the total number of OVA-specific CD8 T cells per spleen was calculated. (C, D) Expression of CD62L was examined on S8L/H-2K^b pentamer positive endogenous OVA-specific CD8 T cells (D) and the total count of CD62^{low} OVA-specific CD8 T cells in the spleen was determined (C). (E-G) Splenocytes, isolated from AdOVA or 9.2s/AdOVA immunized mice, were restimulated with the OVA-specific peptide S8L. IFNγ production of OVA-specific CD8 T cells was measured by intracellular cytokine staining and flow cytometric analysis (E, F) or in splenocyte supernatants by ELISA (G). Representative dot plots or histograms of one mouse out of three are shown. Data presented in bar graphs show mean +/- SEM (n=3 mice per group).

Furthermore, we investigated if a preceding i.v. injection of ssRNA also suppressed transgenic OVA-specific OT-I T cells in response to AdOVA immunization (Hogquist et al., 1994). To this end, we adoptively transferred 5×10^4 OT-I T cells expressing the congenic marker Thy1.1 into naive C57BL/6 wild type recipient mice. Afterwards, mice were either left untreated or injected i.v. with 10 µg of 9.2s/DOTAP or 9.2s/PEI one day before AdOVA immunization. Five days post infection, expansion of transgenic OT-I T cells was examined in spleens by flow cytometric analysis of CD8a and Thy1.1 double-positive lymphocytes. A massive accumulation of OT-I T cells was detected in mice that had been infected with AdOVA alone (2.6% ± 0.14% of splenocytes) and in

those that had received 9.2s/PEI prior to AdOVA administration (2.21% ± 0.16% of splenocytes). Again, only a moderate increase in OT-I T cells was evident (0.45% ± 0.04% of splenocytes), if mice had been pre-injected with 9.2s that had been targeted to the endosome by complexation with DOTAP (Figure 4.8 A). Additionally, we analyzed the activation of OT-I T cells by staining of CD62L. Most OT-I T cells revealed an activated phenotype as reflected by low CD62L expression, if mice had been immunized with AdOVA alone (72.8% ± 0.7) or pre-injected with 9.2s/PEI (57.1% ± 4.9). In contrast, administration of 9.2s/DOTAP prior to AdOVA infection interfered with T cell activation as the majority of OT-I T cells still expressed high levels of CD62L (Figure 4.8 B). Finally, we determined whether OT-I T cells were impaired in IFNγ production upon antigen-specific restimulation. Here, splenocytes were isolated five days post AdOVA or 9.2s/AdOVA administration and restimulated with the OVA-specific peptide S8L. The percentage of IFNγ secreting OT-I T cells was quantified by intracellular cytokine staining and subsequent flow cytometric analysis. Regardless of whether mice were immunized with AdOVA alone or had been previously injected with 9.2s, similar percentages of IFNγ producing OT-I T cells were detected upon restimulation (Figure 4.8 C). In conclusion, these data clearly demonstrate that 9.2s/DOTAP-mediated CTL suppression was not a result of T cell tolerization but rather due to an impaired activation and expansion of antigen-specific CD8 T cells.
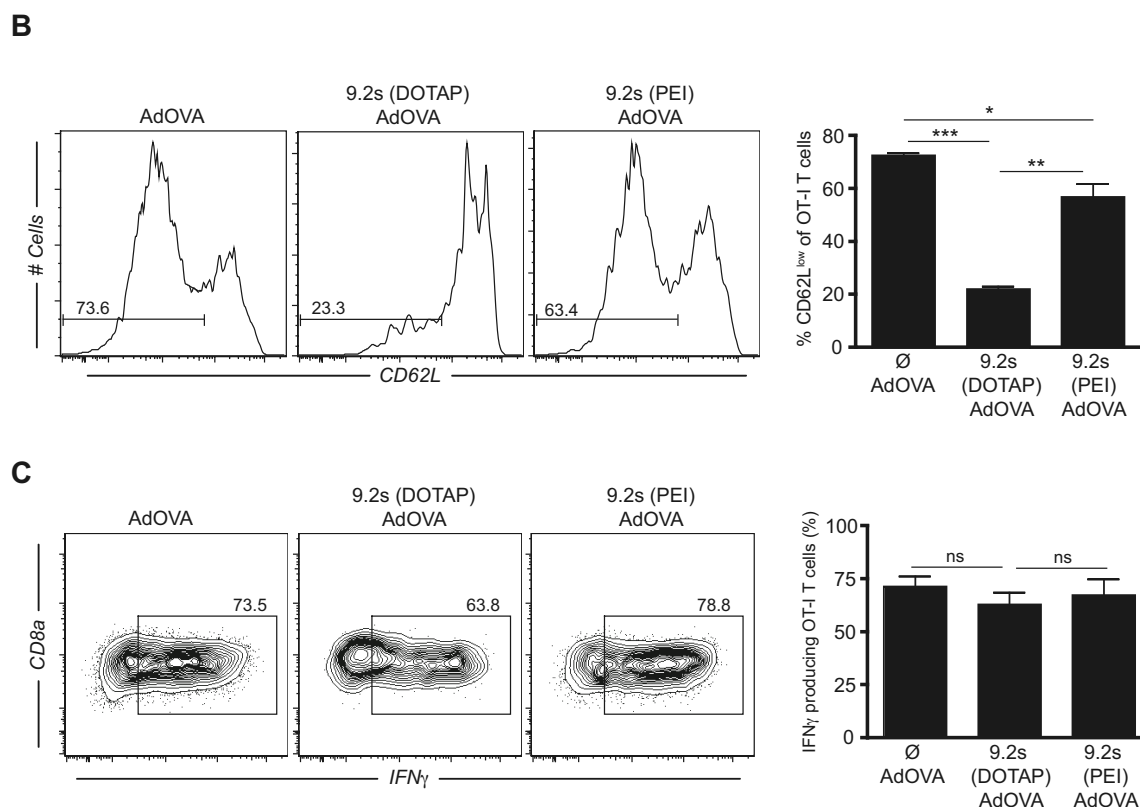
B

C
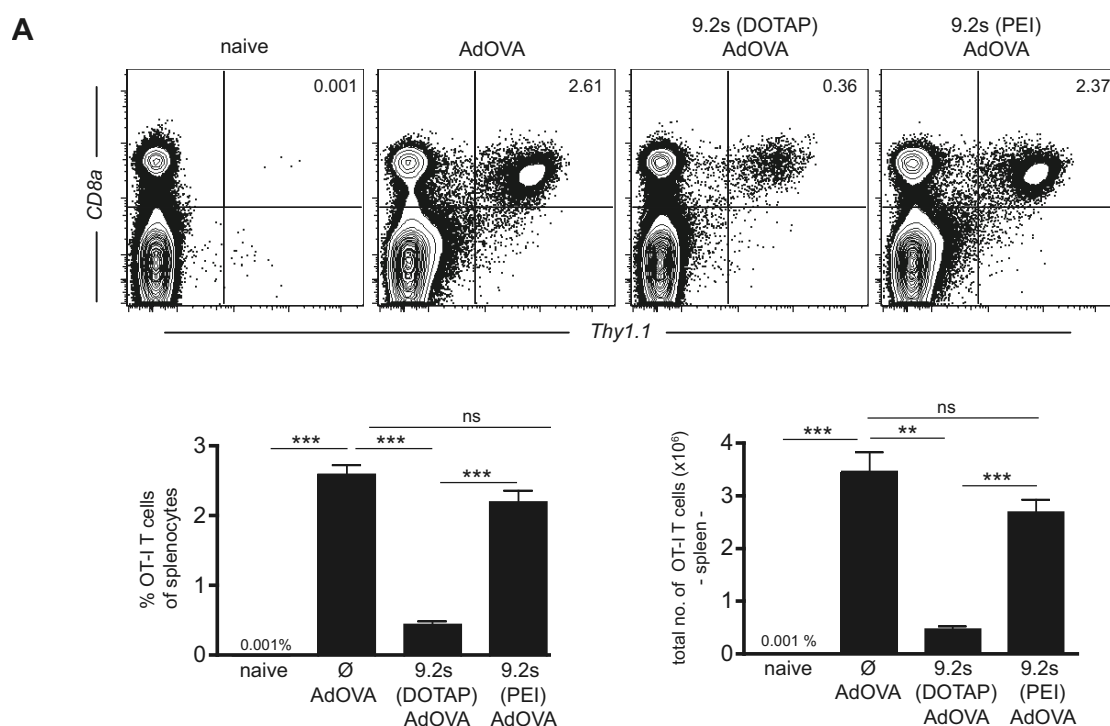
Figure 4.8   Targeting of ssRNA to the endosomes inhibited expansion and efficient activation of transgenic OT-I T cells in response to AdOVA infection.

C57BL/6 wild type mice received 5×10^4 transgenic OT-I/Thy1.1$^+$ T cells. Subsequently, mice were infected i.v. with AdOVA alone or were injected i.v. with 10 µg of 9.2s/DOTAP or 9.2s/PEI one day before adenoviral immunization. (A) Expansion and total number of transgenic OT-I/Thy1.1$^+$ cells was determined in spleens five days post AdOVA administration. (B) CD62L expression was monitored on OT-I/Thy1.1$^+$ cells and the percentage of CD62L^{low} OT-I/Thy1.1$^+$ cells is depicted. (C) Splenocytes were isolated from AdOVA or 9.2s/AdOVA immunized mice and IFNγ production by OT-I/Thy1.1$^+$ CD8 T cells was analyzed by intracellular cytokine staining upon S8L restimulation. Representative dot plots or histograms of one mouse out of three are shown. Data presented in bar graphs are illustrated as mean +/- SEM (n=3 mice per group).

4.1.4   Antigen-specific CD4 T cell responses are impeded by endosomal targeting of ssRNA

It is known, that cognate CD4 T cell help is indispensable to establish an efficient and long-lasting CD8 T cell response. CD4 T cell help was also essential for a strong CTL response against AdOVA, since the cytotoxic activity was significantly impaired in mice that lack CD4 T cells and MHC class II molecules (Figure 4.9 A and B, data kindly provided by Beatrix Schumak).
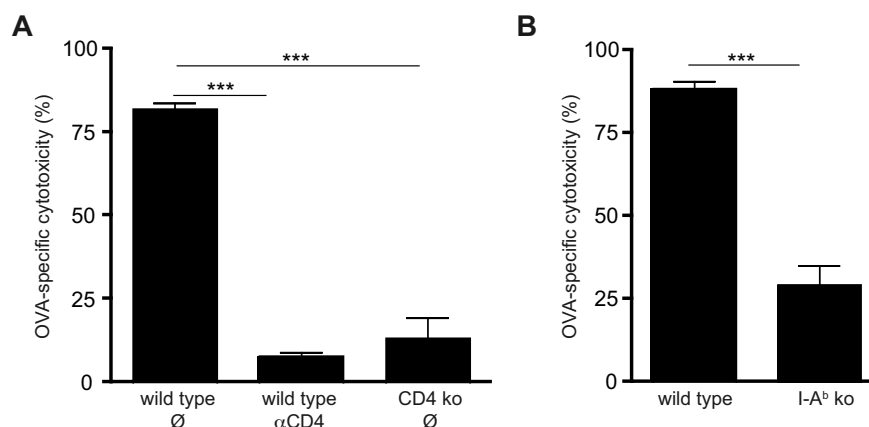
Figure 4.9 Efficient AdOVA-specific CTL response required CD4 T cell help.
OVA-specific CTL responses were examined in spleens of C57BL/6 wild type mice ± CD4 depleting antibody, in CD4 ko (A), and I-A^b ko mice (B) five days post AdOVA infection. 500 µg of CD4 depleting antibody was given i.p. two days before AdOVA challenge (A). Data are shown as mean +/- SEM with n=3 mice per group.

Based on these findings, we asked the question whether systemic injection of ssRNA oligoribonucleotides also affected antigen-specific CD4 T cell responses. First, we investigated if proliferation of OVA-specific CD4 T cells was impaired in response to 9.2s and AdOVA immunization. Here, we adoptively transferred $2x10^6$ CFSE-labeled transgenic OVA-specific CD4 T cells (OT-II) into naive C57BL/6 mice. Two days upon transfer, mice were immunized i.v. with AdOVA alone or indicated groups received 10 µg of 9.2s/DOTAP or 9.2s/PEI one day prior to adenoviral challenge. Proliferation was monitored three days post AdOVA infection by a CFSE dilution assay.
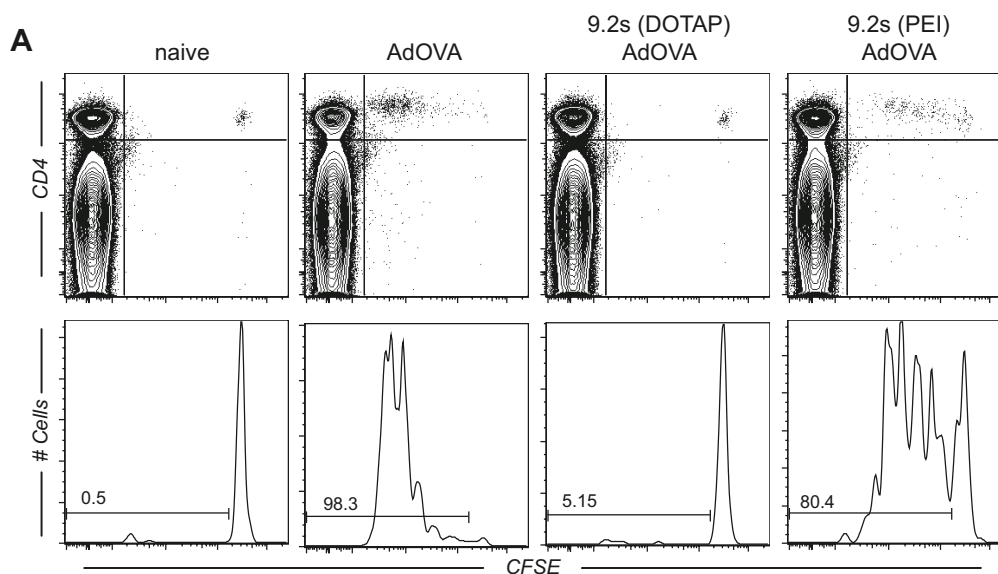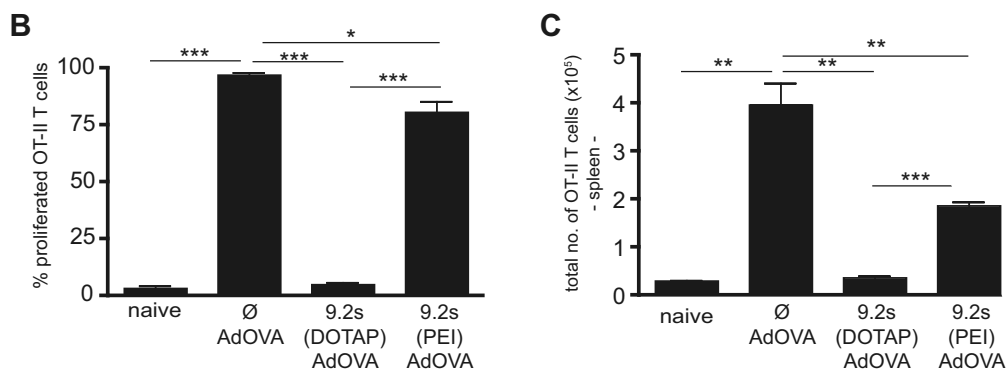
Figure 4.10   DOTAP-complexed ssRNA impaired proliferation and expansion of OVA-specific CD4 T cells.
2×10^6 CFSE-labeled transgenic OVA-specific CD4 T cells (OT-II) were adoptively transferred into naive C57BL/6 wild type mice. Two days upon transfer, mice were immunized i.v. with AdOVA alone or indicated groups received 10 µg of 9.2s/DOTAP or 9.2s/PEI i.v. one day prior to adenovirus administration. (A) Proliferation of OT-II cells was monitored three days post AdOVA challenge by a CFSE dilution assay. In (B) the percentage of proliferated OT-II cells is depicted and the total number of OT-II cells per spleen is shown in (C). The experiment was performed with n=3 mice per group.

Strong proliferation of OT-II T cells was detected in mice that had been infected with AdOVA alone or those that had received a preceding injection of 9.2s/PEI. Although the percentage of proliferated OT-II T cells was similar in AdOVA (97.1% ± 0.9) or 9.2s/PEI and AdOVA (80.7 ± 4.6) immunized mice, obviously less OT-II T cells were counted in spleens of 9.2s/PEI and AdOVA injected mice. However, neither proliferation (Figure 4.10 A and B) nor expansion (Figure 4.10 C) of OT-II T cells was observed in mice that had received DOTAP-complexed 9.2s.

Furthermore, we assayed how systemic RNA oligonucleotides influenced the production of effector cytokines like IFNγ and IL-2 in response to AdOVA immunization. C57BL/6 wild type mice were either left untreated or injected i.v. with 9.2s/DOTAP or 9.2s/PEI one day prior to AdOVA challenge. Splenocytes were isolated five days later and endogenous OVA-specific CD4 T cells were restimulated using an MHC class II restricted OVA-specific peptide (OVA $_{265-279}$; OT3p). IFNγ production by splenic CD4 T cells was investigated by intracellular cytokine staining and subsequent flow cytometric analysis. In addition, the release of IFNγ and IL-2 was measured by ELISA in splenocyte culture supernatants.
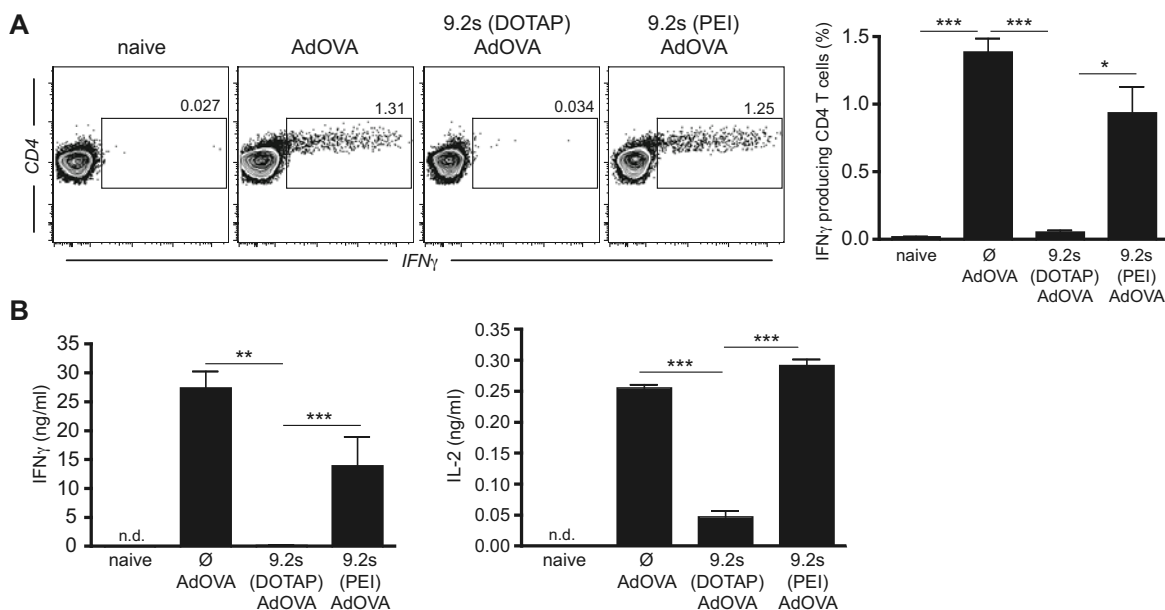
Figure 4.11   DOTAP-stabilized ssRNA impeded effector cytokine production by CD4 T cells upon AdOVA challenge.
C57BL/6 wild type mice were either left untreated or injected i.v. with 10 µg of 9.2s/DOTAP or 9.2s/PEI one day before AdOVA infection. Splenocytes were isolated five days post AdOVA challenge and endogenous OVA-specific CD4 T cells were restimulated using an MHC class II restricted OVA-specific peptide (OVA$_{265-279}$; OT3p). IFNγ production by CD4 T cells was investigated by intracellular cytokine staining and flow cytometric analysis (A). Secretion of IFNγ and IL-2 was measured in splenocyte culture supernatants by ELISA (B). Representative data in bar graphs are shown as mean +/- SEM. A representative dot plot of one mouse is depicted (n=3 mice per group).

Targeting of 9.2s into the endosome by DOTAP complexation impeded effector cytokine production in response to AdOVA stimulation, as no IFNγ producing CD4 T cells were detected (Figure 4.11 A). Moreover, no IFNγ and only little amounts of IL-2 was secreted in splenocyte cultures (Figure 4.11 B). As expected, IFNγ and IL-2 were produced if 9.2s was directed into the cytoplasm by stabilization with *in vivo*-jetPEI^{TM}.

Thus, these data clearly indicated that the systemic injection of RNA oligoribonucleotides impeded the expansion and effector cytokine production of antigen-specific CD4 T cells, if ssRNA was targeted to the endosome by complexation with DOTAP.

T helper cell function is important to ensure an optimal Ig isotype class switch to IgG antibodies (Grewal and Flavell, 1996; Hollenbaugh et al., 1994). Thus, we quantified OVA-specific IgG antibody titers in sera of wild type mice that had been infected with AdOVA alone or had been pre-injected with 9.2s/DOTAP. OVA-specific IgG titers were

remarkably diminished (ca. fourfold reduction) in 9.2s/AdOVA treated mice indicating that CD4 T cell help was compromised (Figure 4.12).
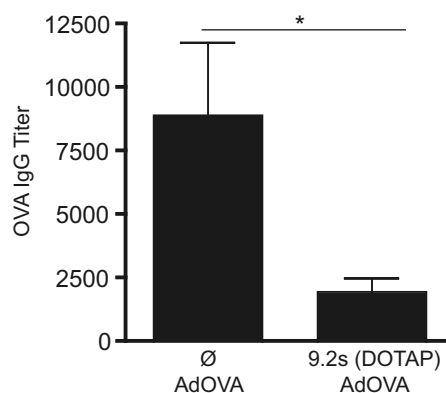

Figure 4.12   Systemic injection of ssRNA interfered with the generation of OVA-specific IgG antibodies.
Titers of OVA-specific IgG antibodies were quantified in sera of C57BL/6 wild type mice 25 days post AdOVA infection. Mice were either left untreated or injected with 10 µg of 9.2s/DOTAP one day prior to AdOVA immunization. Each bar represents the mean +/- SEM with n=5 mice per group. Representative data out of three independent experiments are illustrated.

So far, we have demonstrated that targeting of ssRNA to the endosome interfered with the generation of an antigen-specific CD4 T cell response, thus presumably leading to an impaired CD4 T cell help. Based upon this assumption, we examined whether it might be possible to overcome ssRNA-mediated CTL suppression by providing CD40-CD40L signals. CD40 and CD40L interactions are known to mediate CD4 T cell help and have been implicated in the generation of CTL responses against adenovirus (Bennett et al., 1998; Schoenberger et al., 1998; Toes et al., 1998; Yang et al., 1996). To this end, C57BL/6 wild type mice were immunized i.v. with AdOVA alone or pre-injected i.v. with 10 µg of 9.2s/DOTAP. Indicated groups were injected twice with 100 µg of an anti-CD40 antibody (FGK-45) and OVA-specific lysis was determined in spleens of mice five days post adenoviral challenge. Surprisingly, CD40 stimulation was not sufficient to overcome ssRNA-induced immune suppression (Figure 4.13).
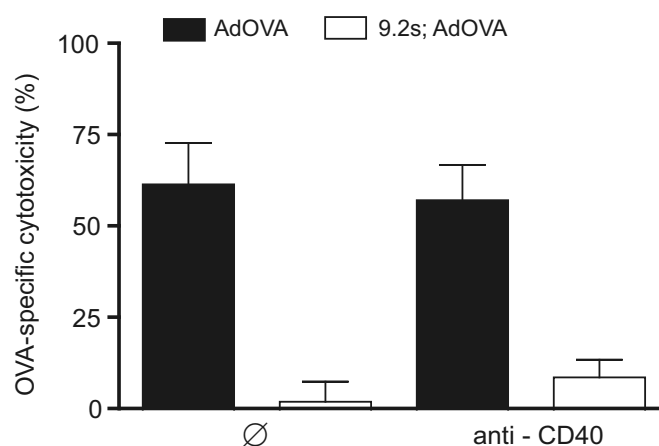
Figure 4.13 CD40 stimulation did not prevent ssRNA-induced CTL suppression.
C57BL/6 wild type mice were immunized i.v. with AdOVA alone or received i.v. 10 µg of
9.2s/DOTAP one day before adenovirus challenge. Indicated groups were injected twice with
100 µg of an anti-CD40 antibody (one injection i.p. on the day of and one injection i.v. one day
after AdOVA infection). OVA-specific cytotoxicity was analyzed five days post AdOVA infection
in spleens of mice. Data are presented as mean +/- SEM (n=3 mice per group).

Furthermore, we addressed the question, if it was possible to prevent ssRNA-induced
CTL suppression in the presence of activated and memory antigen-specific CD4 T cells,
which provide not only CD40-CD40L signals, but license the DCs in an antigen-specific
manner through binding of the TCR to MHC class II molecules and/or by secretion of
cytokines. Endogenous OVA-specific CD4 T cells were activated *in vivo* by s.c.
immunization with 50 µg of $OVA_{265-279}$ peptide (OT3p) in adjuvants six days (for effector
CD4 T cells) or four weeks (for memory CD4 T cells) before AdOVA infection. Indicated
groups were additionally injected i.v. with 9.2s/DOTAP one day before adenovirus
challenge. OVA-specific cytotoxic activity and expansion of OVA-specific CD8 T cells
was analyzed in spleens five days post AdOVA infection. Importantly, ssRNA-induced
abrogation of an AdOVA-specific CD8 T cell response was prevented in the presence of
antigen-specific effector (Figure 4.14 A and C) and memory CD4 T cells (Figure 4.14 B
and D). Thus, suppression of the CD8 T cell response might be overcome in the presence
of CD4 T cell help provided by effector or memory CD4 T cells.
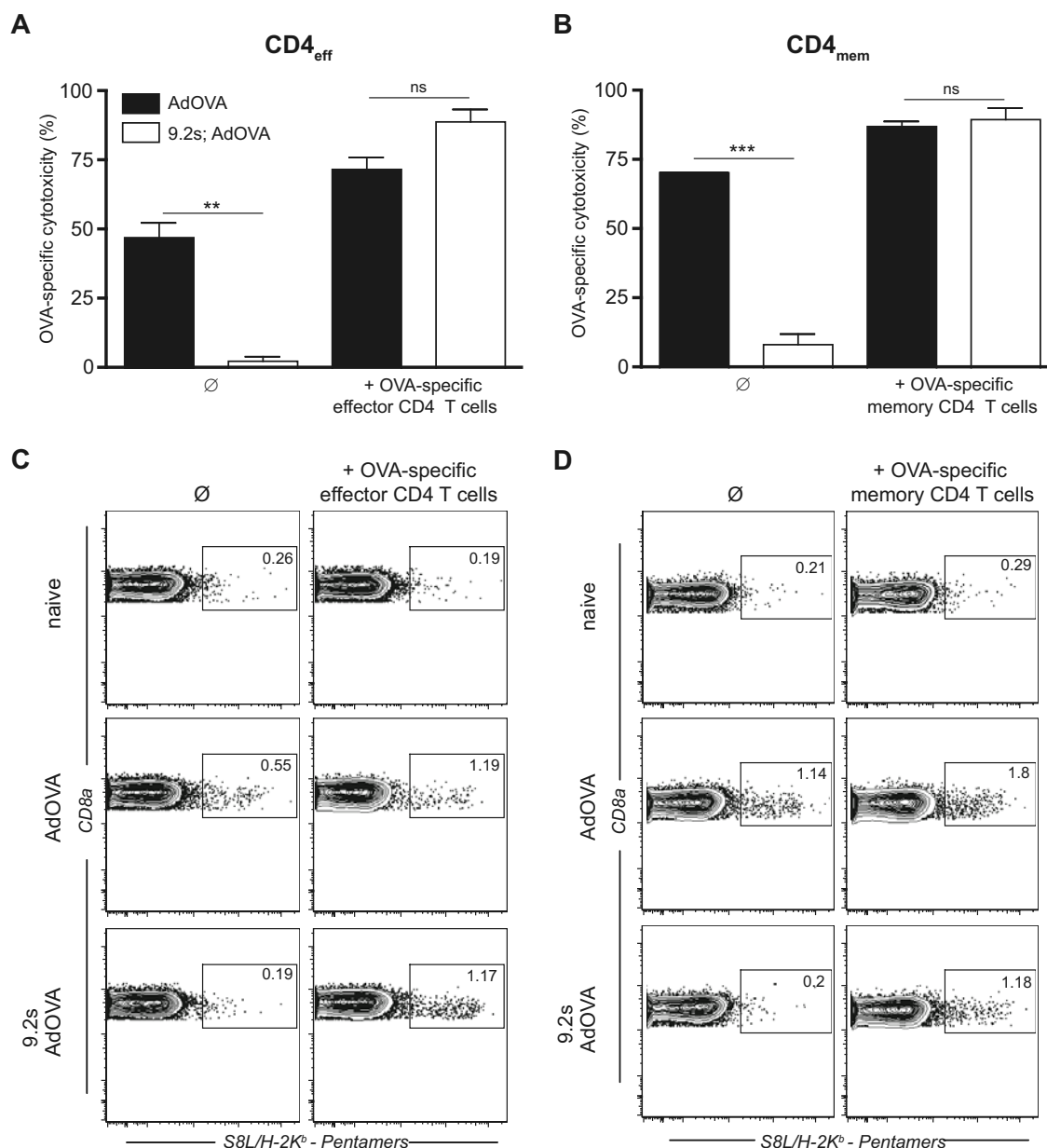
Figure 4.14   Presence of activated and memory antigen-specific CD4 T cells prevented ssRNA-mediated suppression of the CTL response.

Endogenous OVA-specific CD4 T cells were activated *in vivo* by s.c. immunization with 50 µg of OVA$_{265-279}$ peptide, mixed with 5 µg of CpG-1668 and 5 µg of pI:C, six days before AdOVA infection (A, C). For OVA-specific memory CD4 T cell generation, the peptide mixture was given s.c. four weeks before AdOVA challenge (B, D). Indicated groups were injected i.v. with 10 µg 9.2s/DOTAP one day before AdOVA immunization. OVA-specific cytotoxicity (A, B) and the generation of OVA-specific CD8 T cells (C, D) was investigated in spleens of mice five days post AdOVA infection. In bar graphs the mean +/-SEM (with n=3 mice per group) is displayed. Representative S8L/H-2K^{b} - pentamer dot plots of one mouse out of three are presented. Two independent experiments were performed.

4.1.5 IL-10 is not involved in ssRNA-mediated suppression of the CTL response

IL-10 is an anti-inflammatory cytokine, which plays a role in the suppression of immune responses. It is expressed by a variety of immune cells, such as T_H2 cells, T regulatory cells, macrophages, or DCs and suppresses the production of pro-inflammatory cytokines like IL-12, which is important for an efficient T cell response (Fiorentino et al., 1989; Fiorentino et al., 1991; Maynard and Weaver, 2008; Mosser and Zhang, 2008). As we observed suppression of CTL responses upon RNA oligoribonucleotide administration, we speculated whether IL-10 production might be stimulated upon systemic ssRNA administration. To address this question, we used C57BL/6 wild type and IL-10 ko mice, which were infected with AdOVA alone or had been injected i.v. with 10 µg 9.2s/DOTAP one day before adenovirus infection. OVA-specific CTL responses were assessed in spleens five days post infection. 9.2s-induced suppression of an AdOVA-specific CTL response was still observed in IL-10 ko mice, implying that IL-10 was not involved (Figure 4.15).
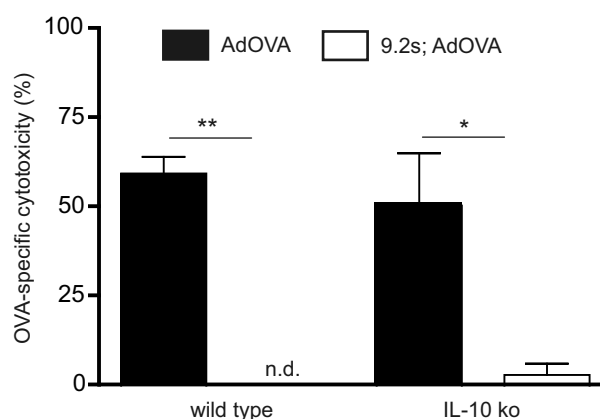
Figure 4.15 ssRNA-mediated CTL suppression was independent of IL-10.
C57BL/6 wild type and IL-10 ko mice were immunized i.v. with either AdOVA alone or injected i.v. with 10 µg of 9.2s/DOTAP one day before adenovirus infection. OVA-specific lysis was measured in spleens five days post AdOVA infection. Data are presented as mean +/- SEM (n=3 mice).

4.1.6 ssRNA-induced CTL suppression depends on type I interferon

It has been reported that detection of viral and non-viral nucleic acids by pattern-recognition receptors results in the induction of type I interferons (IFN), which play a

crucial role in linking innate and adaptive immunity. Type I IFN serves as a signal 3 (in addition to TCR stimulation (signal 1) and costimulation (signal2) to obtain an efficient cytotoxic CD8 T cell response (Curtsinger et al., 2005; Decker et al., 2005; Diebold et al., 2004; Hornung et al., 2005; Lund et al., 2004; Uematsu and Akira, 2007).

Thus, we investigated if systemic administration of 9.2s triggered the release of type I IFNs. To this end, C57BL/6 wild type mice were injected i.v. with 10 μg of 9.2s/DOTAP and type I IFN secretion was monitored at different time points post injection in sera and in splenocyte culture supernatants using a type I IFN bioassay (Nickolaus et al., 1998).
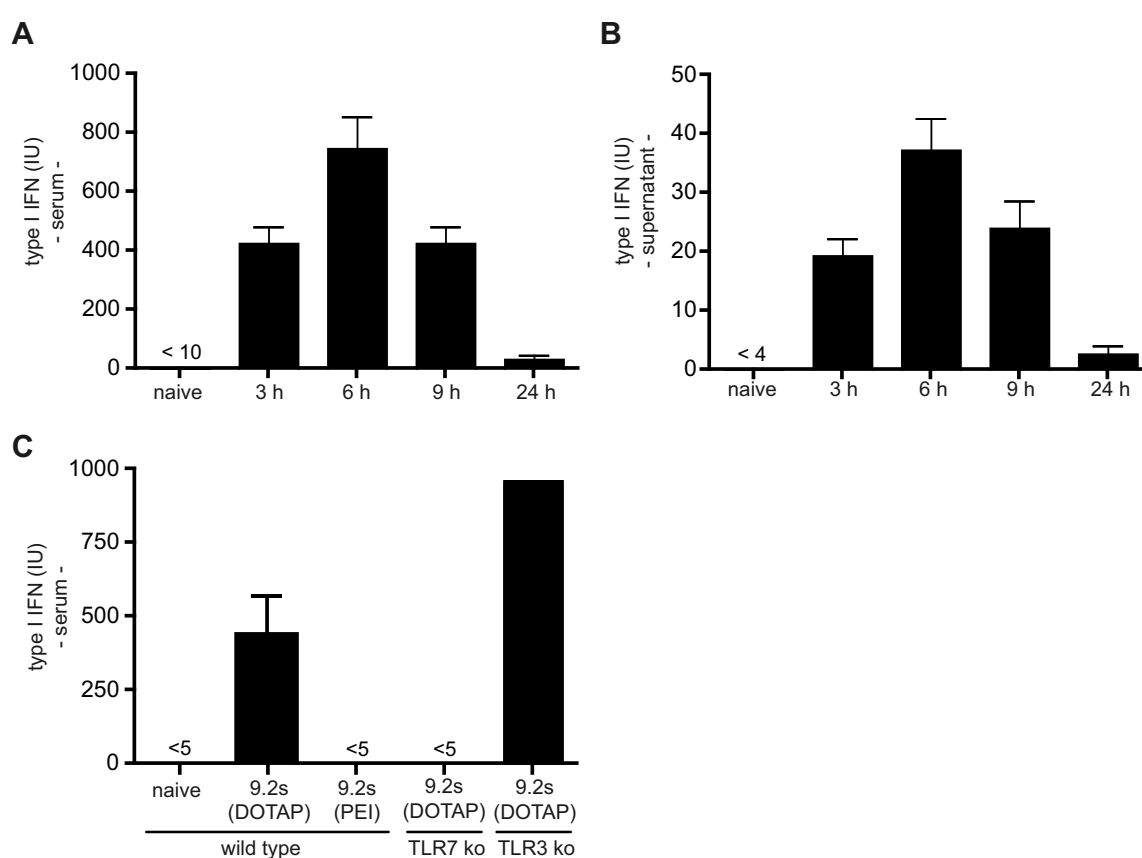
Figure 4.16 DOTAP-complexed ssRNA induced the production of type I interferons.
10 μg of DOTAP-stabilized 9.2s was administered i.v. into mice. (A, B) Type I interferon secretion was analyzed in sera (A) and splenocyte supernatants (B) of C57BL/6 wild type mice at indicated time points post 9.2s injection. (C) Type I interferon production was quantified in sera of wild type, TLR7 ko, and TLR3 ko mice six hours after i.v. injection of DOTAP or *in vivo*-jetPEI[TM] complexed 9.2s.

Type I IFN was detected as early as three hours post 9.2s injection both in sera (Figure 4.16 A) and splenocyte culture supernatants (Figure 4.16 B). Highest levels of type I IFN secretion was recorded six hours after 9.2s challenge, which declined

progressively until almost no type I IFN was measured 24 hours post 9.2s injection (Figure 4.16 A and B). In order to analyze if the initiation of type I IFN required endosomal recognition of 9.2s by TLR7, we performed the experiment in TLR7 ko mice or complexed 9.2s to the carrier *in vivo*-jetPEI^{TM}, which targets 9.2s to the cytoplasm. As expected, we did not detect type I IFN secretion in TLR7 ko mice nor in mice that had received *in vivo*-jetPEI^{TM}-stabilized 9.2. High amounts of type I IFN were found in sera of TLR3 ko mice in response to 9.2s/DOTAP, which was consistent with the result that ssRNA-mediated CTL suppression did not involve TLR3 (Figure 4.16 C).

As 9.2s stimulated the production of type I IFNs, we addressed the question whether the generation of antigen-specific CTL responses was inhibited through the induction of type I IFNs despite their well-described immune-activating properties (Akira et al., 2006; Curtsinger et al., 2005; Uematsu and Akira, 2007). Here, we used IFNAR1-deficient mice, which cannot respond to type I IFNs due to a lack of the receptor (van den Broek et al., 1995a; van den Broek et al., 1995b). C57BL/6 wild type and IFNAR1 ko mice were either left untreated or injected i.v. with 10 μg of 9.2s/DOTAP one day prior to AdLOG immunization. Five days post infection, OVA-specific cytotoxicity was quantified in spleens of mice. Additionally, the generation of OVA-specific CD8 T cells was determined by flow cytometry analysis of S8L/H-2K^b pentamers. Interestingly, 9.2s did not impair the cytotoxic activity (Figure 4.17 A; data kindly provided by Beatrix Schumak) and generation of OVA-specific CD8 T cells (Figure 4.17 B) in IFNAR1 deficient mice, supporting the notion that 9.2s-induced type I IFNs caused the inhibition of subsequent CTL responses.

To further strengthen the hypothesis that type I IFNs act in an immunosuppressive way on adaptive immune responses, we analyzed the influence of recombinant type I IFNs on AdLOG-specific cytotoxic activity in wild type mice. Mice were injected i.v. with 1000U IFNα, IFNβ, or IFNα/β one day before AdLOG immunization. As a positive control, mice received 9.2s/DOTAP. The cytotoxic activity of OVA-specific CD8 T cells was remarkably decreased (reduction in cytotoxic activity of about 50%) in the presence of type I IFN in comparison to mice that had been challenged with AdLOG alone. However, the inhibition of the CTL response was not as severe as if 9.2s was applied, which might be due to lower levels of circulating recombinant type I IFN compared to the amount that was induced by 9.2s administration (Figure 4.17 C).

Finally, we revealed that 9.2s-mediated CTL suppression was not exclusively dependent on IFNβ, as 9.2s administration did not interfere with the OVA-specific CTL response in IFNβ deficient mice (Figure 4.17 D).

Taken together, our results demonstrate that systemic ssRNA application induced the production of type I IFNs, which inhibited antigen-specific CTL responses in the spleen despite its well-known immune stimulatory property.
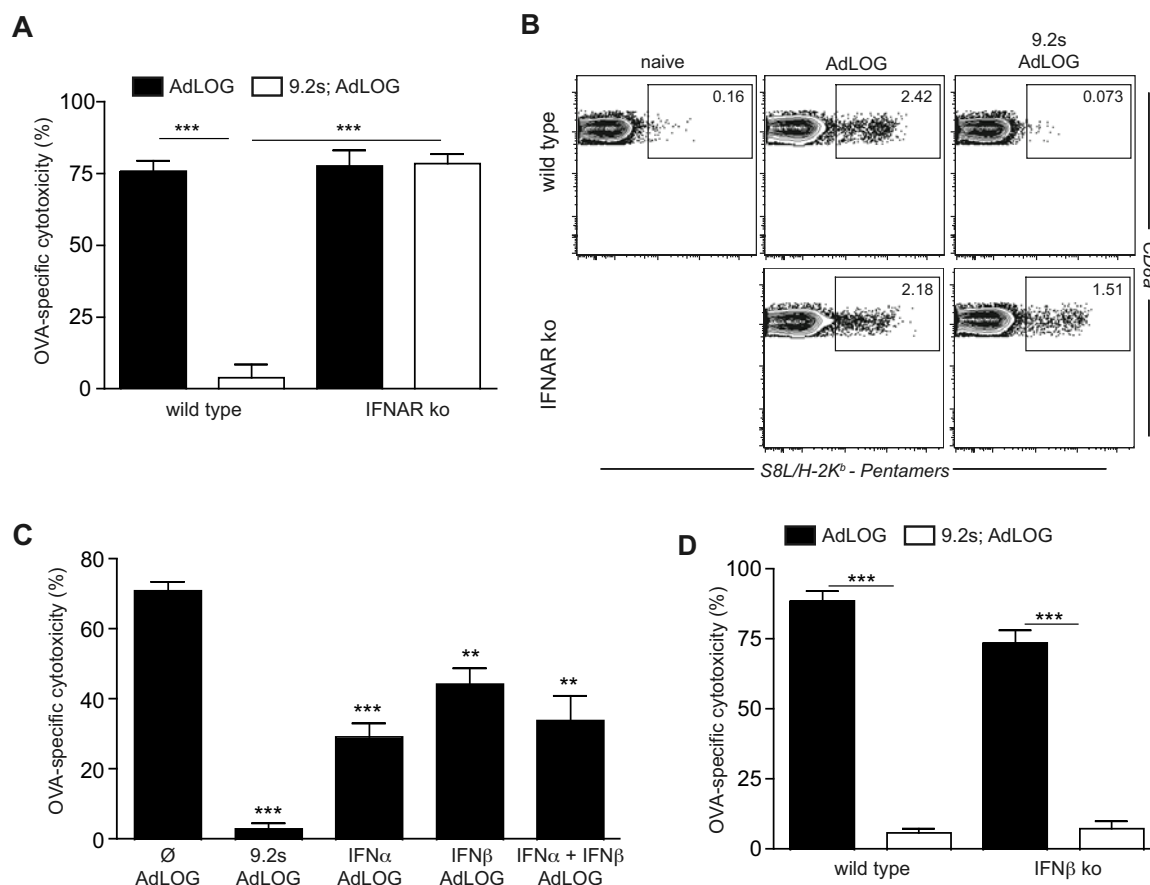
Figure 4.17   ssRNA-mediated inhibition of the CTL response was type I interferon dependent.

(A, B) C57BL/6 and IFNAR1 ko mice were immunized i.v. with AdLOG alone or received an i.v. administration of 10 µg 9.2s/DOTAP one day prior to AdLOG infection. OVA-specific cytotoxicity (A) and the generation of OVA-specific CD8 T cells (B) was determined in spleens five days post AdLOG immunization. (C) The influence of recombinant type I interferon on AdLOG-specific cytotoxic activity was analyzed by i.v. injection of 1000U of IFNα, IFNβ, or IFNα/IFNβ one day before AdLOG administration in wild type mice. As a positive control, mice received 10 µg of 9.2s/DOTAP i.v. before AdLOG vaccination. (D) C57BL/6 and IFNβ ko mice were infected with AdLOG alone or were previously injected i.v. with 10 µg of 9.2s/DOTAP. (C, D) OVA-specific lysis was assayed in spleens of mice five days post adenovirus immunization. Data are shown as mean +/- SEM with n=3 mice per group. (A, B, D) Representative results of at least two independent experiments are depicted.

4.1.7 Plasmacytoid DCs produce type I interferon in response to ssRNA stimulation

It has been reported that a wide variety of cell types are capable of producing type I IFN by using a feed-forward amplification loop that is initiated by paracrine or autocrine binding of secreted IFNα/β to the type I IFN receptor (Bogdan et al., 2004; Decker et al., 2005). Plasmacytoid DCs (pDCs) are known to rapidly release vast quantities of early type I IFN in response to TLR stimulation (especially TLR7 and 9 stimulation) independent of the positive amplification loop (Asselin-Paturel and Trinchieri, 2005; Decker et al., 2005; Honda et al., 2005).

In the following experiment, we sought after the cell type secreting type I IFN in response to 9.2s stimulation. In order to identify the cell population *in vivo* we made use of IFNβ knockin reporter mice, which produce YFP when IFNβ is induced (Scheu et al., 2008). IFNβ-YFP reporter mice were injected i.v. with 10 μg 9.2s/DOTAP and six hours later YFP expression was assayed by flow cytometry of splenocytes. YFP signals, which corresponded to IFNβ production, were detected in splenic pDCs, which were identified by CD11c^{int}PDCA-1^{pos}B220^{pos}CD11b^{neg} expression. No other cell type, such as T or B cells, released IFNβ after 9.2s injection (Figure 4.18 A). Moreover, histological staining was performed on spleen sections to localize IFNβ expression. YFP positive signals were evident in the marginal zone, which had been visualized by counterstaining for MOMA-1 (Figure 4.18 B).

As pDCs were identified to respond rapidly to 9.2s stimulation by releasing type I IFN, we further characterized the role of pDCs in ssRNA-mediated suppression of antigen-specific CTL responses. We depleted pDCs in C57BL/6 wild type mice by using an anti-PDCA-1 antibody and the elimation of pDCs (CD11c^{int}PDCA-1^{pos}B220^{pos} cells) in the spleen was determined 24 hours later by flow cytometry. Depletion of pDCs was only partially efficient as residual cells were still present (approximately 0.2 - 0.3%) (Figure 4.18 C).

Thereafter, we investigated if ssRNA-mediated suppression of an antigen-specific CTL response was circumvented if pDCs were depleted. C57BL/6 wild type mice were injected or not with the anti-PDCA-1 depleting antibody. Subsequently, mice were either left untreated or received an i.v. administration of 10 μg 9.2s/DOTAP one day prior to AdOVA infection. OVA-specific cytotoxic activity was quantified in spleens five days post adenoviral infection. If mice had been pretreated with the anti-PDCA-1 antibody, the OVA-specific cytotoxic activity was augmented by approximately 25% in comparison to mice that were injected with 9.2s/DOTAP and AdOVA (Figure 4.18 D). We assume that

the moderate increase in the CTL response might be attributed in part to an incomplete elimination of pDCs (Figure 4.18 C).

In summary, these data demonstrate that pDCs responded to TLR7-mediated recognition of ssRNA *in vivo* by releasing early IFNβ and contributed to the suppression of an antigen-specific CTL response.
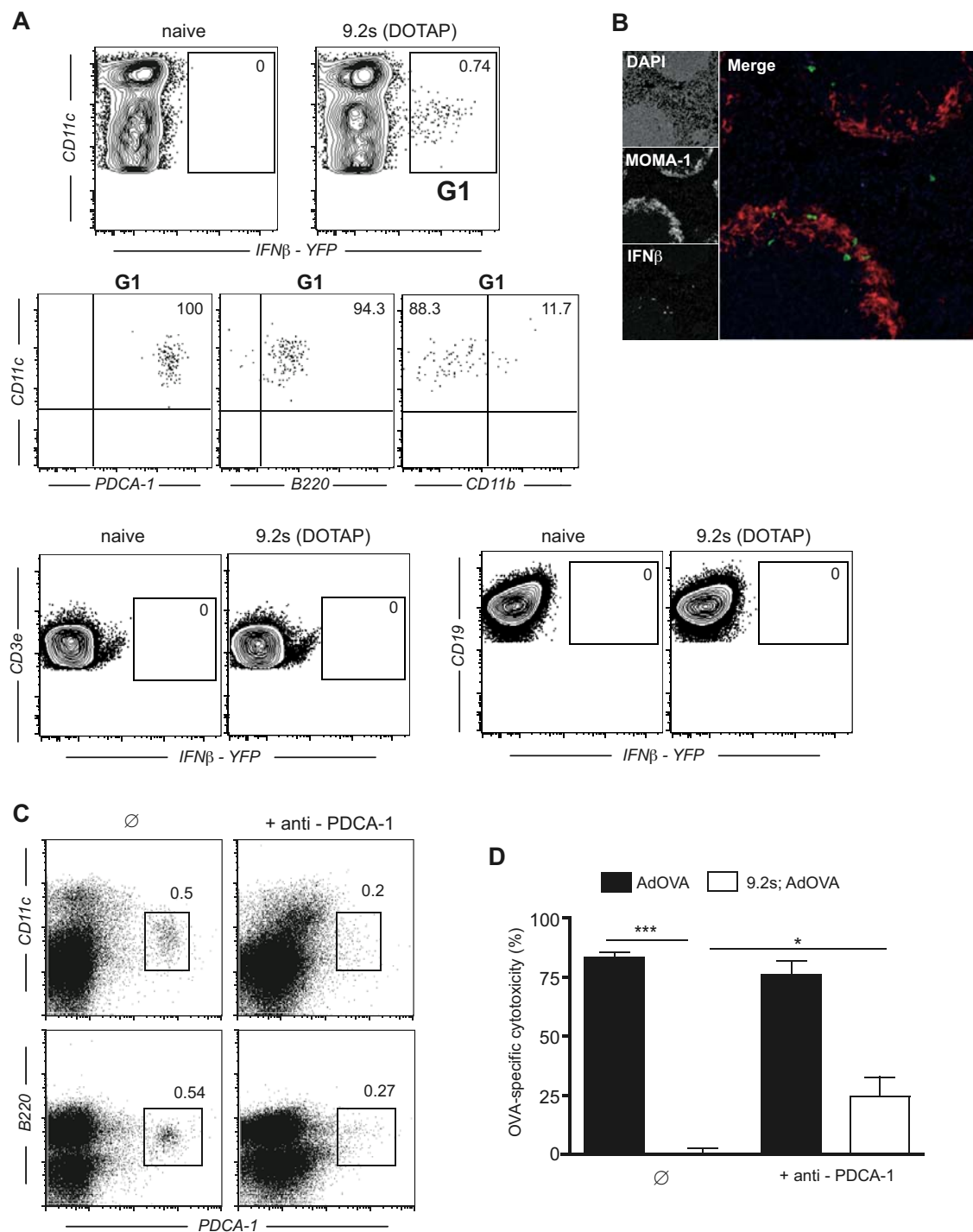

Figure 4.18   Splenic pDCs produced IFNβ in response to ssRNA stimulation and were involved in suppression of the CTL response.

(A, B) IFNβ-YFP reporter mice were injected i.v. with 10 μg of 9.2s/DOTAP. Six hours post injection mice were sacrificed. (A) YFP expression was examined by flow cytometric analysis of splenocytes. pDCs were identified by CD11c^{int}PDCA-1^{pos}B220^{pos}CD11b^{neg} expression (Gate G1), whereas T and B cells were distinguished by CD3e^{pos} and CD19^{pos} expression, respectively. (B) YFP expression was analyzed on histological sections of the spleen, which were counterstained with anti-MOMA-1 and DAPI to visualize the marginal zone and the T/B cell zone. Representative dot plots and histology of one mouse out of three mice of two independent experiments are shown. (D) pDC depletion in spleens was assessed by using an anti-PDCA-1 antibody, which was given i.v. twice (200 μg each). 24 hours after the last injection, pDC depletion efficiency was determined in spleens by staining for CD11c, PDCA-1, and B220 and using flow cytometry. Representative dot plots of one mouse out of two mice are presented. (E) pDCs were depleted as described in D. Subsequently, wild type and pDC-depleted mice were injected i.v. with either AdOVA alone or received i.v. 10 μg 9.2s/DOTAP prior to AdOVA immunization. OVA-specific cytotoxicity was quantified in spleens five days post AdOVA injection. Representative data are shown as mean +/- SEM (n=3 mice per group) out of two independent experiments.

4.1.8 Macrophages and DCs are targets of ssRNA-induced type I interferon

So far, we identified pDCs as one initial cellular source of type I IFN in response to 9.2s stimulation. Thereafter, we were interested in unveiling the cell type that was responding to type I IFNs and contributed to ssRNA-mediated CTL suppression by using Cre-mediated cell-specific IFNAR1 deficient mice (Clausen et al., 1999; Prinz et al., 2008; Stockinger et al., 2009; Varol et al., 2007). C57BL/6 wild type, B cell-specific, T cell-specific, T cell/B cell-specific, macrophage, and DC-specific IFNAR1 deficient mice were immunized i.v. with AdLOG alone or received a preceding i.v. injection of 10 μg 9.2s/DOTAP. As a positive control, complete IFNAR1 ko mice were used in all experiments. OVA-sepcific lysis was determined in spleens five days post AdLOG immunization. 9.2s still caused an abrogation of the CTL response in T cell-specific and/or B cell-specific IFNAR1 deficient mice, indicating that type I IFNs did not act on these cell types (Figure 4.19 A). However, antigen-specific cytotoxic activities were unaffected by systemic ssRNA injection in mice that lack the IFNAR1 receptor on macrophages (LysM-cell) and DCs (Figure 4.19 B and C). Consistent with these results, OVA-specific CD8 T cells were generated in macrophage-specific and DC-specific IFNAR1 ko mice in response to 9.2s and AdLOG injection, while no expansion of S8L/H-2K^{b} pentamer-positive CD8 T cells was observed in spleens of wild type mice (Figure 4.19 D and E). In conclusion, these results demonstrate that ssRNA-induced type I IFNs acted on

macrophages and DCs, which seem to be modulated in their ability to stimulate an efficient CTL response.
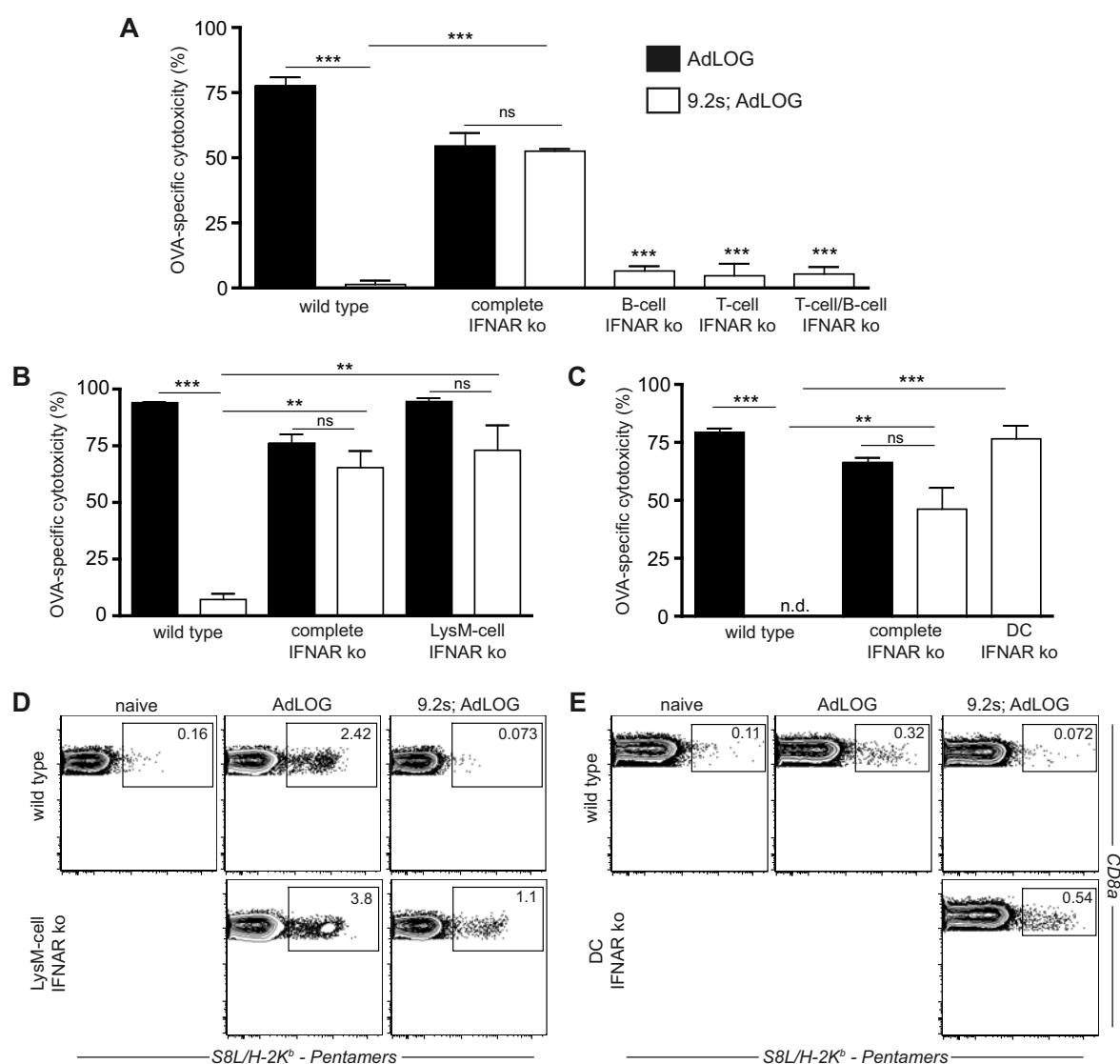
Figure 4.19   Macrophages and DCs were identified to respond to ssRNA-stimulated type I interferons.

Cell-specific IFNAR1 deficient mice were generated by Cre-mediated deletion. (A) C57BL/6 wild type, complete IFNAR1 ko, B cell-specific, T cell-specific, and T cell/B cell-specific IFNAR1 ko were immunized with AdLOG alone or were injected i.v. with 10 µg of 9.2s/DOTAP one day prior to adenoviral challenge. OVA-specific lysis was determined in spleens five days post AdLOG injection. (B, C) Wild type, complete IFNAR1 ko, LysM-specific, and DC-specific IFNAR1 ko were treated as described in A and OVA-specific cytotoxicity was analyzed. (D, E) The generation of OVA-specific CD8 T cells was determined by flow cytometric analysis of S8L/H-2K^b pentamers. OVA-specific cytotoxicity is depicted as mean +/- SEM and a representative pentamer dot plot of one mouse is shown (n=3 mice per group). At least two independent experiments were performed.

4.1.9 ssRNA-mediated CTL suppression involves type I interferon signaling via STAT1 and IRF7

Robust type I IFN production requires a positive feedback amplification, which is initiated through autocrine or paracrine binding of secreted type I IFN to the type I IFN receptor. This receptor is associated with the Janus protein tyrosine kinases JAK1 and Tyk2 that target the STAT transcription factors STAT1 and STAT2 for phoshorylation. Upon phosphorylation, STAT1 and STAT2 heterodimerize, translocate to the nucleus and interact with the DNA-binding protein IRF9. This complex is known as the transactivation complex ISGF3, which initiates the transcription of immediate-early target genes, such as IRF7 and IRF3 which control the production of type IFNs (Decker et al., 2005; Honda and Taniguchi, 2006; Levy et al., 2003; Prakash et al., 2005).

To investigate which signaling molecules downstream of the type I IFN receptor were involved in ssRNA-mediated suppression of the CTL response, we used STAT1, Tyk2, IRF3, and IRF7 deficient mice.

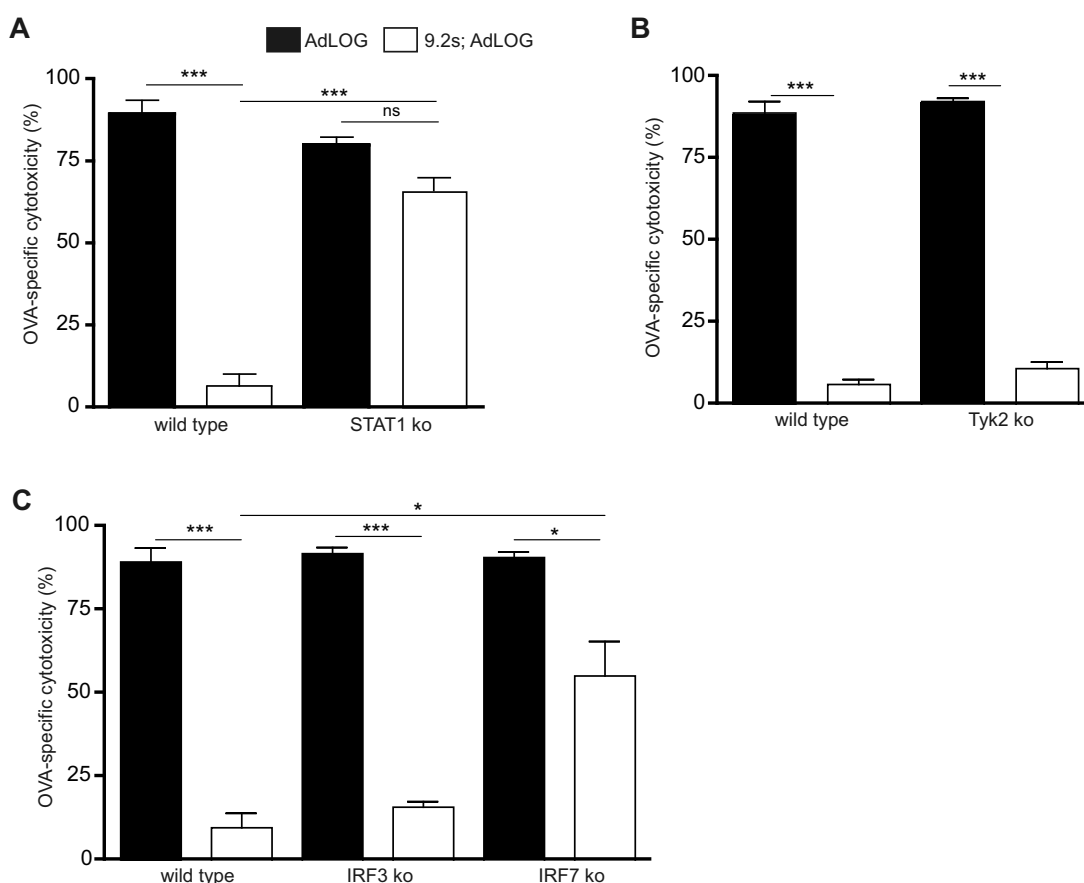**Figure 4.20 ssRNA-mediated CTL suppression involved signaling via STAT1 and IRF7.**
OVA-specific cytotoxic activity was determined in spleens of C57BL/6 wild type (A-C), STAT1 ko (A), Tyk2 ko (B), IRF3 ko, and IRF7 ko (C) mice upon i.v. administration of AdLOG alone or after i.v. pre-injection of 9.2s/DOTAP. Representative data are illustrated as mean +/- SEM of at least two independent experiments (n=3 mice per group).

OVA-specific cytotoxicity was assessed in spleens of mice upon i.v. administration of AdLOG alone or after i.v. pre-injection of 9.2s/DOTAP. 9.2s-induced OVA-specific CTL suppression was still observed in Tyk2 ko (Figure 4.20 B) and IRF3 ko (Figure 4.20 C) mice, whereas a CTL response was generated in STAT1 (Figure 4.20 A) and IRF7 ko (Figure 4.20 C) mice. Consequently, these results imply that type I IFN production might require a positive amplification by signaling through STAT1 and IRF7 downstream of the IFNAR receptor.

4.1.10 ssRNA stimulated DC maturation but impaired IL-12 secretion

Up to here, we have shown that systemic ssRNA affected CD8 and CD4 T cell responses against subsequent infections in a TLR7 and type I IFN dependent manner. In the following, we investigated if systemic ssRNA stimulation interfered with the phenotypic and functional maturation of DCs, which is essential for T lymphocyte activation and differentiation into T_H1 cells, T_H2 cells as well as into cytotoxic effector T lymphocytes (Akira et al., 2006; Banchereau and Steinman, 1998; Iwasaki and Medzhitov, 2004; Kawai and Akira, 2005).

We injected i.v. 10 μg 9.2s/DOTAP into C57BL/6 wild type mice and evaluated the surface expression of activation markers on splenic $CD11c^{high}CD8a^{pos}$ DCs, which are known to cross-present antigen, 24 hours post 9.2s injection. Flow cytometric analysis revealed a moderate but significant reduction (ca. 27%) in the percentage of splenic $CD11c^{high}CD8a^{pos}$ DCs in comparison to naive mice (Figure 4.21 A). Furthermore, costimulatory molecules like CD40, CD80, and CD86 were upregulated on splenic $CD11c^{high}CD8a^{pos}$ DCs in response to ssRNA treatment. Interestingly, we noticed a reduced expression of MHC class II molecules, which was in line with the observation that antigen-specific CD4 T cell responses were impaired by a preceding injection of ssRNA (Figure 4.21 B).
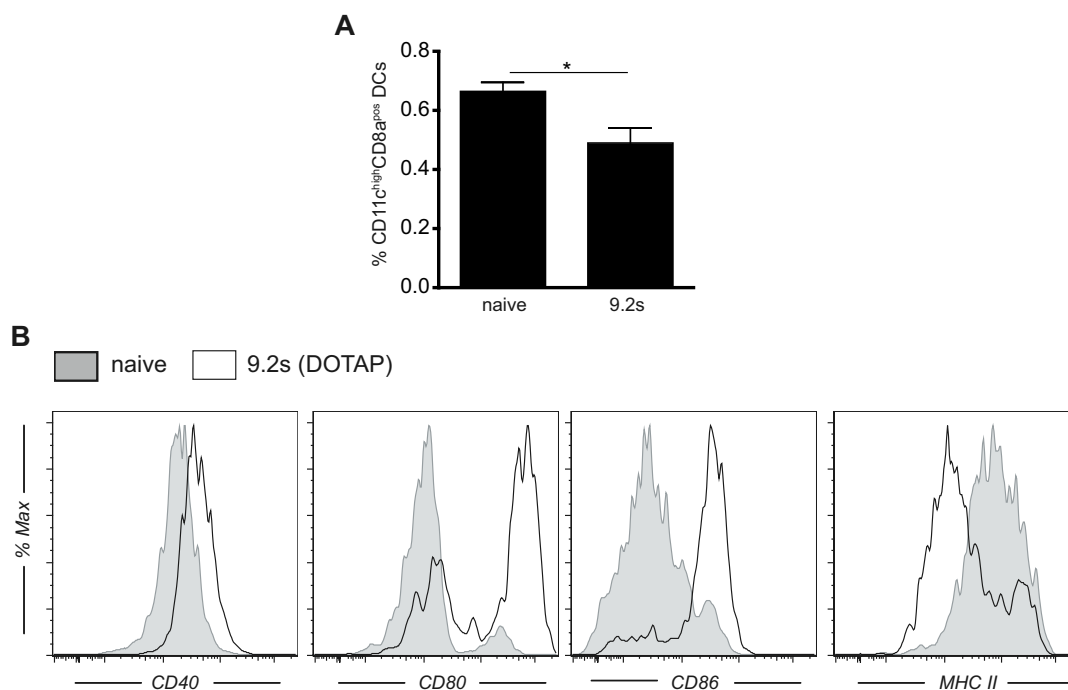
A

B

Figure 4.21   DOTAP-complexed ssRNA induced the upregulation of costimulatory molecules but downregulation of MHC class II molecules expressed by splenic DCs.

C57BL/6 wild type mice were injected i.v. with 10 µg 9.2s/DOTAP. 24 hours later, the percentage of splenic CD11c^{high}CD8a^{pos} DCs was determined (A) and splenic CD11c^{high}CD8a^{pos} DCs were examined for their expression of maturation markers CD40, CD80/86, and MHC class II by flow cytometry (B). The bar graph shows the mean +/- SEM and one representative histogram for each staining is depicted. Two independent experiments were performed with n=3 mice per group.

Pathogen recognition and TLR stimulation leads to the production of pro-inflammatory cytokines, such as IL-12, which is mainly produced by phagocytes and DCs. IL-12, as a "third signal", is a crucial cytokine required for the generation of an efficient and long-lasting T cell response (Curtsinger et al., 2003a; Curtsinger et al., 2003b; Curtsinger et al., 1999; Schmidt and Mescher, 1999; Trinchieri, 1995, 2003). Therefore, we examined whether IL-12 secretion in response to a pathogen was negatively affected by pre-injection of ssRNA. To mimic pathogen infection, we injected mice i.v. with 100 µg CpG-1668. Indicated groups received a preceding injection of 10 µg of 9.2s/DOTAP, which was given i.v. 24 hours prior to CpG administration. IL-12p70 secretion was analyzed in sera of mice by ELISA or by intracellular cytokine staining using flow cytometry. The percentage of IL-12 producing splenic CD11c^{high}CD8a^{pos} DCs was significantly diminished by 45% in mice that had been pre-injected with 9.2s compared to mice that were stimulated with CpG alone (Figure 4.22 A). In addition, also the level of systemic IL-12 was considerably reduced (ca. 85% reduction) in sera of 9.2s and CpG treated mice (Figure 4.22 B).
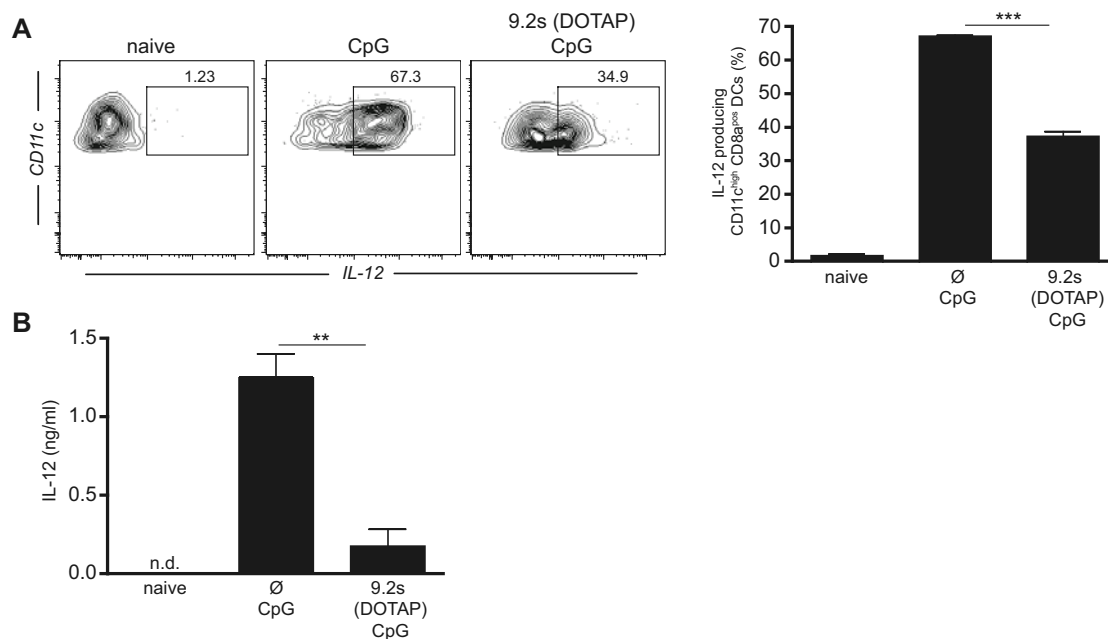
A

B

Figure 4.22   ssRNA pre-activated DCs were impaired in IL-12 secretion in response to CpG stimulation.

C57BL/6 wild type mice received i.v. either 100 µg of CpG-1668 alone or were injected with 10 µg of 9.2s/DOTAP one day prior to CpG administration. Two hours upon CpG injection, IL-12 production by splenic CD11c^{high}CD8a^{pos} DCs was analyzed by intracellular cytokine staining and flow cytometric analysis (A). IL-12 secretion was quantified in sera of mice by ELISA (B). Representative data of two independent experiments are depicted. Results in bar graphs are presented as mean +/- SEM and one representative dot plot of one mouse is shown (n= 3 mice per group).

In conclusion, systemic application of 9.2s induced on the one hand a phenotypic maturation of splenic DCs, which was characterized by the upregulation of costimulatory molecules. On the other hand, MHC class II expression was reduced and IL-12 production was impaired in response to a subsequent pathogenic stimulus.

4.1.11 Impaired cross-priming of antigen-specific CD8 T cells by ssRNA pre-activated splenic DCs

DCs possess the capability to cross-present exogenous antigen, which offers a mechanism for priming of CD8 T cells specific for viruses that do not directly infect DCs. In order to fully activate CD8 T cells by cross-priminig, DCs need to become licensed by appropriate activation signals, which are provided by TLR stimulation, CD4 T cell help through CD40-CD40L interaction, and/or virus-induced type I IFNs (Bennett et al., 1998; Bennett et al., 1997; Iwasaki and Medzhitov, 2004; Le Bon et al., 2003; Schoenberger et al., 1998).

Therefore, we asked whether a preceding injection of ssRNA influenced cross-priming of OVA-specific CD8 T cells (OT-I) by splenic DCs upon AdOVA challenge. C57BL/6 wild type mice were immunized i.v. with AdOVA alone or in combination with 10 µg 9.2s/DOTAP which was given i.v. one day before AdOVA infection. Splenic CD11c positive DCs were purified 20 hours post AdOVA challenge and co-cultured for 24 hours with naive OT-I T cells. Cross-priming of OT-I T cells was monitored by measuring IFNγ and IL-2 secretion in co-culture supernatants by ELISA or by intracellular cytokine staining of OT-I T cells and subsequent flow cytometric analysis. In addition, T cell activation was assayed by investigating CD69 surface expression. Splenic DCs, that had been isolated from AdOVA immunized mice, induced obvious CD8 T cell activation as evidenced by the upregulation of CD69 (Figure 4.23 C) and IL-2 as well as IFNγ production by OT-I T cells (Figure 4.23 A and B). However, CD8 T cell activation and effector cytokine release was compromised if DCs had been pre-activated by systemic ssRNA application prior to AdOVA infection (Figure 4.23).
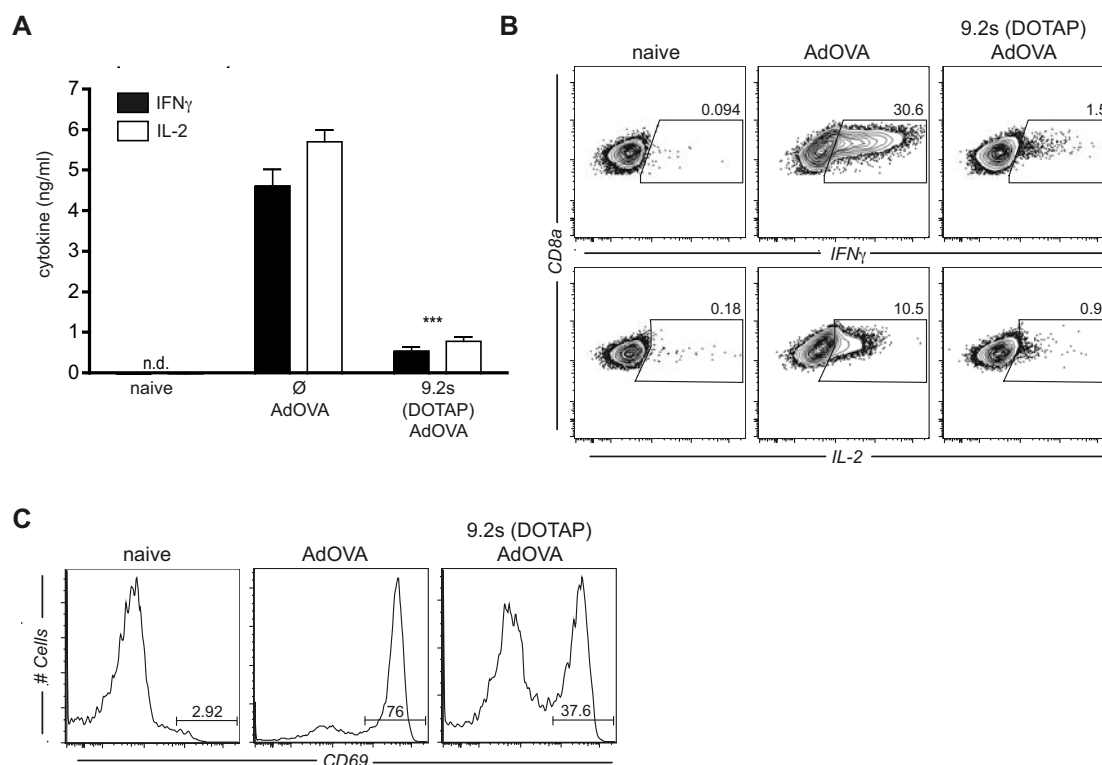
Figure 4.23   Systemic pre-activation of DCs with ssRNA impeded *ex vivo* cross-priming and activation of OVA-specific CD8 T cells.
C57BL/6 wild type mice were immunized i.v. with AdOVA alone or in combination with 10 µg 9.2s/DOTAP given i.v. one day before AdOVA injection. Splenic CD11c positive DCs were MACS-purified 20 hours post AdOVA challenge and co-cultured with naive OVA-specific CD8 T cells (OT-I) for 24 hours. IFNγ and IL-2 production was analyzed in co-culture supernatants by ELISA (A) and by intracellular cytokine staining of OT-I T cells (B). CD8 T cell activation was assayed by staining for CD69 and flow cytometric analysis (C). Representative data of at least three independent experiments with n=3 mice per group are shown.

4.1.12 Impaired cross-priming of antigen-specific CD8 T cells is type I interferon dependent and circumvented by exogenous IL-12

We have shown that systemic injection of ssRNA induced the suppression of the CTL response against a subsequent AdOVA infection through the induction of type I IFNs, which were ultimately acting on DCs (Figure 4.16, 4.17 and 4.19). Thus, we next investigated if the cross-priming ability of ssRNA pre-activated splenic DCs was restored in type I IFN receptor deficient mice. Wild type and IFNAR1 ko mice were treated as described in the previous chapter (see 4.1.11). Indeed, *ex vivo* cross-priming of OVA-specific CD8 T cells (OT-I) by ssRNA pre-activated DCs was unaffected in response to AdOVA infection if DCs were isolated from IFNAR1 deficient mice, as a release of IFNγ was detected in co-culture supernatants (Figure 4.24 A). This observation was in accordance with the results obtained in DC-specific IFNAR1 deficient mice, in which ssRNA-induced CTL suppression was prevented (Figure 4.19).

Figure 4.24 Impaired cross-priming of OVA-specific CD8 T cells by ssRNA pre-activated DCs was dependent on type I interferons and was abolished in the presence of IL-12.
(A) C57BL/6 wild type and IFNAR1 ko mice were immunized i.v. with AdOVA alone or in combination with 10 µg 9.2s/DOTAP given i.v. one day before AdOVA injection. Splenic CD11c positive DCs were MACS-purified 20 hours post AdOVA challenge and co-cultured with naive OVA-specific CD8 T cells (OT-I) for 24 hours. IFNγ production was quantified in co-culture supernatants by ELISA. (B) Wild type mice were treated and splenic DCs were isolated as described in A. Co-culture with OT-I T cells was performed in the absence or presence of 1.25 ng/ml or 5 ng/ml recombinant IL-12p70. IFNγ secretion was determined in co-culture supernatants after 24 hours by ELISA. Representative results of at least two independent experiments are shown with n=3 to 4 mice per group.

As ssRNA pretreatment interfered with the production of IL-12 in response to a subsequent CpG injection, we speculated whether it might be possible to rescue AdOVA-specific cross-priming of OT-I T cells by ssRNA pre-activated DCs in the presence of exogenous

IL-12. Addition of increasing concentrations of recombinant IL-12p70 resulted in an overall enhancement in IFNγ production by OT-I T cells that had been stimulated by splenic DCs isolated from AdOVA only immunized mice (Figure 4.24 B). Importantly, IL-12 supplementation also induced T cell activation by 9.2s pre-activated splenic DCs, as IFNγ secretion by OT-I T cells was comparable to the levels that were secreted, if T cells were primed by DCs from AdOVA immunized mice in the absence of IL-12 (Figure 4.24 B).
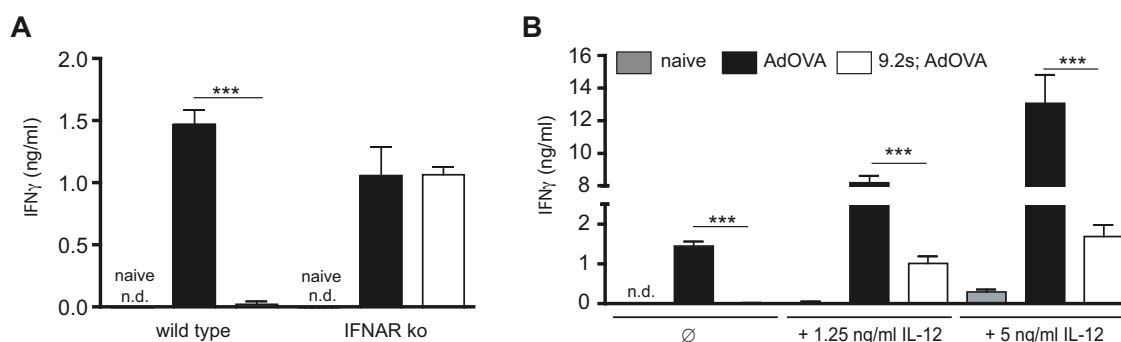
Thus, impaired cross-priming of OVA-specific CD8 T cells by ssRNA-preactivated DCs was dependent on type I IFN and was circumvented by provision of exogenous IL-12.

4.1.13 Summary of chapter 4.1

Our data presented so far showed that the systemic application of ssRNA suppressed subsequent antigen-specific cytotoxic CD8 T cell responses against viral and bacterial pathogens in a TLR7 dependent manner. The absence of antigen-specific cytotoxicity was associated with an impaired expansion and activation of antigen-specific CD8 T cells. Furthermore, we detected that the proliferation and cytokine production of antigen-specific CD4 T cells was impeded, which was associated with a downregulation in MHC class II molecule expression on ssRNA-preactivated splenic DCs. The suppression of T cell responses was avoided if ssRNA was targeted to the cytoplasm by complexation with the carrier *in vivo*-jetPEI^{TM}, thus preventing endosomal recognition by TLR7. Interestingly, we revealed that ssRNA-induced inhibition of an adenovirus-specific CTL response depended on the secretion of type I IFNs, which are generally accepted to have immune stimulatory properties. Furthermore, we identified pDCs to be one initial cellular source of type I IFNs, which then affected macrophages and DCs by modulating their ability to stimulate an efficient CTL response. In addition, we demonstrated that ssRNA-preactivated splenic DCs were impeded in their ability to cross-prime OVA-specific CD8 T cells in response to an AdOVA infection, although they had matured in response to ssRNA stimulation. Finally, we assume that systemic ssRNA interfered with the generation of CD4 T cell help, as the presence of activated as well as memory antigen-specific CD4 T cells circumvented ssRNA-induced CTL suppression. Taken together, ssRNA-induced type I IFNs could also inhibit antigen-specific immune responses in the spleen, despite their well-established immune stimulatory properties.

4.2 Relevance of RNA- and type I interferon-mediated suppression of adaptive CD8 T cell responses

4.2.1 Small-interfering RNA application interferes with an adenoviral-based tumor vaccination in a model of combinatorial anti-tumor therapy

Based on recent insights that small interfering RNA (siRNA) is recognized by TLR7 and induces innate immune stimulation (Judge et al., 2005; Robbins et al., 2008; Robbins et al., 2009; Rossi, 2009; Sioud, 2005, 2006) together with our findings that ssRNA, recognized by TLR7, was capable to interfere with the generation of subsequent CTL responses through the induction of type I IFNs, we speculated what would happen if a siRNA-mediated knockdown approach would be combined with an adenoviral based-tumor vaccintion. To mimic a combinatorial anti-tumor therapy of siRNA application and adenoviral vaccination, we implanted intraportally (i.po.) 5×10^4 luciferase expressing B16 melanoma cells (B16-luc) into C57BL/6 mice. Four days later, tumor bearing mice were vaccinated i.v. with a recombinant adenovirus expressing the melanocyte-specific antigen murine tyrosine-related protein 2 (AdmTRP2). Indicated groups received an i.v. administration of 20 μg DOTAP or *in vivo*-jetPEI[TM] complexed siRNA specific for VEGF (siVEGF) (Filleur et al., 2003; Kornek et al., 2008) one day before adenoviral immunization. Tumor growth was monitored by *in vivo* imaging of bioluminescent luciferase expression (IVIS®200) (Figure 4.25).
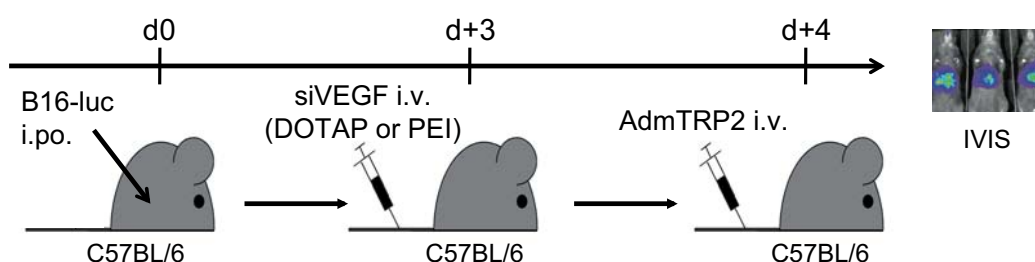
Figure 4.25   Time schedule of melanoma implantation, subsequent siRNA application, and adenoviral vaccination.

Adenoviral therapy alone led to an almost complete tumor regression, whereas tumor expansion was not inhibited in mice pre-injected with siVEGF/DOTAP prior to AdmTRP2 vaccination. As tumors grew so fast in non-treated as well as siVEGF/AdmTRP2 treated mice, they were sacrificed due to ethical reasons (Figure 4.26 A). If siVEGF was stabilized

using the carrier *in vivo*-jetPEI^TM (siVEGF/PEI) and injected i.v. one day before AdmTRP2 immunization, adenoviral based tumor therapy was not impaired (Figure 4.26 B).
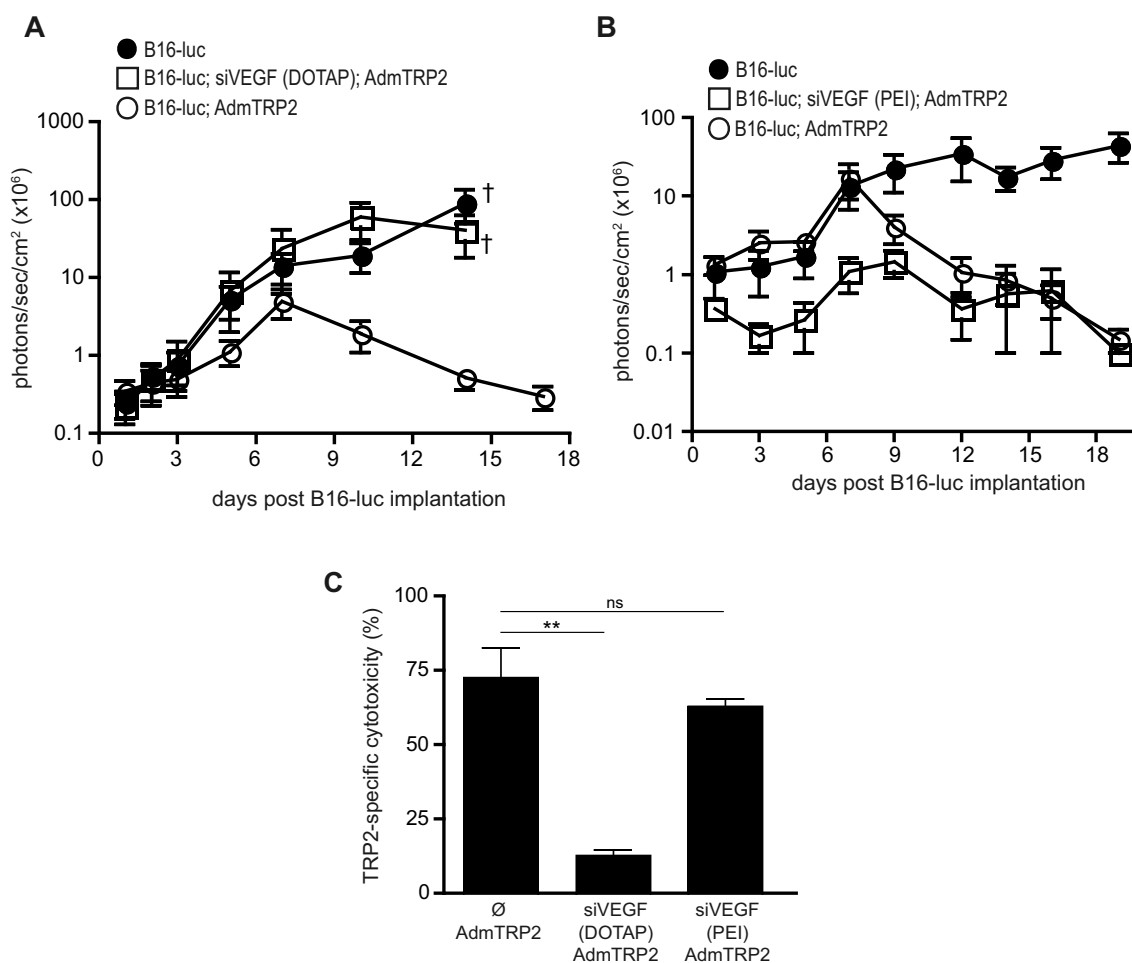
Figure 4.26   DOTAP-complexed siRNA specific for VEGF abrogated adenoviral tumor vaccination due to lack of a tumor-specific CTL response.

(A, B) $5x10^4$ luciferase expressing B16 melanoma cells (B16-luc) were implanted intraportally into C57BL/6 mice. Four days later, tumor bearing mice were vaccinated with AdmTRP2 alone or indicated groups received an i.v. administration of 20 µg DOTAP-complexed siVEGF (siVEGF/DOTAP) (A) or *in vivo*-jetPEI^TM complexed siVEGF (siVEGF/PEI) (B). Tumor growth was monitored by *in vivo* imaging of bioluminescent luciferase expression (IVIS®200). (C) TRP2 tumor-specific cytotoxicity was examined in spleens of wild type mice five days post AdmTRP2 injection. Indicated groups were pre-injected i.v. with 20 µg of siVEGF/DOTAP or siVEGF/PEI. Tumor growth is expressed as photons/sec/cm^2 and is presented as mean +/- SEM (n=4-5 mice per group). † Mice were sacrifized due to massive tumor burden.
OVA-specific lysis is depicted as mean +/- SEM with n=3 mice per group.

Moreover, we determined the mTRP2-specific cytotoxicity in spleens of mice five days post AdmTRP2 immunization and confirmed that the anti-tumor vaccination failed due to a lack of a tumor-specific CTL response when DOTAP-complexed siVEGF was used. In

contrast, an efficient tumor-specific CD8 T cell response was established by AdmTRP2 immunization if siVEGF was stabilized using *in vivo*-jetPEI[TM] (Figure 4.26 C).

4.2.2 Influenza infection inhibits the CTL response against a subsequent adenovirus infection

It has been known for a long time that a temporary lack of cell-mediated immune reactions may occur upon certain viral infections, which is frequently attributed to the effects of virus-induced type I IFNs (De Maeyer et al., 1975; De Maeyer-Guignard et al., 1975; Hahm et al., 2005; Trifilo et al., 2006).

Here, we investigated how an influenza infection, known to trigger type I IFN production (Barchet et al., 2005b; Diebold et al., 2004), influenced the CD8 T cell response against a subsequent adenoviral infection. We infected C57BL/6 wild type mice with $1x10^6$ PFU Influenza A PR8/H1N1 virus one day before infection of those mice with AdLOG. The generation of OVA-specific CD8 T cells and their cytotoxic activity was measured in spleens five days post adenovirus administration. Interestingly, mice that had been infected with Influenza virus were not able to elicit a cytotoxic T cell response against a subsequent adenoviral infection (Figure 4.27 A). The lack of OVA-specific cytotoxic activity was attributed to the absence of OVA-specific CD8 T cells, which were determined by S8L/H-2K[b] pentamer staining (Figure 4.27 B). Furthermore, Influenza-mediated inhibition of the AdLOG-specific CTL response was dependent on type I IFNs, as cytotoxic CD8 T cell responses were equally strong in IFNAR1 deficient mice infected with AdLOG alone or those that had been infected with Influenza before (Figure 4.27 A). Therefore, it seems likely that Influenza virus suppressed subsequent adaptive immune responses in a similar manner as we observed with the TLR7 ligand ssRNA.
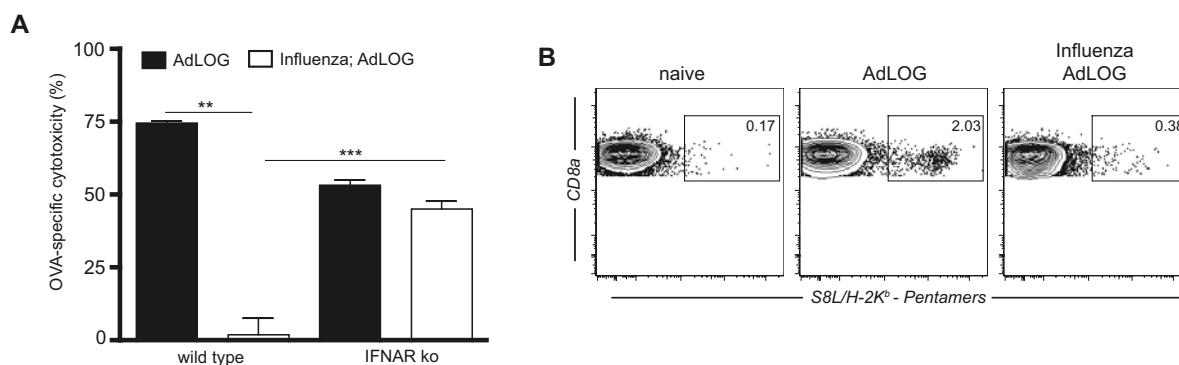

Figure 4.27   Influenza infection suppressed a subsequent adenovirus-specific CD8 T cell response in a type I interferon dependent manner.

C57BL/6 wild type and IFNAR1 ko mice were injected i.v either with AdLOG alone or were infected with 1×10^6 PFU Influenza A PR8/H1N1 virus one day before AdLOG challenge. (A) OVA-specific CTL response was measured in spleens of wild type and IFNAR1 ko mice five days post adenovirus infection. (B) The generation of endogenous OVA-specific CD8 T cells was investigated in spleens of wild type mice by flow cytometric analysis of S8L/H-2K^b pentamers. OVA-specific lysis is illustrated as mean +/- SEM (with n=3 mice per group). Representative data of at least two independent experiments are depicted.

4.2.3   Summary of chapter 4.2

In this chapter we addressed the relevance of RNA- and type I IFN-mediated immune suppression. We investigated in a model of a combinatorial anti-tumor therapy how siRNA application might interfere with the generation of tumor-specific CTL responses induced by adenoviral-based vaccination. Vaccination with an adenovirus expressing the melanoma antigen TRP2 triggered an efficient tumor-specific CTL response and consequently led to an almost complete melanoma regression. If mice received an additional injection of DOTAP-complexed siRNA (specific for VEGF) before AdmTRP2 administration, tumor expansion was not prevented due to an impaired CTL response. However, if siRNA was complexed with the carrier *in vivo*-jetPEI^{TM} instead of DOTAP, AdmTRP2 vaccination induced a strong tumor-specific CTL response which led to a regression of tumor growth. In conclusion, our data demonstrate that a therapeutic application of siRNA might have off-target effects due to the recognition of siRNA by nucleic acid receptors such as TLR7, thus leading to immune stimulation and production of type I IFNs, which might impair subsequent adaptive immune responses.

Furthermore, we described that not only systemic exposure to synthetic ssRNA, but also an Influenza virus infection impaired the generation of a cytotoxic CD8 T cell response against a subsequent adenovirus infection in a type I IFN dependent manner. Consequently, these results imply that viruses, which trigger type I IFNs, might inhibit adaptive immune responses towards following infections in a similar fashion as we observed with the TLR7 ligand ssRNA.

5 Discussion

Toll-like receptors (TLRs) are canonical pattern-recognition receptors (PRRs), which recognize conserved molecular structures of microorganisms. It is known that stimulation of TLRs results in the activation of innate immunity, which is essential for the initiation of an adaptive immune response (Akira et al., 2006; Iwasaki and Medzhitov, 2004; Kawai and Akira, 2005). However, our group has shown that the systemic injection of CpG, a TLR9 ligand, induces immunoregulatory mechanisms that cause suppression of subsequent antigen-specific adaptive immune responses (Wingender et al., 2006), which contradicts the general assumption that stimulation of TLRs results in the induction of host immunity. Furthermore, Beatrix Schumak demonstrated in her PhD thesis, that the suppression of cytotoxic T cell (CTL) responses was not only restricted to CpG, but also other systemically applied TLR ligands impeded antigen-specific CTL responses. She revealed that the systemic injection of single-stranded RNA (ssRNA) oligonucleotides suppressed antigen-specific CTL responses in a TLR7 and type I interferon (IFN) dependent manner (Schumak, 2008). Based on these key findings, we aimed to further unravel the underlying mechanism and relevance of ssRNA- and type I IFN-mediated inhibition of adaptive immune responses.

The results presented in this thesis show that systemic administration of ssRNA leads to the suppression of CTL responses against subsequent infections by viral and bacterial pathogens. The absence of cytotoxicity was associated with impaired expansion and activation of antigen-specific CD8 T cells as well as an impaired proliferation and cytokine production of antigen-specific CD4 T cells. Suppression of adaptive T cell responses was avoided if ssRNA was targeted to the cytoplasm by complexation with the carrier *in vivo*-jetPEI^{TM}, thus preventing endosomal recognition by TLR7. TLR7-mediated recognition of ssRNA triggered the production of type I IFNs, which played a central role in ssRNA-induced CTL suppression. Furthermore, we identified plasmacytoid dendritic cells (pDCs) to respond immediately to ssRNA stimulation by releasing type I IFN. This cytokine then affected macrophages and dendritic cells (DCs) by modulating their capacity to stimulate an efficient CTL response. DCs from ssRNA-pretreated mice were impaired in their ability to cross-prime antigen-specific CD8 T cells upon an adenovirus infection, although they had matured in response to ssRNA stimulation. In addition, ssRNA-induced CTL suppression was circumvented in the presence of activated as well as memory antigen-

specific CD4 T cells, indicating that systemic ssRNA interfered with the generation of CD4 T cell help. This interpretation was supported by the observation that MHC class II expression was downregulated on splenic DCs upon systemic application of ssRNA, thereby impeding CD4 T cell activation.

The second part of this thesis dealt with the relevance and potential risks of RNA- and type I IFN-induced CTL suppression.

In the last decade new strategies in anti-cancer treatment have emerged which aim at the induction of tumor-specific cytotoxic T cell responses and suppression of tumorigenicity by directed post-transcriptional gene silencing using small interfering RNA (siRNA). Mimicking such a combinatorial approach, we reported that, depending on the carrier used, systemic *in vivo* siRNA injection abrogated an adenoviral-based melanoma vaccination due to the lack of a tumor-specific CTL response. In conclusion, our data indicate that a therapeutic application of siRNA might have off-target effects due to the recognition by nucleic acid receptors such as TLR7, thus leading to immune stimulation and production of type I IFNs, which might impair subsequent adaptive immune responses.

Moreover, we could demonstrate that CTL suppression was not restricted to the application of synthetic RNA oligoribonucleotides but rather seems to be true for infections with type I IFN inducing viruses in general. We pointed out that the infection with Influenza virus impeded the generation of the CTL response against a subsequent adenovirus infection in a type I IFN dependent manner. Consequently, these results imply that viruses, known to trigger type I IFN, might inhibit adapative immune responses towards following infections in a similar fashion as we described for systemic ssRNA.

5.1   Mechanisms of ssRNA and type I interferon-mediated suppression of adaptive immune responses

5.1.1   Targeting of ssRNA into the endosome suppresses antigen-specific CD8 T cell responses in a TLR7 dependent manner

In this study, we reported that the systemic application of the TLR7 ligand ssRNA suppressed subsequent antigen-specific CTL responses in the spleen. This result is in line with several reports showing that the systemic exposure to TLR ligands, such as CpG, LPS, or polyI:C can inhibit adaptive T cell immunity despite their well known immune

stimulatory properties (Mellor et al., 2005; Wilson et al., 2006; Wingender et al., 2006). We observed that ssRNA-mediated suppression was not only restricted to adenovirus infection, but impaired CTL responses against various viral and bacterial pathogens (Figure 4.4), indicating a general suppressive effect of systemic ssRNA on the generation of adaptive immunity.

RNA can be recognized by endosomally located TLR3 and TLR7 (Alexopoulou et al., 2001; Diebold et al., 2004; Heil et al., 2004) or by the cytoplasmic receptors MDA5 and RIG-I (Gitlin et al., 2006; Hornung et al., 2006; Schlee et al., 2009; Yoneyama et al., 2005). The RNA oligoribonucleotide used in our studies, namely 9.2, is recognized by TLR7 if complexed to the carrier DOTAP (Hornung et al., 2005). Indeed, the suppression of the CTL response strictly depended on the detection by endosomal TLR7 and was based on the recognition of the single-strand motif of the sense strand (Figure 4.1). In order to circumvent detection by TLR7, we employed an alternative carrier for complexation of RNA. Instead of DOTAP, a cationic liposome, which not only protects RNA from degradation but targets RNA to the endosome (Almofti et al., 2003; Yasuda et al., 2005), we used *in vivo*-jetPEI^{TM} to stabilize RNA for *in vivo* application. *In vivo*-jetPEI^{TM} is a linear polyethylene imine, which facilitates the delivery into the cytoplasm after osmotic rupture of the endosome (Boussif et al., 1995; Brunner et al., 2002; Kichler et al., 1995). In agreement with these studies, we could show that complexation of our RNA using *in vivo*-jetPEI^{TM} promoted the delivery of RNA into the cytoplasm (Figure 4.6 B). TLR7-mediated recognition of ssRNA was thereby prevented, and thus CTL responses against subsequent viral and bacterial infections were not suppressed (Figure 4.5). Moreover, an involvement of RIG-I stimulation in our experiments could be excluded, as the RNA oligonucleotides used lack 5'-triphosphates and blunt ends, which are prerequisites for recognition by RIG-I (Hornung et al., 2006; Schlee et al., 2009).

It is known that TLR stimulation triggers the activation of the innate immune system in order to prevent pathogen spread before the intervention of the adaptive immune system (Akira et al., 2006; Pichlmair and Reis e Sousa, 2007). Therefore, we first investigated whether DOTAP-complexed ssRNA stimulated the innate immune system to induce a fast elimination of the virus, and thereby results in a substantial reduction in antigen load in the organism. However, we could exclude this possibility as ssRNA diminished the viral burden irrespective of whether it was given before, simultaneously with, or one day after adenovirus infection. Importantly, the CTL response was only impaired if ssRNA was given prior to adenovirus infection, indicating that the suppressive impact of ssRNA is not

a consequence of a reduced viral burden but rather involves a regulatory mechanism. Moreover, these data demonstrated that also the timing of ssRNA application played an important role, as only a preceding injection impeded subsequent CTL responses (Figure 4.2). Remarkably, systemic ssRNA interfered with the generation of CTL responses if applied as early as five days prior to an adenovirus infection.

Furthermore, we revealed that systemic injection of DOTAP-complexed ssRNA inhibits the expansion and activation of endogenous antigen-specific CD8 T cells in response to AdOVA infection (Figure 4.7). In spite of an impaired expansion and activation of adoptively transferred OT-I T cells, comparable amounts of IFNγ producing OT-I T cells were detected upon restimulation in mice that had been treated with AdOVA alone or those that had received DOTAP-stabilized ssRNA and AdOVA (Figure 4.8). These results provide evidence that the lack of antigen-specific cytotoxicity is associated with a defective priming of antigen-specific CD8 T cells and not due to tolerization (Steinman et al., 2003) or control of effector CD8 T cells by T regulatory cells (Sakaguchi et al., 1995; Shevach, 2009). However, in order to exclude a definitive role of T regulatory cells in ssRNA-mediated CTL suppression, immunization experiments need to be performed in Foxp3-DTR mice, where T regulatory cells can be selectively depleted by diptheria toxin injection (Hämmerling, unpublished). Furthermore, we could rule out a role of IL-10 in ssRNA-induced CTL suppression (Figure 4.15), which is consistent with the assumption that the absence of cytotoxicity resulted from a defect in CD8 T cell priming by DCs and was not due to a control of effector CD8 T cells by anti-inflammatory cytokines, such as IL-10 or TGFβ (Akdis and Blaser, 2001; Maynard and Weaver, 2008; Shevach, 2009).

In conclusion, our data cleary demonstrate that a preceding systemic injection of ssRNA inhibits subsequent antigen-specific CTL responses against various pathogens in a TLR7-dependent manner. The lack of antigen-specific cytotoxicity is not the consequence of a diminished viral burden due to the stimulation of innate immunity, but is rather accounted for by an impaired priming and activation of naive antigen-specific CD8 T cells.

5.1.2   ssRNA-induced inhibition of antigen-specific CD4 T cell responses results in impaired CD4 T cell help

Cognate CD4 T cell help is essential to establish a strong CD8 T cell response against various pathogens (Bennett et al., 1997; Bevan, 2004; Cardin et al., 1996; Castellino and

Germain, 2006; Keene and Forman, 1982; von Herrath et al., 1996). DCs are licensed by ligation of CD40 and CD40L expressed on CD4 T helper cells, which enhances the DCs ability to stimulate an efficient cytotoxic CD8 T cell response (Bennett et al., 1998; Schoenberger et al., 1998; Toes et al., 1998). Indeed, the data presented in this thesis indicate that CD4 T cell help is impeded by systemic exposure to ssRNA upon TLR7-mediated recognition. This was concluded from observations that injection of DOTAP-complexed ssRNA interfered with the expansion and cytokine production of antigen-specific CD4 T cells upon AdOVA infection (Figure 4.10 and 4.11). Again, impairment of the CD4 T cell response was avoided if ssRNA was targeted to the cytoplasm by complexation with *in vivo*-jetPEI^{TM}. In addition, OVA-specific IgG titers were remarkably diminished in mice that had received DOTAP-complexed ssRNA before AdOVA infection (Figure 4.12). This finding further emphasizes that CD4 T cell help is negatively affected by systemic ssRNA, as T helper cell function is important to ensure an optimal Ig isotype switch to IgG antibodies (Grewal and Flavell, 1996; Hollenbaugh et al., 1994). Moreover, we detected a downregulation in MHC class II expression on splenic DCs upon systemic ssRNA stimulation, which most likely results in a lack of antigen presentation and activation of CD4 T cells. These results coincide with the findings by Young et al, which demonstrate that the systemic activation of DCs by CpG, a TLR9 ligand, inhibits the induction of CD4 T cell responses against subsequently encountered antigens, which is attributed to a downregulation of MHC class II/peptide complex formation (Young et al., 2007).

CD40 and CD40L interactions are known to mediate CD4 T cell help and have been implicated in the induction of cytotoxic T cell responses against adenovirus (Bennett et al., 1998; Schoenberger et al., 1998; Toes et al., 1998; Yang et al., 1996). However, it was impossible to overcome ssRNA-mediated CTL suppression by mimicking CD4 T cell help through CD40 stimulation of DCs (Figure 4.13), indicating that additional signals by CD4 T cells are necessary to prevent inhibition of the CTL response. Indeed, the suppressive effect of DOTAP-complexed ssRNA was avoided in the presence of effector and memory antigen-specific CD4 T cells (Figure 4.14), which not only provide CD40-CD40L signals, but license DCs in an antigen-specific manner through binding of the T cell receptor (TCR) to MHC class II molecules and/or secretion of cytokines. Future experiments are required to elucidate the exact mechanism and signals provided by CD4 T helper cells, which prevent ssRNA-induced suppression of cytotoxicity. A preliminary *ex vivo* DC/T cell coculture experiment already indicated, that activated

CD4 T cells have to be present during priming of CD8 T cells by DCs, as no OT-I T cell activation was observed, if splenic DCs were isolated from mice, that had been immunized with ssRNA and AdOVA in the presence of activated OVA-specific effector CD4 T cells. Based on our findings that intravenous injection of DOTAP-complexed ssRNA interfered with the induction of CD4 T cell help together with the fact that CD4 T cell help is crucial for the generation of long-lived and functional CD8 T cell memory (Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003; Williams and Bevan, 2007), it will be interesting to investigate if systemic TLR ligand treatment prior to the primary infection results in an impaired memory response upon re-encounter with the pathogenic antigen. We observed in a preliminary experiment that mice suffered from a high bacterial burden in response to OVA-expressing *Listeria monocytogenes (LmOVA)* rechallenge, if they had received DOTAP-complexed ssRNA prior to the primary infection with AdOVA. In contrast, mice that were immunized with AdOVA alone or had received *in vivo*-jetPEI^{TM}-complexed ssRNA prior to AdOVA infection, had a lower bacterial burden, implying that the memory response was impaired in ssRNA/DOTAP-treated mice. However, further experiments have to be designed in order to characterize how systemic TLR ligands affect the generation of antigen-specific memory responses.

5.1.3 ssRNA-induced CTL suppression requires type I interferon signaling

Detection of viral nucleic acids by PRRs results in the induction of type I IFNs, which play a crucial role in directing antiviral defense as well as in linking the innate and adaptive immune response (Lund et al., 2004; Uematsu and Akira, 2007). Furthermore, the recognition of nonviral single-stranded RNA and siRNA by TLR7 leads to the production of type I IFNs (Diebold et al., 2004; Hornung et al., 2005; Judge et al., 2005). In agreement with these publications we detected a TLR7-dependent production of type I IFNs in sera and splenocyte supernants upon systemic injection of ssRNA (Figure 4.16).

Moreover, we revealed that the suppression of the CTL response was ascribed to the induction of type IFNs (Figure 4.17 A-C). This finding constrasts the general association of type I IFNs with immune stimulation, as type I IFNs enhance DC maturation and promote the activation as well as differentiation of CD8 T cells by providing signal 3 (Curtsinger et al., 2005; Le Bon et al., 2003; Luft et al., 1998; Montoya et al., 2002;

Nguyen et al., 2000; Nguyen et al., 2002). Despite their well-described stimulatory properties, also anti-inflammatory functions of type I IFNs have been described in several models of autoimmunity, such as multiple sclerosis or systemic lupus erythematosus (Hron and Peng, 2004; Karp et al., 2000; Katakura et al., 2005; Touil et al., 2006; Yarilina et al., 2007). Furthermore, a negative regulation of CTL responses has been reported by the groups of Welsh and Reimann (Bahl et al., 2006; Bochtler et al., 2008; Dikopoulos et al., 2005).

As type I IFNs played an important role in ssRNA-induced suppression of the immune response, we aimed to further elucidate the underlying mechanisms. Using IFNβ-reporter mice (Scheu et al., 2008), we identified pDCs to immediately respond to TLR7-mediated recognition of ssRNA *in vivo* (Figure 4.18 A). Nevertheless, it is likely that pDCs additionally secrete IFNα, as they express high constitutive levels of IRF7, a transcription factor described to initiate IFNα production (Barchet et al., 2002; Coccia et al., 2004; Dai et al., 2004; Kawai et al., 2004). Our observation is in line with several studies reporting that pDCs produce vast amounts of early type I IFNs upon TLR stimulation, especially TLR7 and 9 (Asselin-Paturel et al., 2001; Asselin-Paturel and Trinchieri, 2005; Barchet et al., 2005b). Moreover, we could show that the elimination of pDCs augmented the CTL response in ssRNA and AdOVA immunized mice (Figure 4.18 C and D). These results substantiate the contribution of pDCs in ssRNA-induced suppression of antigen-specific CTL responses. However, pDC depletion did not result in a full recovery of the CTL response, which we assume is attributed at least in part to an incomplete depletion of pDCs. In addition, we cannot exclude that also other splenic cell populations, such as TLR7-expressing macrophages, react to ssRNA stimulation and produce early type I IFNs, especially IFNβ and IFNα4 through activation of pre-existing IRF3, which is constitutively expressed (Applequist et al., 2002; Barchet et al., 2005b; Hassan et al., 2009). We failed to detect IFNβ-producing macrophages in IFNβ-reporter mice, which might be due to low levels of IFNβ secreted by macrophages or due to production of IFNα4, which cannot be detected in IFNβ-reporter mice.

Next, we were interested in unveiling the cell types responding to ssRNA-induced type I IFNs. Several studies report a severe lymphopenia in early stages upon virus infection, which is ascribed to virus-induced type I IFNs (Bahl et al., 2006; Jiang et al., 2005; McNally et al., 2001). Using Cre-mediated cell-specific type I IFN receptor deficient mice we could exclude a direct inhibitory effect of type I IFN on T cells. Rather, we revealed that ssRNA-induced CTL suppression was prevented in CD11c-specific as well as

LysM-specific IFNAR1 deficient mice (Figure 4.19). In the spleen, LysM expression is restricted to monocyte-derived macrophages. Therefore, LysM-specific IFNAR1 deficient mice lack the type I IFN receptor on splenic macrophages but not on DCs, which do not differentiate from monocyte precursors but rather develop from lymphoid origin (Jakubzick et al., 2008; Prinz et al., 2008; Stockinger et al., 2009; Varol et al., 2007). Thus, these results clearly showed that type I IFNs affected splenic macrophages and DCs.

In addition, we analyzed which signaling molecules downstream of the type I IFN receptor were involved in the transmission of type I IFN-mediated inhibition of CTL responses. We detected that STAT1 and IRF7 played a crucial role, as CTL responses were present in STAT1 and IRF7 deficient mice in response to systemic DOTAP-stabilized ssRNA injection and adenovirus infection (Figure 4.20 A and C). Synthesis of early type I IFNs, presumably low levels of IFNα4 and IFNβ, initiates a positive feedback loop via paracrine or autocrine stimulation of the JAK-STAT pathway through the type I IFN receptor. Signaling via STAT1 results in the induction and activation of the transcription factor IRF7, which in turn binds to the promoter regions of many IFNα genes and induces the production of several delayed IFNα isoforms (Levy et al., 2003; Marie et al., 1998; Prakash et al., 2005; Sato et al., 1998). Based on our result that CTL suppression was absent in LysM-specific type I IFN receptor deficient mice, we assume that the synthesis of IFNα subtypes occurs via this amplification loop in splenic macrophages, which subsequently mediate the suppression of the CTL response. A role of splenic macrophages in type I IFN amplification is underlined by a recent study showing that type I IFNs in response to *Listeria monocytogenes* are amplified by a splenic cell type with the cell surface markers characteristic of macrophages (Stockinger et al., 2009). Nevertheless, further experiments are required to prove the involvement of splenic macrophages in the amplification of type I IFN in our studies. In future experiments, we will determine type I IFN levels upon systemic ssRNA stimulation in complete IFNAR1 deficient and LysM-specific IFNAR1 deficient mice. If the amplification of the type I IFN response depends on a feed-forward loop through the type I IFN receptor expressed by macrophages, low levels of systemic type I IFN, especially IFNα, are expected in LysM-specific IFNAR1 deficient mice. In addition, we will monitor MxA expression, which is induced by type I IFNs (Arnheiter et al., 1996; Roers et al., 1994; Staeheli et al., 1993), by histological stainings performed on spleen sections. A visible ring of MxA expression upon ssRNA stimulation in the marginal zone, where most splenic macrophages are

located (Mebius and Kraal, 2005; Mebius et al., 2004), would be a further indication that splenic macrophages are important for the amplication of the type I IFN response.

A dominant role of IFNα in ssRNA-induced immune suppression is substantiated by the observation that injection of ssRNA did not impede the CTL response in IFNβ and IRF3 deficient mice. Strinkingly, the systemic injection of recombinant IFNα (subtype A) did not result in a complete inhibition of the CTL response. We suppose that the moderate suppression of the CTL response might be either due to lower levels of circulating type I IFN compared to the amount that is stimulated by i.v. injection of ssRNA or due to a different subtype of IFNα that is produced through the amplification loop. As there exist several IFNα isoforms, it will be difficult to identify which specific IFNα subtype mediates the suppressive effect on adaptive immunity.

5.1.4   ssRNA-induced type I interferons impair IL-12 secretion and cross-priming of antigen-specific CD8 T cells by splenic DCs

Phenotypic and functional maturation of DCs is essential for T lymphocyte activation and differentiation into T helper as well as into cytotoxic T lymphocytes (Akira et al., 2006; Banchereau and Steinman, 1998; Iwasaki and Medzhitov, 2004; Kawai and Akira, 2005). Our data obtained in transgenic mice lacking the type I IFN receptor on DCs, where CTL suppression by ssRNA was prevented, cleary demonstrated that ssRNA-induced type I IFN acted on DCs, which were negatively modulated in their ability to generate CTL responses. Although a slight decrease in splenic CD11c^{high}CD8a^{pos} DCs, those known to cross-present antigens *in vivo* (den Haan et al., 2000), was detected upon ssRNA treatment, we assume that this had a minor influence on the lack of the CTL response as the number of DCs was still sufficient to stimulate a CD8 T cell response. Activation of T cells requires the maturation of DCs, which is characterized by the upregulation of costimulatory molecules CD80/86 and expression of CD40 on DCs. The latter is necessary to receive CD4 T cell help via CD40 and CD40L interactions. We observed that a systemic injection of ssRNA induced the upregulation of CD80, CD86, and CD40 on splenic CD11c^{high}CD8a^{pos} DCs (Figure 4.21 B). Thus, we could rule out that an impaired DC maturation contributed to the inhibition of the CTL response. This is in agreement with recent studies reporting that systemic TLR ligands or type I IFN impede the generation of T cell responses despite a maturation of DCs (Longman et al., 2007; Wilson et al., 2006; Young et al., 2007).

Interestingly, we noticed a downregulation in MHC class II molecule expression on splenic DCs in response to ssRNA injection, which is in line with our finding that the induction of antigen-specific CD4 T cell responses was impeded.

In addition to antigen presentation (signal 1) and costimulation by DCs (signal 2), the production of IL-12, as a signal 3, is crucial for the generation of an efficient and long-lasting CD8 T cell response (Curtsinger et al., 2003a; Curtsinger et al., 2003b; Curtsinger et al., 1999; Schmidt and Mescher, 1999; Trinchieri, 1995, 2003). Indeed, IL-12 secretion by ssRNA pre-activated DCs was considerably diminished in response to CpG stimulation (Figure 4.22), which is consistent with reports describing that type I IFNs inhibit IL-12 secretion by murine and human DCs (Cousens et al., 1997; McRae et al., 1998). It is likely that the reduction in IL-12 production after CpG stimulation is mediated by ssRNA-induced type I IFNs. To confirm this assumption, we will analyze IL-12 secretion in IFNAR1 deficient mice upon systemic administration of DOTAP-complexed ssRNA and CpG administration.

Beside the downregulation in MHC class II molecule expression and the reduction in IL-12 production, we revealed that ssRNA pre-activated splenic DCs were impaired in their ability to cross-prime antigen-specific CD8 T cells *ex vivo* (Figure 4.23), which was overcome by exogenous provision of recombinant IL-12 (Figure 4.24 B). Again, the inability to activate antigen-specific CD8 T cells was dependent on type I IFNs, as cross-priming of CD8 T cells by ssRNA pre-activated DCs isolated from IFNAR1 deficient mice was unaffected in response to AdOVA infection (Figure 4.24 A). These results strengthen the finding that ssRNA-induced type I IFN acts on DCs, thereby modulating their capacity to activate CD8 T cells. Our findings are supported by observations of Longman et al, showing in an *in vitro* system that immature human DCs exposed to type I IFNs are impaired in their ability to activate CD8 T cells via the cross-presentation pathway. In contrast, already matured DCs enhance T cell activation in the presence of type I IFNs (Longman et al., 2007). Likewise, the injection of DOTAP-complexed ssRNA after adenovirus infection did not inhibit the generation of a CTL response in our experiments. Our results and the findings by Longman et al suggest, that type I IFNs can exert contrasting effects on adaptive immunity depending on the timepoint. The opposing effects of type I IFN are not attributed to a differential expression of the type I IFN receptor (Longman et al., 2007), but rather seem to be mediated by distinct intracellular signaling pathways downstream of the type I IFN receptor. It has been demonstrated that inhibitory effects of type I IFN on immature DCs are mediated by signaling via STAT1, whereas the

immune stimulatory effects on mature DCs are triggered through signaling via STAT4 (Longman et al., 2007). This view was supported by the observation that type I IFNs negatively regulate IFNγ expression in mice by activation of STAT1 (Nguyen et al., 2000), whereas STAT4 is involved in the transmission of stimulatory effects of type I IFNs and triggers the production of IFNγ in response to viral infections (Nguyen et al., 2002). Whether the suppressive effect of type I IFN on splenic DCs in our experiments is indeed transmitted via STAT1 will be difficult to investigate, as STAT1 deficient mice lack the positive feedback loop, which is essential to ensure the production of IFNα, which leads to the suppression of the CTL response. In order to address this question, it will be necessary to use Cre-mediated DC-specific STAT1 deficient mice, which are not available at present. The inhibitory effect of ssRNA-induced type I IFN might be additionally attributed to differences in antigen uptake. So far, we have not examined whether antigen capture as such or time-kinetics of antigen uptake are altered. However, the absence of a CD8 T cell response cannot be soley ascribed to a substantial decrease in antigen uptake in ssRNA-preactivated DCs, as we were able to circumvent ssRNA-induced CTL suppression in the presence of antigen-specific effector or memory CD4 T cells. Furthermore, supplementation of recombinant IL-12 in *ex vivo* DC/T cell coculture experiments induced T cell activation by ssRNA pre-activated splenic DCs. These results imply that the amount of antigen presented on MHC class I must have been sufficient for T cell activation at least in the presence of CD4 T cell help. Nevertheless, it will be interesting to compare antigen uptake and the amount of antigen cross-presented by immature or ssRNA-activated splenic DCs in response to AdOVA infection.

5.1.5 ssRNA- and type I interferon-mediated suppression of antigen-specific T cell responses – Summary and conclusion

In Figure 5.1, our findings are summarized and we suggest a potential mechanism how systemic ssRNA stimulates the production of type I IFNs, which mediates the suppression of subsequent antigen-specific T cell responses in the spleen.

(A) Upon systemic injection, DOTAP-complexed ssRNA is recognized in the endosome by TLR7-expressing pDCs in the spleen, which leads to the production of immediate-early IFNα/β (1). Secreted IFNα/β binds in a paracrine manner to the type I IFN receptor expressed by splenic macrophages, which initiates a positive feeback amplification loop

through the JAK-STAT pathway (2, 3). Signaling via STAT1 induces the formation of the transactivation complex ISGF3, which translocates to the nucleus and induces the transcription factor IRF7, which in turn stimulates the transcription and production of delayed IFNα subtypes (4). Amplified IFNα binds to the type I IFN receptor expressed on splenic DCs, thereby stimulating on the one hand the upregulation of costimulatory molecules CD80/86 as well as CD40, and on the other hand the downregulation of MHC class II expression (5). Upon encounter with a pathogen (e.g. adenovirus, HSV-1, Influenza virus, *Listeria monocytogenes*), ssRNA-activated DCs are impaired in IL-12 production, in their ability to activate CD4 T cells, and to cross-prime CD8 T cells (6), which ultimately results in the lack of an antigen-specific CTL response (7).
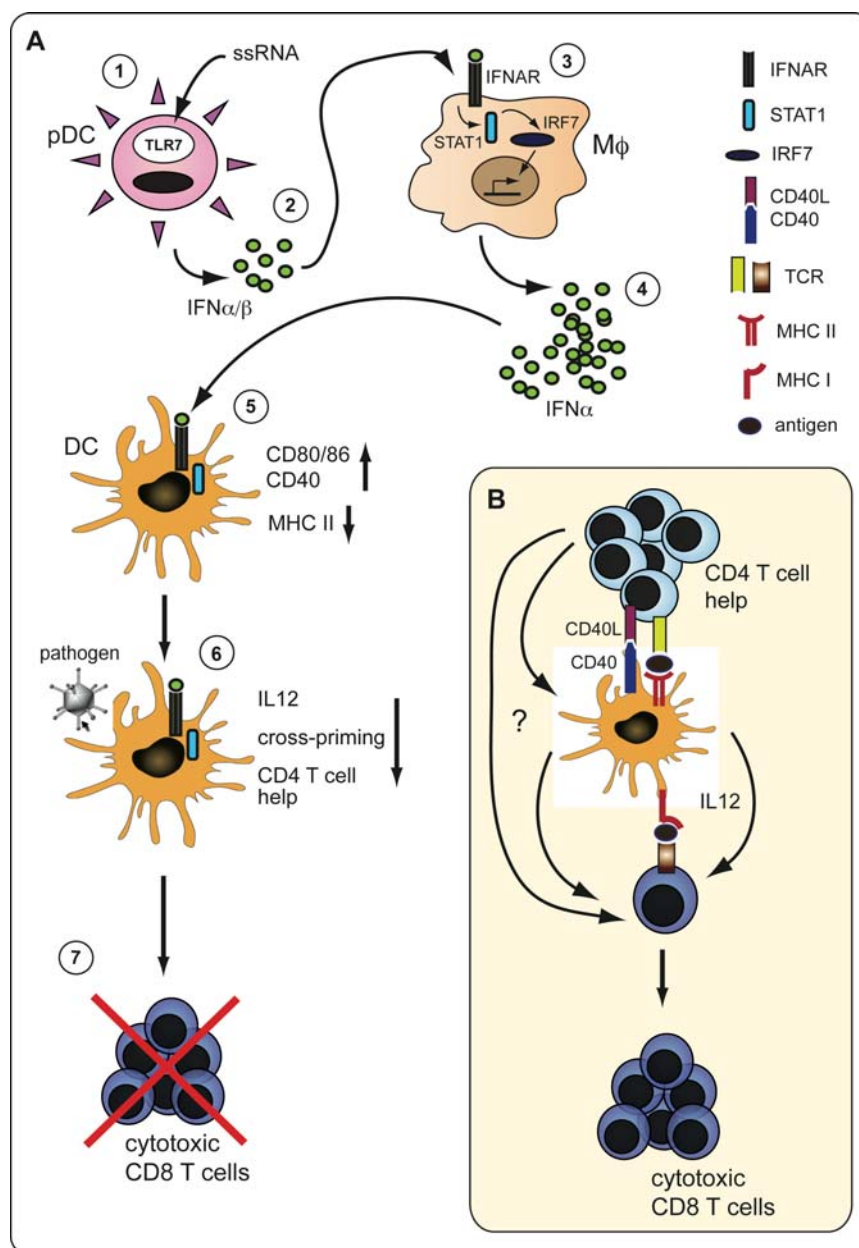

Figure 5.1 Summary and mechanisms of ssRNA- and type I interferon-mediated CTL suppression in the spleen.

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(B) In the presence of CD4 T cell help, provided by activated antigen-specific effector or memory CD4 T cells, ssRNA-induced inhibition of CTL responses is circumvented. We suppose that effector or memory CD4 T cells provide CD40-CD40L signals, but also license the DC in an antigen-specific manner through binding of the TCR to MHC class II molecules and trigger the secretion of important cytokines, such as IL-12 or IL-2. However, we do not know, which additional signals might be provided by activated CD4 T cells, that prevent ssRNA-induced CTL suppression. Possible candidates might be chemokines, which are secreted to recruit CD8 T cells to the site, where antigen-specific DC/CD4 T cell interaction occurs (Castellino et al., 2006). How exactly activated CD4 T cells help to prevent ssRNA-induced suppression of cytotoxicity remains to be determined.

5.2 Relevance of RNA- and type I interferon-mediated suppression of adaptive CD8 T cell responses

5.2.1 Therapeutic application of siRNA harbors the risk of immune suppression – Interference of siRNA application with induction of tumor-specific CTL responses

Traditional ways of anti-cancer treatment include surgery, irradiation, and chemotherapy. However, recent studies offer a successful suppression of tumorigenicity by using siRNA mediated post-transcriptional gene silencing. For instance, it has been recently published that mitosis in cancer cells is disrupted by targeting of cell cycle proteins, such as KSP or PLK1 (Judge et al., 2009), and that apoptosis of cancer cells is initiated by silencing the anti-apoptotic protein Bcl-2 (Poeck et al., 2008). Another promising target that has come into the focus of cancer therapy is the vascular endothelial growth factor (VEGF), which is involved in tumor angiogenesis. Indeed, several studies have used gene silencing targeting VEGF-A mRNA in distinct tumor models in order to block angiogenesis (Filleur et al., 2003; Jia et al., 2007; Kim et al., 2006; Kornek et al., 2008; Mulkeen et al., 2006; Shen et al., 2007; Shen et al., 2006; Takei et al., 2004). Recently, a dual function of siRNA application in tumor therapy has been discussed, which aims at the induction of innate immune stimulation in addition to a target-specific gene knockdown (Poeck et al., 2008). Indeed, numerous reports have revealed that unmodified siRNAs are recognized by nucleic

acid receptors, such as TLR7, and stimulate the innate immune system to produce pro-inflammatory cytokines, like type I IFNs, TNFα, and IL-6 (Judge et al., 2005; Robbins et al., 2008; Rossi, 2009; Sioud, 2005, 2006).

Moreover, the induction of tumor-directed CTL responses constitutes a promising strategy for the prevention and treatment of malignancies. Induction of an effective tumor-specific CTL response can be achieved by vaccination with antigen-loaded DCs or with recombinant adenoviruses expressing tumor-associated antigens (Chen et al., 2003; Gallo et al., 2005; Gallo et al., 2007; Steitz et al., 2002; Steitz et al., 2001a; Steitz et al., 2001b; Steitz et al., 2006; Toes et al., 1997; Tuting et al., 1999).

Owing to their plasticity, tumors tend to evade single-targeted therapeutic approaches. Therefore, it is reasonable that a combinatorial tumor-directed approach using siRNA-mediated gene-silencing and innate immune stimulation together with an adenoviral-based vaccination would provide an efficient opportunity in tumor therapy.

Importantly, we observed in a model of a combinatorial anti-tumor therapy that, depending on the carrier used, systemic *in vivo* siRNA injection (Filleur et al., 2003; Kornek et al., 2008) abrogated a subsequent adenoviral-based tumor vaccination due to the lack of a tumor-specific CTL response, indicating that systemic siRNA application might result in undesired off-target effects (Figure 4.26). Positive off-target effects of siRNA application due to innate immune stimulation have been recently described in the study of Kleinman which shows, that siRNA targeting VEGF reduces choroidal neovascularization, a hallmark of age-related macula degeneration (AMD), which was not ascribed to gene silencing but was rather a consequence of TLR3-mediated recognition of siRNA and induction of IFNγ (Kleinman et al., 2008). In order to avoid unwanted off-target effects of siRNA we propose to use a carrier, such as *in vivo*-jetPEI^{TM}, which targets siRNA to the cytoplasm and therby prevents recognition of siRNA by endosomally located TLR3 and TLR7. Another opportunity to prevent the failure of the adenovirus-based tumor vaccination would be to inject DOTAP-complexed siRNA after adenovirus immunization, as we have shown that the timing of systemic RNA oligonucleotide injection plays a crucial role whether a CTL response is generated or not. A preliminary experiment already revealed that systemic siRNA application post adenovirus injection promoted tumor regression. Nevertheless, this strategy may offer only limited success and is questionable, as the application temporally after the adenovirus immunization would eliminate the tumor, but may occur before a subsequent unknown infection, and thus is likely to cause immune suppression again. Recent publications offer another elegant possibility to

circumvent siRNA-mediated stimulation of the innate immune system by making use of chemically modified siRNAs, where 2'-O-methyl residues are incorporated into the siRNA's backbone (Judge and MacLachlan, 2008; Judge et al., 2006; Robbins et al., 2007; Sioud et al., 2007).

However, as mentioned above, new strategies are currently emerging where innate immune stimulation is desired in addition to gene silencing. In a study by Poeck et al a bifunctional siRNA was designed, which silenced Bcl-2 and additionally activated RIG-I (Poeck et al., 2008), due to triphosphates attached to the 5'end (Hornung et al., 2006). Systemic treatment with this triphosphate-siRNA (pppsiRNA) resulted in a significant reduction in metastatic growth of a melanoma, which was attributed to the synergistic effects of RIG-I-mediated innate immune stimulation and siRNA-mediated Bcl2 silencing that provoked massive apoptosis in tumor cells (Poeck et al., 2008). A major drawback of a systemic application of pppsiRNA, which was not considered in this publication, is that the intravenous injection of pppsiRNA results in the suppression of subsequent cytotoxic T cell responses due to the induction of type I IFNs by RIG-I activation (unpublished observation). Therefore, we attempt to circumvent a suppression of adaptive immune responses by targeting pppsiRNA exclusively to the liver. Indeed, it has been shown that LDL-cholesterol-modified siRNA is efficiently taken up in the liver and silences apoB protein expression *in vivo* (Wolfrum et al., 2007). Using similar approaches, we intend to induce a local production of type I IFNs, which may result in a synergistic effect of innate immune stimulation and target-specific gene knockdown, without interfering with subsequent adaptive immune responses, offering a powerful treatment of liver tumors or chronic viral liver infections.

Taken together, we demonstrate that systemic siRNA application might lead to undesired off-target effects such as immune suppression. In order to avoid these unwanted side effects we suggest to carefully consider how to use siRNA in therapeutic applications.

5.2.2 Influenza virus infection inhibits the CTL response against a subsequent adenovirus infection in a type I interferon dependent manner

It is known that certain viral infections result in a temporary lack in cell-mediated immune responses, which is frequently ascribed to the effects of virus-induced type I IFNs (De Maeyer et al., 1975; De Maeyer-Guignard et al., 1975). Several reports demonstrate, that

viral infections can elicit negative impacts on DCs by interfering with their differentiation, expansion, and maturation or by induction of apoptosis (Andrews et al., 2001; Hahm et al., 2005; Li et al., 2002; Salio et al., 1999; Sevilla et al., 2004). However, not only DCs are the targets of virus-induced immunosuppression, as virus-induced type I IFNs are able to induce apoptosis in T cells or inhibit T cell proliferation upon measles virus infection (Bahl et al., 2006; Hahm et al., 2003; Jiang et al., 2005; McNally et al., 2001; Sun et al., 1998). We could show that an infection with Influenza virus inhibited the generation of a cytotoxic T cell response against a subsequent adenovirus infection in a type I IFN dependent manner (Figure 4.27). Consequently, these results imply that viruses, known to trigger type I IFNs, might inhibit adaptive immune responses towards following infections in a similar fashion as we have described for systemic ssRNA injection. It remains to be determined, whether Influenza virus infection impedes subsequent adaptive immune responses due to an impaired cross-priming of CD8 T cells and CD4 T cell help.

5.3   Concluding Remarks

The findings presented within this thesis demonstrate that ssRNA- and virus-induced type I IFNs cannot only stimulate, but also inhibit antigen-specific T cell responses in the spleen, depending on the timing of administration. Although immune suppression is generally not desired, it might be that the induction of immune suppression represents a type of "emergency shutdown" that is initiated when circumstances of TLR activation or infection poses a serious danger by excessive inflammation for the host. Furthermore, immune suppression against a subsequent infection may enable the immune system to cope with the initial infection first before fighting against the following one.

In addition, we indicate that a therapeutic application of siRNA, which can be recognized by nucleic acid receptors, might have off-target effects that result in immune suppression. Our findings will contribute to a better understanding how to design and apply siRNA in the clinics in order to avoid undesired off-target effects.

Moreover, we think that TLR-dependent induction of immune suppression by recognition of endogenous ligands, as it has been observed for immunocomplexed "self" RNA or DNA by TLR7/8 or TLR9, respectively, represents an ongoing physiological mechanism to prevent potentially undesired or even harmful immune responses.

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7 Table of Figures

8 Abbreviations

AdLOG	adenovirus expressing luciferase, ovalbumin, green fluorescent protein
AdOVA	adenovirus expressing ovalbumin
Al647	Alexa647
AMD	age-related macula degeneration
APC	antigen presenting cell
BCR	B cell receptor
BMDC	bone-marrow derived DC
BSA	bovine serum albumin
C	Celsius
CCL	CC-chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
cDC	conventional DC
CFU	colony forming unit
cm	centimeter
CpG	cytosine-phosphate-guanosine
CTL	cytotoxic T lymphocyte
CXCL	CXC-chemokine ligand
DC	dendritic cell
DMSO	Dimethylsulfoxide
DMSO	Dimethyl sulfoxide
dsRNA	double-stranded RNA
DTR	diptheria toxin receptor
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMCV	encephalomyocarditis virus
ER	endoplasmatic reticulum

FACS	fluorescence activated cell sorting
FCS	fetal calf serum
g	gram
g	gravity
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage-colony stimulating factor
h	hours
HSP	heat shock protein
HSV	Herpes Simplex Virus
i.p.	intraperitoneally
i.po.	intraportally
i.v.	intravenously
IFN	interferon
IFNAR	type I interferon receptor
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
IRF	interferon regulatory factor
IRFBS	IRF-binding site
ISGF	interferon-stimulated gene factor
ISRE	interferon-stimulates response element
JAK	Janus kinase
ko	knock out
l	liter
LCMV	lymphocytic choriomeningitis virus
Lm	Listeria monocytogenes
LPS	lipopolysaccharide

MACS	magnetic activated cell sorting
MBL	mannose-binding lectin
MCMV	murine cytomegalovirus
MDA-5	melanoma differentiation-associated gene 5
MHC	major histocompatibility complex
min	minutes
ml	milliliter
mm	millimeter
mRNA	messenger RNA
mTRP2	murine tyrosine-related protein 2
ng	nanogram
NK cells	natural killer cells
NOD	nucleotide-binding oligomerization domain
nt	nucleotides
OD	optical density
ODN	oligonucleotide
OPD	*o*-Phenylenediamine dihydrochloride
ORN	oligoribonucleotide
OVA	ovalbumin
PALS	periarteriolar lymphoid sheaths
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
pDC	plasmacytoid DC
PEI	polyethylene imine
PFA	paraformaldehyde
PFU	plaque forming unit
PRR	pattern recognition receptor
RIG-I	retinoic acid-inducible gene I
RISC	RNA-inducing silencing complex
RNA	ribonucleic acid
rpm	rounds per minute

s.c.	subcutaneously
S8L	SIINFEKL
SEM	standard error of the mean
Siglec-1	sialic acid-binding immunoglobulin-like lectin-1
siRNA	small interfering RNA
ssRNA	single-stranded RNA
STAT	signal transducers and activators of transcription
T_{CM}	T central memory cells
TCR	T cell receptor
T_{EM}	T effectot memory cells
TGFβ	transforming growth factor beta
Th1	T helper 1 cells
Th2	T helper 2 cells
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Tyk	tyrosine kinase
VP	virus particles
YFP	yellow fluorescence protein
µg	microgram
µl	microliter
µm	mircrometer

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Carl Hilty
Schweizer Rechtsgelehrter und Schriftsteller (1833-1909)